Mixture toxicity

Empirical studies with defined chemical mixtures in rats

Diana Jonker

Hypericum perforatum

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Mixture toxicity

Empirical studies with defined chemical mixtures in rats

Mengseltoxiciteit
Empirisch onderzoek met gedefinieerde mengsels bij de rat

(met een samenvatting in het Nederlands)

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Contents

Abbreviations

ACGIH	American Conference of Governmental Industrial Hygienists
ADI	Acceptable Daily Intake
ALAT	Alanine aminotransferase
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ASAT	Aspartate aminotransferase
DOTC	Di-*n*-octyltin dichloride
EPA	U.S. Environmental Protection Agency
GGT	γ-Glutamyl transferase
HCBD	Hexachloro-1,3-butadiene
HI	Hazard Index
LAL	Synthetic lysinoalanine
LDH	Lactate dehydrogenase
LONEL	Lowest-Observed-Nephrotoxic-Effect Level
LSD	Least Significant Difference
MNEL	Minimum-Nephrotoxic-Effect Level
MOAEL	Minimum-Observed-Adverse-Effect Level
NAG	N-acetyl-β-glucosaminidase
NNEL	No-Nephrotoxic-Effect Level
NOAEL	No-Observed-Adverse-Effect Level
NONEL	No-Observed-Nephrotoxic-Effect Level
NTP	U.S. National Toxicology Program
TCTFP	1,1,2-Trichloro-3,3,3-trifluoropropene
TEF	Toxicity Equivalency Factor
TETRA	Tetrachloroethylene
TLV	Threshold Limit Value
TRI	Trichloroethylene

Chapter 1

General Introduction

1.1 Introduction

The work described in this thesis concerns the toxicity of chemical mixtures. A key question concerning the toxicity of a mixture of chemicals is whether the effects of a mixture deviate from what could have been expected from the known effects of the individual chemicals. This question was addressed as early as 1870, when Fraser introduced a graphical tool (the isobologram) to determine whether the effect of a combination of drugs was 'less than expected' (Calabrese, 1991, p. 26). For many years thereafter, there was no general agreement on the interpretation of mixture data, which has led to situations in which different conclusions were drawn from the same data set. To resolve this problem, Bliss (1939) introduced a conceptual framework in which he defined three types of joint action: independent joint action, similar joint action, and synergistic action. These basic concepts are still widely used today (Mumtaz *et al.*, 1994; Feron and Groten, 2002) and are explained further in section 1.2.

The early work on chemical mixtures was undertaken to understand whether the joint application of two pesticides or of two or more drugs led to outcomes that were greater than the sum of the outcomes predicted on the basis of individual applications of the substances (National Research Council, 1988). It was not focused on the unintended, usually low-level exposure to chemicals in the environment. As far as environmental health is concerned, the field of toxicology and risk assessment which addresses the toxicological consequences of exposure to chemical mixtures has exploded in the 1980s (Calabrese, 1991). The studies described in this thesis are an example of the efforts made to gain insight in the toxicological impact of exposure to chemical mixtures at levels around the toxicity threshold of the individual chemicals.

The rationale for concern regarding potential toxicity from the exposure to multiple chemicals is obvious. First, people's actual exposures are to a complex and ever-changing mixture of environmental agents in the air they breathe, the water they drink, the food and beverages they consume, the surfaces they touch, and the consumer products they use (Sexton *et al.*, 1995). Second, in contrast to the reality of multiple chemical exposure, toxicity testing to predict effects on humans has traditionally studied substances one at a time and the vast majority of established exposure standards are for single compounds. To illustrate, a rough estimation by Yang (1994a), based on a cursory survey of toxicology literature and chronic toxicity/carcinogenicity studies from the U.S. National Toxicology Program, indicated that over 95% of the resources in toxicology is devoted to single chemical studies. Third, many studies in experimental animals exposed to chemical mixtures have shown unexpected responses, i.e. toxicity greater or less than that predicted from the constituents, and, though limited, there is

some evidence for the occurrence of such a phenomenon in humans (for reviews, see Calabrese, 1991; Krishnan and Brodeur, 1991,1994; Schilling, 1987; WHO, 1981; Yang, 1994b).

1.2 Terminology

When reviewing the literature on the toxicity of chemical mixtures, the reader may easily become confused by the many terms which are inconsistently used to describe the joint action of chemicals (see overviews in Calabrese, 1991; Kodell and Pounds, 1991; Könemann and Pieters, 1996). The same term may be used to describe different types of joint action (e.g. the term 'additivity' generally implies absence of synergism, but has sometimes been used to indicate a special case of synergism). Also, different terms are used to describe the same phenomenon. The fact that many investigators do not clearly define their terminology further adds to the confusion.

In this thesis, a (chemical) mixture is defined as any combination of two or more chemicals, regardless of source or of spatial or temporal proximity. Combined or joint action is defined broadly as any outcome of the exposure (via the same or different routes) to a mixture.

To evaluate the safety of chemical mixtures, Feron *et al.* (1995) recommend to distinguish between simple and complex mixtures. A simple mixture is defined as a mixture that consists of a relatively small number of chemicals, say ten or less, the composition of which is qualitatively and quantitatively known (e.g. a cocktail of pesticides, a combination of medicines, or the mixtures studied in this thesis). A complex mixture is defined as a mixture that consists of tens, hundreds, or thousands of chemicals, and its composition is qualitatively and quantitatively not fully known (e.g. diesel exhaust, cigarette smoke, a workplace atmosphere, drinking water or food).

Based on the pioneering conceptual framework described by Bliss (1939), the various types of joint action are often classified into the following three categories (Mumtaz *et al*, 1994):

1) Independent joint action:
Independent joint action (Bliss, 1939) is also referred to as simple independent action (Finney, 1971), dissimilar joint action (Plackett and Hewlett, 1952) or response addition (EPA, 1986, 2000). This type of joint action is non-interactive, i.e. the chemicals in the mixture do not affect the toxicity of one another. In other words, the

chemicals are assumed to behave independently of one another, so that the body's response to the first chemical is the same whether or not the second chemical is present. The modes of action and possibly the nature and site of the toxic effect differ among the chemicals in the mixture.

The toxicity of the mixture can be predicted (calculated) from the dose-response curves of the individual chemicals. Response is expressed as the probability that an effect is elicited (viz. the likelihood of an individual showing effects, or the proportion of a population showing effects).

The response to a mixture depends not only on the dose, but also on the correlation of the tolerances, which can vary between -1 and +1. In case the individual most sensitive to chemical 1 is also most sensitive to chemical 2, the susceptibilities (tolerances) are considered to show complete, positive correlation ($r = 1$). In this case, the response to the mixture of chemicals 1 and 2 would be equal to that attributed to the most toxic chemical (e.g. if the amounts of chemicals 1 and 2 in the mixture would elicit responses of, respectively, 25% and 10% when given alone, the response to the mixture would be 25%).

In contrast, if the correlation of tolerances would be completely negative ($r = -1$; the individual most sensitive to chemical 1 is least sensitive to chemical 2), the response to the mixture would be equal to the sum of the responses of the individual chemicals (in the above example the mixture response would be $25 + 10 = 35\%$). This formula is the most 'conservative' form of independent joint action and is equivalent to dose addition. It is extensively used for assessing the risk of mixtures of carcinogens (EPA, 2000).

If there is no correlation of tolerances ($r = 0$), then the chemicals are assumed to produce toxicity independently, and the mixture response is calculated by the standard formula for statistical independence:

$P_{mix}(d_1,d_2) = P_1(d_1) + P_2(d_2) - [P_1(d_1) * P_2(d_2)]$

$P_1(d_1)$, $P_2(d_2)$ and $P_{mix}(d_1,d_2)$ are the probabilities that an effect is elicited by, respectively, chemical 1 at dose d_1, chemical 2 at dose d_2, and their mixture. This formula is also used to calculate the probability of an effect in an individual exposed to a mixture of independently acting chemicals.

An important characteristic of response addition is that a chemical does not contribute to the mixture response when it is present at a level below its individual effect threshold. So, if the doses of all chemicals in a mixture are below their respective thresholds, their individual responses will be zero and the response to the mixture will also be zero.

2) Similar joint action:

Similar joint action (Bliss, 1939; Plackett and Hewlett, 1952) is also referred to as simple similar action (Finney, 1971; Plackett and Hewlett, 1952), dose addition (EPA, 1986, 2000) or concentration addition (Kodell and Pounds, 1991). Like independent joint action, similar joint action is non-interactive. The chemicals produce similar but independent effects, so that one chemical can be substituted at a constant proportion for the other. Under the narrow definition of dose addition[1] (as given by Bliss, 1939), the chemicals are assumed to behave similarly in terms of mode of action and primary physiologic processes (uptake, metabolism, distribution, elimination), and to have similarly shaped (parallel) dose-response curves and complete positive correlation of individual susceptibilities. The chemicals differ only in their potencies. In practice, since information on the mode of action and toxicokinetics is often lacking, the requirement of toxicological similarity is usually relaxed to that of similarity of target organs (EPA, 2000).

[1] Broader definitions allow for non-linear or non-parallel dose-response curves, imperfect correlation of tolerances, or different modes (not sites) of primary action (Calabrese, 1991; Kodell and Pounds, 1991; Svendsgaard and Hertzberg, 1994).

The toxicity of the mixture can be calculated using summation of the doses of the individual chemicals after adjustment for the differences in potencies:
$P_{mix}(d_1,d_2) = f(d_1 + t*d_2)$ where 't' is the relative potency of chemical 2 to 1, and the mixture response P_{mix} at dose d_1 of chemical 1 and dose d_2 of chemical 2 is given in terms of the equivalent dose and dose-response function (f(d)) for chemical 1.

The adding of doses implies that the summed dose can be high enough to induce a toxic effect even when the dose of each individual chemical is at a level below its individual effect threshold (recall that response addition at subthreshold levels would result in a zero response for the mixture). The higher the number of chemicals in a mixture, the higher the likelihood that addition of low (subthreshold) doses predicts a mixture response which is of health concern.

It should be realized that the above theoretical distinction between dose and response addition generally does not hold so strictly in whole organisms due to the complexity and interdependence of their physiological systems. When a mixture contains many chemicals, it is unlikely that the mode of toxic action is the same for all chemicals and the application of full dose addition would overestimate the toxicity of the mixture. On the other hand, it is also unlikely that all chemicals in such a mixture

have completely different modes of action. It is more reasonable to assume an intermediate form of non-interactive joint action. Chemicals with different mechanisms for their primary, specific effect may have a common mechanism for some less specific, secondary effect. In case of such overlapping mechanisms partial dose additivity may occur. This phenomenon is well known in the aquatic toxicology where chemicals have been found to contribute to a narcotic mode of action at levels below the threshold for their specific mode of action. This narcotic effect (also termed baseline toxicity) is considered due to general membrane perturbation or membrane depolarisation (ECETOC, 2001).

3) Interaction:

In a broad sense, interaction is characterised by one chemical influencing the biological action of the other (Kodell and Pounds, 1991). Operationally, interaction can be defined as the type of joint action showing a mixture response which deviates from that expected on the basis of (dose or response) addition. Interactive joint actions can be less than additive (e.g. antagonistic, inhibitive, infra- or sub-additive) or greater than additive (e.g. synergistic, potentiating, supra-additive). Though synergistic interactions can result in novel effects, such effects seem to be rare. Instead, combinations of agents acting synergistically tend to do so through an increase in the effects of one of the agents (National Research Council, 1989).

Note that in the above definition the term interaction has a different meaning than in the physiological sense where it describes biological interference with a cellular target molecule or receptor.

Terms such as antagonism or synergism indicate in which direction a response to a mixture differs from what is expected under the assumption of additivity. However, they provide no information about mechanisms or quantitative aspects of interactions. Moreover, it should be emphasized that the nature of the interaction may change with altered exposure conditions (e.g. dose, duration, sequence). For example, upon chronic exposure the neurotoxicity of the solvent *n*-hexane was potentiated by the non-neurotoxic solvent methyl ethyl ketone (as seen in glue sniffers which developed neuropathy after methyl ethyl ketone was added to an *n*-hexane containing glue). On the other hand, acute (15 minutes) co-exposure to these solvents was associated with decreased formation of *n*-hexane's neurotoxic metabolite 2,5-hexanedione, indicating an antagonistic interaction (Engelen *et al*, 1997).

1.3 Mechanisms of interaction

The mechanisms underlying interactions are often divided into three categories: direct chemical-chemical, toxico/pharmacokinetic, and toxico/pharmacodynamic mechanisms. A short explanation of these mechanisms follows below. Numerous examples can be found in the extensive literature on drug-drug interactions (e.g. Aarons, 1986; Calabrese, 1991) and in several reviews focused on chemicals of occupational or environmental concern (e.g. chapter 5 in Calabrese, 1991; chapter 4 in Vouk *et al*, 1987; World Health Organization, 1981).

In direct chemical-chemical mechanisms, one chemical directly interacts with another, causing a chemical change in one or more of the compounds. In many cases this mechanism results in decreased toxicity (less than additive effect), and it is one of the common principles of antidotal treatment. Chemical-chemical interactions leading to greater than additive effects have been much less frequently documented. A well known example of the latter type is the formation of carcinogenic nitrosoamines in the stomach through the reaction of non-carcinogenic nitrite (from drinking water or food) with amines (e.g. from fish protein).

Toxicokinetic mechanisms involve alterations in metabolism (biotransformation) or disposition of a chemical and are often divided into effects on absorption, distribution, metabolism and excretion. Essentially, toxicokinetic interactions alter the amount of the toxic agent(s) reaching the cellular target site(s) without qualitatively affecting the toxicant-receptor site interaction. With respect to their toxicological consequences at low doses, interactions in the process of metabolism (enzyme induction or inhibition) are considered most relevant. The impact of alterations in absorption, distribution or excretion is expected to be small at low dose levels (Könemann and Pieters, 1996).

Toxicodynamic interactions do not directly affect the metabolism or disposition of a chemical, but affect a tissue's response or susceptibility to toxic injury. They include, for example, immunomodulation, alterations in protective factors (depletion or induction), and changes in tissue repair or hemodynamics (EPA, 2000). Alternatively, toxicodynamic interactions are described as interactions occurring at or among cellular receptor sites. Interactions at the same receptor site resulting in antagonism have been termed 'receptor antagonism' (e.g. the antagonistic effect of oxygen on carbon monoxide). Interactions resulting from different chemicals acting on different receptor sites and causing opposite effects have been termed 'functional antagonism' (e.g. opposing effects of histamine and noradrenaline on vasodilatation and blood pressure) (Mumtaz and Hertzberg, 1993).

1.4 Experimental approaches to assess the toxicity of mixtures

Given the diversity of mixture exposure scenarios, the questions to be addressed in mixture studies vary widely and, consequently, there is no 'one size fits all' design and analysis suitable for all mixture studies (Simmons and Gennings, 1996). Factors that influence study design and analysis include the number of chemicals in the mixture, the availability of the mixture for testing in its entirety, the extent to which the toxicity of the mixture needs to be characterised in terms of dose-response relationship or departure from additivity, and available resources.

1.4.1 Detecting interaction

There are several graphical or statistical/mathematical methods to detect and characterise interactions (departures from additivity) between mixture components. A few commonly used methods are presented in this section. Experimental designs which can be used to generate the data required for detecting interaction are the subject of the next section.

The isobolographic method is the classical approach to determine whether two chemicals interact (Calabrese, 1991; Carter and Gennings, 1994; Gessner, 1995; Loewe and Muischnek, 1926; Tallarida, 2000). An isobologram is a graphical representation of the joint effect of two chemicals in which the doses of chemicals 1 and 2 are given on the x- and y-axis, respectively, and the experimentally determined dose combinations of 1 and 2 which all cause the same effect (e.g. 50% mortality) are plotted and connected by a line: the iso-effect line or isobole (or contour of constant response). This experimentally determined line is then compared with the theoretical iso-effect line based on the assumption of additivity. Differences between these lines indicate departure from additivity. Isoboles below the line of additivity indicate synergism (in the presence of chemical 1, less of chemical 2 is required to generate the specified response than would be the case under additivity), those above it indicate antagonism. A major disadvantage of the isobolographic method is its extensive data demand. Also, because of its graphical nature and use of perpendicular axes, isobolograms are unsuitable for mixtures of more than three chemicals.

The interaction index method proposed by Berenbaum (1981, 1985) is related to the isobolographic method (both use iso-effective doses) but does not require plotting of the isobologram and can be used for mixtures of any number of chemicals. Under Berenbaum's definition of additivity, non-interactive combinations satisfy the equation $d_1/D_1 + d_2/D_2 + \ldots + d_i/D_i = 1$.

D_1, D_2, ... D_i are doses of the individual chemicals 1, 2, ... i that produce some specified effect when given alone, and d_1, d_2, ...d_i are their doses in a combination that produces the same effect. The sum of the fractions on the left side of the equation is termed the interaction index which is <1 for synergy, 1 for zero interaction (additivity), and >1 for antagonism. These criteria are independent of the type of effect under consideration, the shapes of the dose-response curves or the homogeneity of the target population, and do not require any assumptions about mechanisms of action of the chemicals. The interaction index covers combinations of an active chemical with a chemical that does not affect the endpoint of interest (for the inactive chemical D_i may be assumed to be infinite so that d_i/D_i is zero).

A problem with both the isobolographic and the interaction index method is that it is not clear how large the difference between an isobole and the line of additivity must be, or how different from unity the interaction index must be before a departure from additivity is likely to be 'real' (as opposed to the result of the biological and experimental variation in the data used to construct the isobole or to calculate the index).

A method which permits the use of well known and readily available statistical methods to analyse the data from mixture studies, and thus accounts for variability in the data, is response surface analysis (Carter and Gennings, 1994; Carter, 1995). This method assumes the existence of a mathematical relationship (a statistical model or equation) between the response variable (effect) and the concentrations of the chemicals in the mixture. This concentration-response relationship describes the mathematical 'surface' of a mixture. An example for a 2-component mixture is given below:

$E = ß_0 + ß_1 d_1 + ß_2 d_2 + ß_{12} d_1 d_2$.

E is the effect produced by the mixture, d_1 and d_2 are the concentrations of chemicals 1 and 2, respectively; $ß_0$ is an unknown constant representing the control situation (concentration zero for each chemical); $ß_1$ and $ß_2$ are unknown constants associated with the effect of chemicals 1 and 2, respectively; $ß_{12}$ is an unknown constant associated with the interaction between the two chemicals (this constant has been shown to be linked to the interaction index). The analysis consists of estimating the unknown constants (parameters) in the model from the experimental results (e.g. by multiple linear regression), testing the goodness of fit of the model to the data, and testing the statistical significance of the constants. If the interaction parameter $ß_{12}$ deviates significantly from zero, the joint effect of the two chemicals differs significantly from additivity. In addition, $ß_{12}$ indicates the direction and magnitude of the interaction.

When a mixture contains more than two chemicals, the response surface analysis method enables detection and characterisation of two-factor interactions between specific pairs of chemicals as well as possible higher order interactions (e.g. a three-factor interaction in case chemical 3 modulates the interaction between chemicals 1 and 2). In contrast, the index method only indicates whether the overall effect of the mixture departs from additivity.

1.4.2 Experimental designs

Whether or not the toxicity of a mixture needs to be characterised in terms of departure from additivity, and, if so, which method is desired for detection of such departures, has major implications for the design of a mixture study. This section describes a few widely used experimental designs with their applications and limitations.

In a whole mixture study, a given mixture is tested as a whole, the mixture may be viewed as a 'single chemical'. This approach has been recommended for complex mixtures with a poorly characterised, but stable composition (e.g. industrial effluents, municipal waste), for semicharacterised mixtures (e.g. jet fuels, diesel, gasoline), and for specially designed mixtures with completely characterised composition (Mumtaz *et al.*, 1993). Obviously, the results of whole mixture studies do not support definite conclusions on the presence or nature of possible interactions among components of the mixture, nor do they enable identification of toxic components or fractions. A whole mixture study can be conducted as an initial screening for toxic effects and followed by testing of submixtures and, eventually, individual chemicals to tease out the component(s) responsible for toxic effects or interactions. Such an approach is called a top-down approach. Conversely, the investigation can start with the toxicity of the individual components, i.e. a bottom-up approach (Yang *et al*, 1995).

A factorial design is a design in which each level (concentration) of each factor (chemical) is combined with each level (concentration) of every other factor (chemical). The number of groups in a factorial design is m^k where k is the number of chemicals and m the number of concentrations of each chemical. The simplest form of factorial design is a 2x2 design which measures the responses to the control situation (concentration zero for both chemicals), to one dose of each of two chemicals, and to the same doses of these two chemicals combined (Calabrese, 1991; Michaud *et al.*, 1994). The 2x2 design has been used in many mixture studies and can support the conclusion that the chemicals interact antagonistically. However, it does not permit definite conclusions on whether the joint action is synergistic or additive. In case the

response to the mixture is greater than that to the individual chemicals, proving synergism or additivity requires knowledge of the responses to the individual chemicals at higher dose levels.

If proper identification of interactions is required, larger factorial designs can be used since these permit estimation of the interaction parameters in a concentration-response surface model. The required number of dose levels in such designs increases with the complexity of the mathematical model, e.g. when terms for higher order interactions are included (Carter, 1995). The number of treatment groups in a full factorial design increases so rapidly with the number of chemicals that it is unfeasible when a mixture contains more than four or five chemicals, especially in case of complex and costly *in vivo* studies.

The number of groups can be limited by considering specific fractions of the complete factorial design in a so-called fractional factorial design. A good experimental illustration hereof is the fractional two-level factorial design used by Groten and colleagues (1996, 1997). They examined the toxicity of a mixture of nine chemicals (chosen because of their relevance to the general population) in a subacute rat study, using 16 carefully selected combinations (out of the 512 required for a full factorial design) and were able to identify cases of non-additivity a well as causative chemicals of effects on specific endpoints. Other efficient designs which sample only part of the mixture's response surface include ray designs and (augmented) central composite designs (Gennings, 1996; Michaud *et al*, 1994; Svendsgaard and Hertzberg, 1994).

Instead of modeling the concentration-response relationship for a mixture of chemicals to detect departures from additivity, Berenbaum (1989) and Gennings (1995, 1996) have proposed methods based on the concentration-response relationships of each individual chemical. In this 'additivity approach' the concentration-response relationships of the individual chemicals are used to calculate the expected response for a given combination, under the assumption of additivity. Then the predicted response is compared with the response for that combination observed experimentally. For a mixture of k chemicals, the number of groups required would be $(m * k) + 1$ (m levels of k chemicals + the combination) which is much less than the m^k groups required for a complete factorial design. A disadvantage of this additivity approach is that it can only show whether the effect of the whole mixture deviates from additivity, it is unable to identify the chemicals which cause the interaction.

1.5 Repeated-dose mixture toxicity studies at low dose levels

Most of the published mixture toxicity studies involve simple mixtures of only two or three chemicals and acute exposure to high, often lethal, dose levels (Yang and Rauckman, 1987; EPA, 1990; Mumtaz and Hertzberg, 1993). Though these studies have clearly demonstrated that combinations of chemicals may act synergistically, and thus raise concern about unexpected toxicity upon exposure to chemical mixtures, their use for (quantitatively) assessing health risk from the usual long-term exposure of humans to low levels of multiple chemicals is highly questionable (EPA, 1986). Unfortunately, however, toxicological data on more realistic exposure conditions are scarce. In the late 1980s, when the studies described in this thesis were initiated, only very few studies had addressed the adverse effects of repeated or long-term exposure to more than two chemicals at low dose levels. As illustrated below, the experimental approaches in these studies differed widely, both in terms of the complexity of the mixture and the exposure level or duration.

1.5.1 Sub-acute and sub-chronic studies with contaminants in the Great Lakes

For several decades now, research has been undertaken on mixtures of chemical contaminants found in the Great Lakes region of North America (Villeneuve *et al.*, 1994). This research included animal studies using simple chemical mixtures and studies in which rats were fed contaminated Great Lakes fish.

A study of the first category is the 28-day feeding study in rats which aimed to determine whether mirex-related compounds (mirex, photomirex or kepone) potentiated the toxic responses produced by halogenated biphenyls (Aroclor 1254, Aroclor 1260 or polybrominated biphenyl) and vice versa (Chu *et al.*, 1980). The dietary concentrations used were selected to be at or close to the lowest levels reported to produce toxic effects. Each chemical was given alone and in a binary mixture (each mirex-related compound was combined with each of the three biphenyls). Growth, food consumption and routine hematology/clinical chemistry parameters were not affected by any treatment. When given alone, the biphenyls induced liver enlargement, increases in hepatic microsomal mixed-function oxidases and histopathological changes in the liver and thyroid, whereas the mirex-related compounds induced changes in liver morphology only. The results of the mixture groups showed that the chemicals acted in an additive manner. Remarkably, the levels of mirex and photomirex in the livers of rats co-exposed to biphenyls were four to six times

increased compared with rats fed (photo)mirex alone, whereas the toxic response in the liver was not increased as a result of this increased tissue accumulation.

In a subsequent study, Chu and coworkers investigated the toxic effects of a mixture of 11 heavy metals and trace inorganic substances, at three dose levels, in a sub-chronic (13-week) drinking water study in rats (Chu *et al.*, 1981). At the lowest level, the concentration of each element was equal to that specified by the Great Lakes Water Quality Objectives (established to protect the most sensitive species which, in most cases, is an aquatic organism). The concentrations at the other levels were 5 and 25 times higher, respectively. For most elements, the concentrations at the 25x level exceeded those recommended to be safe levels for drinking water. None of the mixtures induced any sign of toxicity as shown by the results of routine toxicological examinations. In another sub-chronic study, mixtures of 15 persistent contaminants (mostly organochlorine pesticides) were fed to rats at levels of 1, 10, 100 and 1000 times the Great Lakes Water Quality Objectives (Côté *et al.*, 1985). No toxic effects were observed and hepatic microsomal aniline hydroxylase and aminopyrine demethylase activities in the mixture groups were within the control range. These results indicate that the water quality objectives established for single chemicals afford some measure of safety when exposure to mixtures occurs.

A limitation of the above studies is that the synthetic mixtures may not accurately reflect the qualitative or quantitative pattern of contamination that exists in the environment. As an alternative approach, laboratory rats were fed diets containing 5-20% (on a wet weight basis) contaminated Lake Ontario salmon for 4 or 13 weeks (Villeneuve *et al.*, 1981; Chu *et al.*, 1984). This way, the rats were exposed to environmentally relevant levels of the whole range of organic and inorganic contaminants which may accumulate in food chains. Control groups received diet without salmon or diet with salmon from the Pacific Ocean. In general, the contaminant levels in Pacific salmon were two orders of magnitude lower than those in Lake Ontario salmon. The results showed dose-dependent and reversible, minor histological changes in the liver and thyroid, increases in hepatic microsomal mixed function oxidase activities, and a gradual build-up of organochlorine residues in the liver upon prolonged exposure. Because the levels of polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-doxin in the contaminated fish were far below the minimum required for enzyme induction, the authors speculated that 'the enzyme induction was probably associated with the presence of a multitude of the contaminants rather than the only individual one found in Lake Ontario fish' or 'due to the presence of some unidentified contaminants'.

The above fish studies used traditional toxicity endpoints which may not be sensitive enough to detect subtle effects of constant low level exposure to environmental contaminants. On account of lessened psychomotor development observed in children of mothers who ate about 0.5 kg of Lake Michigan fish per month (and had increased levels of polychlorinated biphenyls in their cord blood), some further rat studies with contaminated Lake Ontario salmon addressed behavioral changes. Rats fed the contaminated fish showed increased reactivity to aversive events, but not to positive events (Daly *et al.*, 1989; Daly, 1991). Though some of the contaminants found in the fish and rat brain have been shown to cause similar behavioral changes, it remains unclear which chemical or combination of chemicals caused these changes in rats fed Lake Ontario salmon.

1.5.2 Lifespan studies with drinking water contaminants

Early in the 1980s, two lifespan studies in rats were conducted to investigate the toxicity of contaminants found in drinking water in the Netherlands. The first study was conducted with a mixture of eleven volatile halogenated hydrocarbons which were chosen on the basis of their occurrence in ground wells used for drinking water supply (Wester *et al.*, 1985). During 25 months, the rats received drinking water containing a mixture consisting of equal quantities of the eleven contaminants. The mixture was studied at three dose levels, the highest level being three orders of magnitude higher than found in several water wells. The results revealed no toxic or carcinogenic effects.

Whereas the mixture in the first study was chemically defined and not mutagenic, the mixture in the second study was a complex drinking water concentrate of unknown organic composition and positive in the Ames test (Kool *et al.*, 1985). The organic concentrate (in dimethylsulphoxide, prepared by XAD-4/8 concentration procedure) was given in the drinking water, at three dose levels, for nearly 25 months. The highest dose was at least 40 times higher than the expected human exposure level. Lifespan consumption of the concentrate, which represented only about 1% of the total organic material present in drinking water, did not result in an increased tumor incidence.

1.6 Scope of the work described in this thesis

In 1985, the Health Council of The Netherlands published an advisory report on the setting of health-based standards for non-carcinogenic substances (Health Council of The Netherlands, 1985). One of the issues addressed in this advisory report was the question as to whether (uncertainty about the toxicological consequences of) combined

action should be incorporated in the standards for individual substances. The Health Council concluded that there was no immediate reason to take combination effects into account at concentrations well below the standards for the individual substances. Furthermore, the Council was not aware of useful data on the effects of prolonged exposure to combinations of substances at concentrations around the experimental No-Observed-Adverse-Effect Levels (NOAELs) of the individual substances and, therefore, recommended research to fill this data gap.

A few years thereafter, the Dutch Ministry of Housing, Spatial Planning and Environment funded a research programme on combination toxicology with the general aim to obtain a better understanding of the importance of mixture toxicity, and, if possible, to obtain enough information to support some general statements that can be helpful to responsible authorities (Zorge, 1996). The research programme was started by Feron at TNO Nutrition and Food Research, and included, among others, the studies described in chapters 2-5 of this thesis. One of the major aims of the programme was to test the hypothesis that as a rule exposure to mixtures of chemicals at (low) non-toxic doses of the individual chemicals is of no health concern (Feron *et al*, 1995).

Following the Health Council's recommendation, we initially carried out a repeated-dose (4-week) oral toxicity study in rats with a combination of eight chemicals with different primary target organs (chapter 2). The objective of this study was to determine whether simultaneous administration of the chemicals at dose levels equal to the NOAEL of each of the individual chemicals would result in a NOAEL or and adverse-effect level for the combination. This study and the next studies were empirical studies intended to examine the toxicity of the mixtures as a whole. They were not designed to detect interactions between specific pairs of mixture components, to identify components responsible for specific mixture effects, or to investigate mechanisms underlying these effects.

As a sequel to this study with chemicals acting on different target organs, a 4-week repeated-dose study (chapter 3) and an acute study (chapter 4) in rats were conducted with mixtures of four chemicals with the same target organ, viz. the kidney, but presumably different modes of action. Again the objective was to examine whether the combined action of the four chemicals at dose levels equal to their individual 'No-Observed-Nephrotoxic-Effect Level' would result in an effect level.

The next study was conducted with mixtures of three or four chemicals which affect the same target organ, again the kidney, through a similar mode of action (chapter 5). The toxicity of a mixture of similarly acting systemic toxicants is commonly predicted by a model based on dose addition (EPA, 1986). Though the assumption of dose addition for such mixtures has been shown to be valid in acute, high-dose studies in

rats, its validity under conditions more relevant to human environmental exposure has been examined poorly. The major goal of our study was, therefore, to test the additivity assumption (dose addition) under conditions of concurrent, repeated exposure (32 days) at non-toxic dose levels slightly below the 'Lowest-Observed-Nephrotoxic-Effect Level' of the individual chemicals.

In chapter 6, the above studies are discussed and an overview is given of approaches to risk assessment of chemical mixtures. This chapter also contains a summary of (complementary) low-dose studies conducted by other investigators and ends with the conclusions drawn.

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Chapter 2

4-Week oral toxicity study of a combination of eight chemicals in rats: comparison with the toxicity of the individual compounds

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4-WEEK ORAL TOXICITY STUDY OF A COMBINATION OF EIGHT CHEMICALS IN RATS: COMPARISON WITH THE TOXICITY OF THE INDIVIDUAL COMPOUNDS

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Abstract—In a 4-wk oral toxicity study, 4-wk-old male and female Wistar rats were exposed to a combination of arbitrarily chosen chemicals comprising sodium metabisulphite, Mirex, Loperamide, metaldehyde, di-*n*-octyltin dichloride, stannous chloride, lysinoalanine and potassium nitrite. The dose levels used were based on the "no-observed-adverse-effect level" (NOAEL) and the "minimum-observed-adverse-effect level" (MOAEL) of the individual compounds obtained in similar studies with Wistar rats previously performed at TNO–CIVO, and comprised 0 (controls), 1/10 and 1/3 of the NOAEL, the NOAEL and the MOAEL. In comparison with the adverse effects of the individual compounds, both more severe and less severe adverse effects were observed at the MOAEL of the combined compounds, indicating interaction of effects at this exposure level. Slightly decreased haemoglobin content and slightly increased relative kidney weight were the only treatment-related adverse effects seen in the NOAEL group. In the 1/10 and 1/3 NOAEL groups no untoward effects were found that could be related to treatment. The present study clearly demonstrates absence of a simple additive effect, and provides some, but no convincing, evidence for an increased risk from exposure to a combination of chemicals when each chemical is administered at its own individual NOAEL. At lower dose levels no increased risk appears to exist. These generalizations may not be fully justifiable from a purely scientific point of view but are the most important practical lesson learnt from the present study.

INTRODUCTION

Toxicological research for the assessment of hazards associated with chemical exposure has traditionally focused on single substances in isolation. However, in reality, humans are simultaneously exposed to a multitude of chemical substances, of both natural and synthetic origin, which necessitates knowledge of the health effects of combined exposures. There is some experience of exposure to combinations, for example of drugs (Szmigielski, 1983), metals (Chmielnicka, 1983), solvents (Ikeda, 1983 and 1988) and pesticides (Kaloyanova, 1983; Kommission für Pflanzenschutz, Pflanzenbehandlungs- und Vorratsschutzmittel, 1975). Combined exposure studies encountered in the literature normally use only two or three compounds and relate to acute toxicity or to carcinogenicity. In fact, very little is known about the adverse effects of subacute or subchronic exposure to combinations of more than three chemicals at concentrations around the "no-observed-adverse-effect level" (NOAEL).

In the present study eight different chemicals, of which the subacute oral toxicity in rats had previously been studied in our laboratory, were simultaneously administered to male and female rats by the oral route for a period of 4 wk. Each compound was administered at its "minimum-observed-adverse-effect level" (MOAEL), at its NOAEL, and at one-third and one-tenth of the NOAEL. The choice of the chemicals was neither based on similar or dissimilar action or target organ nor on expected interaction or expected independent action (Plackett and Hewlett, 1948 and 1952). In fact the choice was fully arbitrary with respect to type of action, target organ and (un)expected interaction. The major criteria for selecting a chemical were: (1) the existence of freely available data on subacute oral toxicity in rats produced in our laboratory; and (2) the absence of markedly reduced food or water intake at the MOAEL. Eight chemicals were considered suitable for use in the present study.

The main purpose of this study was to determine whether simultaneous administration of the compounds at a concentration equal to the NOAEL for each of the chemicals would result in a NOAEL or an adverse-effect level for the combination. In case this level would indeed turn out to be an adverse-effect level for the combination, it would be highly relevant to know whether such interactive effects would also occur at lower dose levels. Therefore, the 1/3 and 1/10 NOAEL groups were included in this study. Finally, the MOAEL group was included to ensure the possibility of studying interactive toxic effects; such information was considered necessary for the interpretation of the results obtained at the lower dose levels.

Abbreviations: ALAT = alanine aminotransferase; ALP = alkaline phosphatase; ANOVA = analysis of variance; ASAT = aspartate aminotransferase; DOTC = di-*n*-octyltin dichloride; LAL = synthetic lysinoalanine; LSD = least significant difference; MOAEL = minimum-observed-adverse-effect level; NOAEL = no-observed-adverse-effect level.

MATERIALS AND METHODS

Source of test compounds

Nitrite (KNO_2; at least 97% pure by analysis) was obtained from E. Merck (Darmstadt, FRG),

stannous chloride ($SnCl_2 \cdot 2H_2O$; 97%) from Analar, BDH Chemicals Ltd (Poole, Dorset, UK), sodium metabisulphite ($Na_2S_2O_5$; at least 95% pure) from Brocades-acf (Maarssen, The Netherlands), Mirex from Riedel-de-Haën (Seelze-Hannover, FRG), Loperamide (Loperamide hydrochloride) from Janssen Pharmaceutica (Goirle, The Netherlands), and metaldehyde (technical grade, 99.5%) from AAgrunol (Groningen, The Netherlands). DOTC (di-*n*-octyltin dichloride; purity at least 98%) was a gift from Dr E. J. Bulten (Institute for Applied Chemistry, TNO, Utrecht, The Netherlands). Free synthetic lysinoalanine·2HCl (LAL) was synthesized in the former Organic Synthesis section of TNO–CIVO, following the directions of Okuda and Zahn (1965).

Animals and maintenance

Weanling, Wistar-derived SPF-bred male and female rats (Bor:WISW) were obtained from F. Winkelmann (Institute for the Breeding of Laboratory Animals GmbH & Co. KG, Borchen, FRG). They were housed in groups of five males or females in stainless-steel cages with wire-mesh floors in an animal room maintained at $22 \pm 2°C$, a relative humidity of at least 40%, and a 12-hr light/dark cycle. The percentage composition of the Institute's cereal-based, open formula diet, used as basal diet in this study, was as follows: soya-bean meal 11, fish meal 7, meat and bone scraps 4, wheat 36, maize 29.7, brewers' yeast 3, alfalfa meal 3, soya-bean oil 3, whey powder 2, defatted bone meal 0.4, trace mineral salt 0.5, vitamin B mixture 0.1, and vitamin ADEK mixture 0.3. To compensate for destruction of vitamin B_1 by sulphite, the basal diet was fortified with 10 mg vitamin B_1/kg diet. Test diets were prepared every 2 wk by blending the test compounds and basal diet in a Stephan cutter, and were then stored in a freezer at $-20°C$ until use. Test solutions were prepared every 2 days by dissolving potassium nitrite in tap-water, and stored in a refrigerator at 4°C.

Experimental design

Following a 6-day acclimatization period, during which basal diet and tap-water were available *ad lib.*, the rats were allocated randomly to groups of 10 males and 10 females each, such that the mean body weights were about the same in all groups (56 g for males, range 48 to 65 g; 62 g for females, range 57 to 67 g). Next, the rats were allowed free access to test diets and test solutions for 4 wk. The study included one control group, given basal diet and tap-water without test compounds, and four test groups. Rats of each test group received eight test compounds simultaneously in their drinking-water (nitrite) and feed (stannous chloride, sulphite, metaldehyde, Loperamide, Mirex, LAL and DOTC). In the highest dose group (indicated by MOAEL) all test compounds were administered at levels that had been found to induce minimal effects when administered separately. The levels given in the next lower dose group (indicated by NOAEL) were found to be NOAELs on separate administration. The levels in the two remaining test groups amounted to one-third (indicated by 1/3 NOAEL) and one-tenth (indicated by 1/10 NOAEL) of those in the NOAEL group. The concentrations of the test compounds in water and feed are presented in Table 1.

Observations and analyses

The rats were weighed weekly and observed daily for condition and behaviour. Food intake was measured weekly and fluid intake daily, on a cage basis, by weighing feeders or drinking-bottles, respectively.

Haematology and clinical chemistry. Blood samples were collected from the tip of the tail of all males on day 22 and of all females on day 23, and examined for haemoglobin and methaemoglobin concentration, packed cell volume, prothrombin time, counts of erythrocytes, reticulocytes, thrombocytes, and total and differential leucocytes. Whole blood taken from the tip of the tail from each rat after overnight fasting on day 25 was examined for glucose (Boehringer Glucoquant No. 245-178; Boehringer Mannheim GmbH, Mannheim, FRG). At autopsy (for males on day 28 and for females on day 29), blood samples collected from the abdominal aorta of all rats were centrifuged at 1250 ***g*** for 15 min and then analysed for alkaline phosphatase (ALP), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), total protein, albumin, total bilirubin, urea, creatinine, cholesterol, triglycerides, phospholipids, inorganic phosphate and calcium (Cobas-Bio Centrifugal Analyzer), chloride (Chloro Counter), and sodium and potassium (Electrolyte-2-Analyzer).

Urinalysis. In wk 4, rats were deprived of water for 24 hr and of food for 16 hr. Urine was collected for the last 16 hr of the deprivation period and its volume (calibrated tubes) and density (Bellingham and Stanley refractometer) were determined. Semi-quantitative observations in the individual urine samples included appearance (visual inspection), protein, glucose, ketones, occult blood, urobilinogen and bilirubin (Combur-7-Test strips; Boehringer

Table 1. Concentrations of test compounds in food or drinking-water for various dose groups in a 4-wk oral toxicity study of a combination of 8 chemicals in rats

Test compound	Concentration (ppm) in food or drinking-water* given to the group indicated by:			
	NOAEL/10	NOAEL/3	NOAEL	MOAEL
KNO_2	10	33	100	300
$SnCl_2 \cdot 2H_2O$	100	330	1000	3000
$Na_2S_2O_5$	500	1670	5000	20000
Metaldehyde	20	70	200	1000
Loperamide	0.5	1.7	5	25
Mirex	0.5	1.7	5	80
Lysinoalanine	3	10	30	100
DOTC	0.6	2	6	30

*Only KNO_2 was administered in the drinking-water.

Mannheim GmbH, Mannheim, FRG). The sediment of pooled samples (one sample per group) was examined microscopically for red and white blood cells, epithelial cells, amorph material, crystals, casts, bacteria, worm eggs and sperms. Urinary pH was determined (pH meter) in early-morning, 2-hr urine samples collected on day 24 from unfasted rats.

Pathology. On day 28 (males) or 29 (females) the rats were killed by exsanguination from the abdominal aorta whilst under light ether anaesthesia, and a thorough autopsy was performed. The weights of adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes, thymus and thyroid were recorded and the organ-to-body-weight ratios were calculated. Samples of these organs and of the epididymides, mesenteric lymph nodes, pancreas, prostate, seminal vesicles, stomach and small intestine were fixed in 4% neutral buffered formalin, and processed for histopathological examination. Sections were cut at 5 μm and stained with haematoxylin and eosin.

Statistical analysis

Body weights were evaluated by one-way analysis of covariance followed by Dunnett's multiple comparison tests. Water intake was evaluated by analysis of variance (ANOVA) repeated measures design. Data on food intake and food conversion efficiency were evaluated by one-way ANOVA followed by least significant difference (LSD) tests, and haematology, clinical chemistry, organ weights and urinary volume and density by one-way ANOVA followed by Dunnett's multiple comparison tests. Differential white blood cell and reticulocyte counts and the results of urinalysis, except for volume and density, were analysed by the Mann–Whitney U-test. The histopathological changes were examined by Fisher's Exact Probability Test.

RESULTS

No mortality occurred. Clinical signs of ill-health were limited to the MOAEL group, in which all male and female rats exhibited emaciation and abdominal distension from wk 2 onwards. Other signs of impaired health observed in this group included rough fur, serous or haemorrhagic discharge from the nose, encrustations around the nose, focal alopecia and red, scaly paws.

Compared with controls, rats of the MOAEL group showed growth retardation, reduced food and water consumption and lower food efficiency (Table 2). At the lower dose levels there were no significant changes in these parameters.

Haemoglobin concentration, mean corpuscular haemoglobin and prothrombin time were lower in males and females of the MOAEL group than in controls (Tables 3 and 4). Female rats of the MOAEL group also showed a lower mean corpuscular haemoglobin concentration, and higher counts of total white blood cells and lymphocytes. A slightly

Table 2. Mean values of body weight, food intake, food efficiency and water intake of rats given a combination of 8 chemicals in the food (7 chemicals) or drinking-water (1 chemical) for 4 wk

Parameter	Values for rats in the various dose groups				
	Control	NOAEL/10	NOAEL/3	NOAEL	MOAEL
			Males		
Body weight (g)					
on day 0	56 ± 1.4	56 ± 1.4	56 ± 1.5	56 ± 1.4	56 ± 1.6
7	90 ± 1.9	91 ± 2.4	92 ± 1.7	91 ± 1.7	77 ± 2.2**
14	127 ± 2.4	129 ± 3.2	132 ± 2.2	129 ± 2.1	98 ± 3.1**
21	163 ± 2.9	167 ± 3.9	171 ± 2.7	164 ± 2.7	120 ± 4.3**
28	197 ± 4.2	199 ± 4.7	203 ± 3.0	196 ± 3.9	136 ± 7.1**
Food intake (g/rat/day)					
in wk 1	12.2	12.7	13.2*	12.4	8.8***
2	16.9	17.1	17.4	16.3	10.6***
3	17.5	17.9	18.2	17.5	12.9***
4	16.0	16.3	16.4	16.2	12.4***
Food efficiency over 4 wk†	0.32	0.32	0.32	0.32	0.26**
Water intake (g/rat/day)					
over 4 wk	19.3	19.2	20.5	21.2**	17.2**
			Females		
Body weight (g)					
on day 0	62 ± 0.9	62 ± 0.8	62 ± 0.9	62 ± 0.9	62 ± 0.9
7	93 ± 1.4	92 ± 1.2	92 ± 1.2	92 ± 1.9	81 ± 1.0**
14	119 ± 1.8	119 ± 1.6	117 ± 1.9	118 ± 2.7	96 ± 1.8**
21	139 ± 2.6	138 ± 1.8	132 ± 2.1	135 ± 3.3	110 ± 2.7**
28	157 ± 2.7	154 ± 2.3	151 ± 2.2	153 ± 3.3	119 ± 4.6**
Food intake (g/rat/day)					
in wk 1	12.4	13.0	12.7	11.6	9.2**
2	14.8	15.4	14.1	14.4	11.9
3	14.4	14.9	14.5	13.7	11.4***
4	13.1	13.4	11.8	12.6	10.1**
Food efficiency over 4 wk†	0.25	0.23	0.24	0.25	0.19
Water intake (g/rat/day)					
over 4 wk	18.9	20.0	18.8	18.5	16.3

†Weight gained (g)/food consumed (g).

Body-weight values are means ± SEM for groups of ten rats. Food and water intake are the means for two cages of five animals each. Although water intake was recorded daily, only the mean over 4 wk is presented in the table. The values marked with asterisks differ significantly (body weight: Covariance–Dunnett's test; food intake and efficiency: ANOVA–LSD test; water intake: ANOVA-repeated measures design) from the control value (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Table 3. Effects on haematological and plasma biochemical parameters in male rats given a combination of 8 chemicals in the food (7 chemicals) or drinking-water (1 chemical) for 4 wk

Parameter	Values for rats of the group indicated by:				
	Control	1/10 NOAEL	1/3 NOAEL	NOAEL	MOAEL
RBC (10^{12}/litre)	6.0 ± 0.1	5.8 ± 0.1	6.0 ± 0.1	5.8 ± 0.1	6.0 ± 0.1
Hb (mmol/litre)	8.3 ± 0.1	8.3 ± 0.1	8.2 ± 0.1	8.0 ± 0.1*	7.9 ± 0.1**
PCV (litre/litre)	0.433 ± 0.004	0.427 ± 0.004	0.438 ± 0.005	0.428 ± 0.005	0.422 ± 0.004
MCV (fl)	72.7 ± 0.9	73.7 ± 0.8	72.7 ± 0.9	74.0 ± 1.0	70.0 ± 1.1
MCH (fmol)	1.40 ± 0.01	1.43 ± 0.01	1.36 ± 0.01	1.39 ± 0.01	1.32 ± 0.02**
MCHC (mmol/litre)	19.2 ± 0.1	19.3 ± 0.1	18.7 ± 0.2	18.8 ± 0.2	18.8 ± 0.2
PTT (sec)	46.1 ± 0.5	45.5 ± 0.5	46.4 ± 0.3	44.8 ± 0.5	41.3 ± 0.8**
WBC (10^9/litre)	12.7 ± 1.0	10.6 ± 0.7	11.4 ± 0.5	12.2 ± 0.8	15.2 ± 2.0
Neutrophils (10^9/litre)†	1.0 ± 0.1	1.1 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	1.3 ± 0.2
Lymphocytes (10^9/litre)†	11.6 ± 1.0	9.4 ± 0.7	10.6 ± 0.5	11.4 ± 0.8	13.7 ± 1.8
Glucose (mmol/litre)	3.1 ± 0.0	3.3 ± 0.1	3.6 ± 0.1*	3.5 ± 0.1*	3.8 ± 0.1**
ALP activity (U/litre)	334.2 ± 18.9	317.9 ± 16.0	376.6 ± 26.4	328.9 ± 13.6	266.8 ± 14.5*
ALAT activity (U/litre)	40.8 ± 1.6	40.5 ± 1.4	41.0 ± 2.2	43.0 ± 3.0	102.6 ± 26.8**
ASAT activity (U/litre)	56.8 ± 1.9	61.5 ± 1.8	58.2 ± 1.9	61.9 ± 2.1	123.9 ± 29.0**
Total protein (g/litre)	55.6 ± 0.4	56.0 ± 0.3	56.0 ± 0.6	55.9 ± 0.5	51.5 ± 0.9**
Albumin (g/litre)	33.9 ± 0.4	33.5 ± 0.3	34.2 ± 0.4	33.6 ± 0.3	28.9 ± 0.7**
Urea (mmol/litre)	5.32 ± 0.16	5.43 ± 0.28	5.20 ± 0.31	5.37 ± 0.22	5.63 ± 0.31
Creatinine (μmol/litre)	60.5 ± 1.1	60.7 ± 1.7	57.1 ± 2.1	61.4 ± 1.3	50.1 ± 1.3**
Cholesterol (mmol/litre)	2.09 ± 0.05	2.10 ± 0.05	2.13 ± 0.11	2.31 ± 0.09	2.67 ± 0.16**
Triglycerides (mmol/litre)	1.09 ± 0.18	0.98 ± 0.12	1.09 ± 0.15	0.94 ± 0.14	0.46 ± 0.04**
Phospholipids (mmol/litre)	1.60 ± 0.07	1.57 ± 0.05	1.66 ± 0.07	1.54 ± 0.07	1.93 ± 0.11*
Calcium (mmol/litre)	2.71 ± 0.02	2.73 ± 0.02	2.72 ± 0.03	2.76 ± 0.02	2.68 ± 0.04
Potassium (mmol/litre)	3.60 ± 0.05	3.75 ± 0.10	3.67 ± 0.04	3.66 ± 0.08	4.02 ± 0.09**
Sodium (mmol/litre)	140.6 ± 0.6	140.9 ± 0.6	141.4 ± 0.4	141.7 ± 0.7	139.0 ± 0.5
Chloride (mmol/litre)	100.3 ± 0.5	100.9 ± 0.8	101.5 ± 0.6	101.3 ± 0.5	98.2 ± 0.6
Inorganic phosphate (mmol/litre)	2.56 ± 0.03	2.64 ± 0.04	2.70 ± 0.04	2.78 ± 0.03	2.60 ± 0.10

RBC = red blood cell count Hb = haemoglobin concentration PCV = packed-cell volume MCV = mean corpuscular volume MCH = mean corpuscular haemoglobin MCHC = mean corpuscular haemoglobin concentration PTT = prothrombin time WBC = white blood cell count

†Neutrophils and lymphocytes: calculated from total and differential white blood cell counts.

The values are means ± SEM for groups of ten rats. The values marked with asterisks differ significantly (ANOVA–Dunnett's test) from the control value (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). All other haematological and plasma biochemical parameters measured showed no differences from the controls.

Table 4. Effects on haematological and plasma biochemical parameters in female rats given a combination of 8 chemicals in the food (7 chemicals) or drinking-water (1 chemical) for 4 wk

Parameter	Values for rats of the group indicated by:				
	Control	1/10 NOAEL	1/3 NOAEL	NOAEL	MOAEL
RBC (10^{12}/litre)	6.1 ± 0.1	6.0 ± 0.1	6.2 ± 0.1	6.0 ± 0.1	6.2 ± 0.1
Hb (mmol/litre)	8.6 ± 0.1	8.6 ± 0.0	8.7 ± 0.1	8.5 ± 0.1	8.1 ± 0.1**
PCV (litre/litre)	0.447 ± 0.004	0.448 ± 0.004	0.452 ± 0.005	0.444 ± 0.006	0.435 ± 0.005
MCV (fl)	73.6 ± 0.6	75.0 ± 1.0	72.8 ± 1.2	73.8 ± 1.4	70.5 ± 0.9
MCH (fmol)	1.42 ± 0.01	1.44 ± 0.02	1.40 ± 0.02	1.42 ± 0.02	1.31 ± 0.02**
MCHC (mmol/litre)	19.3 ± 0.1	19.1 ± 0.2	19.3 ± 0.1	19.2 ± 0.2	18.6 ± 0.2*
PTT (sec)	42.2 ± 0.9	40.9 ± 1.0	42.7 ± 0.4	40.2 ± 0.5	38.9 ± 0.8*
WBC (10^9/litre)	9.6 ± 0.4	9.7 ± 0.4	9.7 ± 0.7	8.8 ± 0.4	13.6 ± 0.5**
Neutrophils (10^9/litre)†	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.1
Lymphocytes (10^9/litre)†	8.7 ± 0.4	8.9 ± 0.4	9.0 ± 0.6	8.0 ± 0.3	12.6 ± 0.5**
Glucose (mmol/litre)	4.0 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	4.0 ± 0.1	3.8 ± 0.1
ALP activity (U/litre)	244.3 ± 10.7	206.6 ± 9.1*	247.2 ± 14.5	206.3 ± 8.8*	188.3 ± 7.4**
ALAT activity (U/litre)	41.7 ± 1.2	40.8 ± 1.6	36.7 ± 1.8	33.6 ± 1.2*	46.8 ± 3.2
ASAT activity (U/litre)	60.2 ± 1.8	58.2 ± 1.7	58.3 ± 1.9	55.0 ± 1.5	72.3 ± 4.5**
Total protein (g/litre)	56.4 ± 0.7	55.7 ± 0.3	55.6 ± 0.7	56.8 ± 0.6	53.0 ± 0.8**
Albumin (g/litre)	36.4 ± 0.3	36.3 ± 0.4	36.7 ± 0.7	36.5 ± 0.4	31.2 ± 0.8**
Urea (mmol/litre)	5.86 ± 0.25	6.56 ± 0.35	6.72 ± 0.51	5.99 ± 0.37	6.31 ± 0.46
Creatinine (μmol/litre)	55.1 ± 1.4	55.8 ± 1.8	53.3 ± 1.3	55.5 ± 2.0	48.7 ± 1.3*
Cholesterol (mmol/litre)	1.88 ± 0.08	1.96 ± 0.06	1.77 ± 0.03	2.05 ± 0.07	3.19 ± 0.23**
Triglycerides (mmol/litre)	0.70 ± 0.07	0.79 ± 0.08	0.58 ± 0.04	0.75 ± 0.10	0.49 ± 0.04
Phospholipids (mmol/litre)	1.64 ± 0.05	1.72 ± 0.04	1.55 ± 0.04	1.68 ± 0.04	2.42 ± 0.08**
Calcium (mmol/litre)	2.60 ± 0.02	2.62 ± 0.02	2.57 ± 0.03	2.66 ± 0.02	2.72 ± 0.02**
Potassium (mmol/litre)	3.59 ± 0.08	3.59 ± 0.15	3.36 ± 0.09	3.59 ± 0.08	3.93 ± 0.09
Sodium (mmol/litre)	144.2 ± 0.5	142.5 ± 0.5	143.2 ± 0.5	143.9 ± 0.5	143.3 ± 0.8
Chloride (mmol/litre)	101.6 ± 0.4	101.5 ± 0.4	102.2 ± 0.4	101.5 ± 0.4	98.8 ± 0.4**
Inorganic phosphate (mmol/litre)	2.44 ± 0.10	2.36 ± 0.06	2.32 ± 0.08	2.35 ± 0.05	2.39 ± 0.08

RBC = red blood cell count Hb = haemoglobin concentration PCV = packed-cell volume MCV = mean corpuscular volume MCH = mean corpuscular haemoglobin MCHC = mean corpuscular haemoglobin concentration PTT = prothrombin time WBC = white blood cell count

†Neutrophils and lymphocytes: calculated from total and differential white blood cell counts.

The values are means ± SEM for groups of ten (haematology and glucose) or nine (biochemistry) rats.

The values marked with asterisks differ significantly (ANOVA–Dunnett's test) from the control value (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). All other haematological and plasma biochemical parameters measured showed no differences from the controls.

decreased haemoglobin concentration in males of the NOAEL group was the only change observed at lower dose levels.

Urinalysis revealed a decreased pH in males and females of the MOAEL group (males: control 6.24, MOAEL 5.89; females: control 6.56, MOAEL 6.02; $P < 0.02$), and brownish-yellow urine in males of this group. Other semi-quantitative observations and urinary volume and density did not show differences among the various groups.

The results of clinical chemistry are given in Tables 3 and 4. In the MOAEL group, many statistically significant differences from control values were observed. They occurred in both sexes, unless indicated otherwise, and included decreased values for the activity of ALP and the plasma concentrations of total protein, albumin, creatinine, triglycerides, and chloride (females only), and increased values for blood glucose (males only), the activities of ALAT (males only) and ASAT, and the plasma concentrations of cholesterol, phospholipids, calcium (females only) and potassium (males only). In the lower dose groups only a few changes were noted—decreased ALP activity in females of the NOAEL and 1/10 NOAEL groups, decreased ALAT activity in females of the NOAEL group, and increased concentration of blood glucose in males of the NOAEL and 1/3 NOAEL groups. The changes in blood glucose are believed to be unrelated to treatment, because there was no clear dose-effect relationship or (microscopic) evidence of adverse effects on liver or pancreas, organs involved in regulating blood glucose in fasted rats.

With a few exceptions, the absolute organ weights were decreased in rats of the MOAEL group compared with those of the controls. The exceptions were increased absolute liver weight in rats of both sexes, and unchanged absolute weights of the adrenals (in males only), brain and thyroid. The organ-to-body-weight ratios are shown in Table 5. The relative weights of the testes, thyroid, adrenals (in males only), kidneys (in males only), brain and liver were statistically significantly higher in rats of the MOAEL group than in controls, whereas the relative weights of the ovaries, thymus (in males only) and spleen (in males only) were significantly lower. Moreover, relative kidney weights were increased in males of the NOAEL group and in females of the 1/3 NOAEL group.

Gross examination at autopsy revealed a pronounced limiting ridge of the stomach in four males and two females of the MOAEL group, and swollen and/or dark livers in five male and seven female rats of the MOAEL group. These liver changes were also seen in three males of the NOAEL group. Treatment-related histopathological changes were observed in the MOAEL group only (Table 6). In the livers of all rats of this group hepatocellular swelling accompanied by vacuolation and/or single-cell necrosis was observed. The forestomach of five males and six females showed epithelial hyperplasia accompanied by hyperkeratosis of the limiting ridge. Lymphoid depletion occurred in the thymus of seven male rats. The number of corpora lutea was decreased in six out of ten females, while the epididymides of six out of ten males showed accumulation of a few to several multinucleated giant cells. Although special attention was paid to the adrenals, kidneys, pancreas, prostate, spleen and small intestine, which are target organs for one or more of the test compounds, no morphological changes were detected that could be related to treatment.

DISCUSSION

To facilitate the evaluation of the results of the present study, the data of this study as well as those of the studies with the individual chemicals have been summarized in Table 7.

As expected, a wide range of adverse effects was found at the MOAEL. In view of the toxicity data of the individual compounds we indeed expected to see growth retardation, reduced food and water intake, reduced food efficiency, decreased haemoglobin and

Table 5. Relative organ weights of rats given a combination of 8 chemicals in the food (7 chemicals) or drinking-water (1 chemical) for 4 wk

Organ	Relative organ weights (g/kg body weight) in the group indicated by:				
	Control	1/10 NOAEL	1/3 NOAEL	NOAEL	MOAEL
			Males		
Testes	12.44 ± 0.28	12.64 ± 0.29	12.16 ± 0.27	12.94 ± 0.29	15.82 ± 1.12**
Thyroid	0.084 ± 0.003	0.089 ± 0.004	0.090 ± 0.004	0.092 ± 0.006	0.121 ± 0.006**
Adrenals	0.174 ± 0.008	0.190 ± 0.006	0.192 ± 0.007	0.193 ± 0.004	0.254 ± 0.012**
Kidneys	7.31 ± 0.16	7.51 ± 0.13	7.38 ± 0.10	7.86 ± 0.12*	8.39 ± 0.20**
Thymus	2.93 ± 0.19	2.56 ± 0.18	2.70 ± 0.15	2.96 ± 0.12	1.83 ± 0.21**
Brain	8.54 ± 0.21	8.48 ± 0.19	8.37 ± 0.09	9.00 ± 0.19	11.98 ± 0.52**
Spleen	2.42 ± 0.10	2.35 ± 0.09	2.41 ± 0.07	2.30 ± 0.15	1.97 ± 0.11*
Heart	3.73 ± 0.08	3.96 ± 0.06	3.78 ± 0.09	3.87 ± 0.10	3.72 ± 0.11
Liver	46.2 ± 1.0	46.4 ± 0.8	48.6 ± 0.6	50.8 ± 1.1	84.8 ± 2.3**
			Females		
Ovaries	0.456 ± 0.020	0.425 ± 0.020	0.441 ± 0.031	0.453 ± 0.018	0.327 ± 0.020**
Thyroid	0.111 ± 0.004	0.108 ± 0.006	0.108 ± 0.006	0.111 ± 0.005	0.151 ± 0.007**
Adrenals	0.291 ± 0.032	0.319 ± 0.013	0.335 ± 0.013	0.327 ± 0.013	0.294 ± 0.007
Kidneys	7.81 ± 0.13	7.90 ± 0.20	8.42 ± 0.18*	7.76 ± 0.15	8.39 ± 0.15
Thymus	2.87 ± 0.10	3.00 ± 0.10	2.89 ± 0.06	2.78 ± 0.09	2.53 ± 0.17
Brain	10.03 ± 0.14	10.42 ± 0.17	10.58 ± 0.31	10.35 ± 0.22	13.17 ± 0.35**
Spleen	2.43 ± 0.08	2.38 ± 0.09	2.22 ± 0.09	2.37 ± 0.08	2.31 ± 0.05
Heart	4.19 ± 0.09	4.21 ± 0.12	4.21 ± 0.08	4.08 ± 0.09	4.16 ± 0.12
Liver	43.9 ± 1.0	45.2 ± 0.9	43.8 ± 0.4	46.3 ± 0.8	81.6 ± 2.1**

The values are means ± SEM for groups of ten rats. Those marked with asterisks differ significantly (ANOVA–Dunnett's test) from the corresponding control weights (*$P < 0.05$; **$P < 0.01$).

Table 6. Histopathological changes in rats given a combination of 8 chemicals in the food (7 chemicals) or drinking-water (1 chemical) for 4 wk

Type of change	No. of rats showing the change in the various groups (10 rats examined/group)									
	Control	NOAEL/10	NOAEL/3	NOAEL	MOAEL	Control	NOAEL/10	NOAEL/3	NOAEL	MOAEL
			Males					**Females**		
Epididymides										
Single multinucleate giant cell	4	0	0	0	2					
Few multinucleate giant cells	1	1	0	0	4					
Several multinucleate giant cells	0	0	0	0	2					
Spermatogenic reduction	0	0	0	0	1					
Liver										
Centrilobular swollen hepatocytes										
Very slight	0	0	0	0	1	0	0	0	0	0
Slight	0	0	0	0	0	0	0	0	0	4
Moderate	0	0	0	0	9**	0	0	0	0	6*
Hepatocellular vacuolation										
Very slight	0	0	0	0	0	0	0	0	0	1
Slight	0	0	0	0	3	0	0	0	0	4
Moderate	3	0	0	0	0	0	0	0	0	0
Single cell necrosis										
Very slight	0	0	0	1	2	0	0	0	0	1
Slight	0	0	0	0	3	0	0	0	0	7**
Moderate	0	0	0	0	2	0	0	0	0	0
Ovaries										
Loss of corpora lutea										
Moderate						0	0	0	0	1
Severe						0	0	0	0	2
Very severe						0	0	0	0	3
Stomach (limiting ridge)										
(Papillary) epithelial hyperplasia	0	0	0	0	5*	0	0	0	0	6*
Hyperkeratosis										
Slight	1	0	0	2	4	0	0	0	0	4
Moderate	0	0	0	0	1	0	0	0	0	2
Thymus										
Lymphoid depletion	0	0	0	0	7**	0	0	0	0	0

Values marked with asterisks differ significantly (Fisher's Exact Test) from the controls (*$P < 0.05$; **$P < 0.01$).
All other organs examined showed no treatment-related changes.

Table 7. Summary of changes observed on exposure to 8 chemicals separately and in combination

	Changes found on exposure to the individual compounds																			Changes found on combined exposure			
	KNO_2			$SnCl_2$		$Na_2S_2O_5$		Metaldehyde		Loperamide			Mirex		Lysinoalanine		DOTC						
Parameter dose (ppm)	MOAEL 300	1000	3000	MOAEL 3000	10000	MOAEL 20000	60000	MOAEL 1000	2500	MOAEL 25	100	400	MOAEL 80	320	MOAEL 100	1000	MOAEL 30	150	MOAEL	NOAEL	1/3 NOAEL	1/10 NOAEL
Body weight	0	0	–	–	– –	0	–	0	0	–	–	– –	–	– – –	0	–	0	–	– –	0	0	0
Food intake	0	0	0	–	– –	0	–	–	–	–	–	– –	–	– – –	0	–	0	–	– –	0	0	0
Food efficiency	0	0	0	0	–	0	–	0	0	0	0	–	–	– – –	0	–	0	–	–	0	0	0
Water intake	0	0	– –							– f	–	– –							–	0	0	0
Haematology																						
Red blood cells	0	–	– –	0	0	–	– –	0	0	0	0	+	0	+	0	0	0	0	0	0	0	0
Haemoglobin	0	–	– m	–	– –	–	– –	0	0	0	0	0	0	+ m	0	0	0	0	–	– m	0	0
MCV	0	0	+ f			0	–	0	0	0	0	–	– m	– –	0	0	0	0	0	0	0	0
MCH	0	0	+ f			0	–	0	0	0	0	– m	–	– –	0	0	0	0	–	0	0	0
MCHC	0	0	0			0	–	0	0	0	0	0	0	0	0	0	0	0	– f	0	0	0
Prothrombin time	0	– f	0																–	0	0	0
White blood cells	0	0	0	0	0	0	+	0	0	0	0	0	0	+	0	0	0	–	+ f	0	0	0
Clinical chemistry																						
Glucose	0	0	0					0	0	0	+	+	0	0			0	– f	+ m	+ m	+ m	0
ALP	0	0	– f	–				0	– f	0	0	+	– f	– m	0	0	+	+ +	–	– f	0	– f
GPT (ALAT)	0	0	0	0				0	– f	0	0	+	0	0	0	0	0	– m	+ m	– f	0	0
GOT (ASAT)	0	0	0	0				0	0	0	0	+	0	+ f	0	0	0	– m	+	0	0	0
Total protein	0	0	0					0	0	0	0	–	0	– –			0	–	–	0	0	0
Albumin	0	0	0					0	0	0	0	0	– f	– –			0	–	–	0	0	0
Urea	0	0	+ m					0	0	0	0	0	+	+ +			+ f	+	0	0	0	0
Creatinine	0	0	0					0	0	0	0	–	0	0					–	0	0	0
Urinalysis																						
pH															0	0	0	0	–	0	0	0
Appearance															0	0	0	0	+	0	0	0
Relative organ weights																						
Testes	0	0	0					0	0	0	0	0	+	0			0	0	+	0	0	0
Ovaries	0	0	0					0	0	0	0	0	0	–			0	0	–	0	0	0
Thyroid	0	0	0					0	0	0	0	0	+ m	+ m			0	+ f	+	0	0	0
Adrenals	0	0	0					0	+ f	0	0	+ m	0	+ m			0	+ m	+ m	0	0	0
Kidneys	0	0	+	0	0	0	0	0	0	0	0	0	0	+	0	0	0	+ f	+ m	+ m	+ f	0
Thymus	0	0	0					0	0	0	0	0	0	– m					– –	– – –	– m	0	0
Brain	0	0	+ m							0	+ f	+ +					0	+ +	+ +	0	0	0
Spleen	0	0	+ f	0	0	0	+ +	0	0	0	0	0	0	0	0	0	0	0	– m	0	0	0
Liver	0	0	0	0	0	0	0	+ m	+ m	0	0	– m	+ +	+ + +	0	0	0	+	+ +	0	0	0
Morphology																						
Adrenals	+	+	+ +					0	0	0	0	0	0	0	0	0	0	0	0			
Epididymides	0	0	0							0	+	+ +			0	0	0	0	+	0	0	0
Kidneys	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+ +	0	0	0			
Liver	0	0	0	+	+ +	0	+	0	0	0	+ f	+	+	+ +	0	0	0	0	+ +	0	0	0
Ovaries	0	0	0							0	0	–	0	+			0	0	–	0	0	0
Pancreas	0	0	0	+											0	0	0	0	0			
Prostate	0	0	0							0	0	+			0	0	0	0	0			
Seminal vesicles										0	+	+ +					0	0	0			
Small intestine	0	0	0	+															0			
Spleen	0	0	0	0	0	0	+	0	0	0	0	0	0	–	0	0	0	–	0			
Stomach	0	0	0			+	+								0	0	0	0	+	0	0	0
Thymus	0	0	0							0	0	0	0	+	0	0	+	+ +	+ m	0	0	0

0 = not affected + = slightly increased/affected – = slightly decreased/affected blank = not measured + + = moderately increased/affected – – = moderately decreased/affected m/f = observed in males/females only + + + = markedly increased/affected – – – = markedly decreased/affected

albumin content, increased relative testes and thyroid weights, increased liver weights, swollen and vacuolated hepatocytes, hyperplasia and hyperkeratosis of the forestomach, and reduced weight and lymphoid depletion of the thymus. However, several effects, for example growth retardation, reduced food intake and liver damage, were more severe than seen with the individual compounds. Moreover, the clinical signs of impaired health observed in the present study were absent or less pronounced with the individual compounds, indicating a rather severe effect on the general health condition of the animals at the MOAEL of the combination. On the other hand, changes in weight and morphology of the thymus were less pronounced on combined exposure than after exposure to DOTC alone. A large number of adverse effects seen at the MOAEL such as decreased prothrombin time, clearly increased ALAT and ASAT activity in blood plasma, increased kidney weight, reduced number of corpora lutea in ovaries, and increased numbers of multinucleated giant cells in the epididymides had not been seen at all or had been encountered at levels higher than the MOAEL of the individual chemicals. Although the toxicological relevance of some of the alterations is questionable because they may be related to the rather severe growth retardation, and several of the affected parameters had not been included in each of the studies with the individual compounds, these findings do suggest some kind of interaction resulting in a slightly more severe and maybe broader range of toxic response.

In terms of human health risk, interactions leading to increased toxicity are more relevant than those resulting in decreased toxicity. However, from a scientific point of view, it is of interest to note that several adverse effects such as decreased number of red blood cells, increased urea blood levels, nephrocytomegaly, hypertrophy of the zona glomerulosa of the adrenals, necrosis of pancreatic acinar cells, and swollen intestinal epithelium cells seen at the MOAEL on exposure to individual compounds were not observed at all at the MOAEL of the combination. The absence of these effects in the MOAEL group indeed may indicate decreased toxicity of some of the compounds.

Though comparison of the results of the present study with those previously obtained with the individual compounds is hampered by slight dissimilarities in experimental conditions, it seems fully justifiable to conclude that the simultaneous administration of the eight chemicals in effective (toxic) doses resulted in positive (more severe) and negative (less severe) interactive effects. The fact that these chemicals were arbitrarily chosen with respect to mechanism of action or target organ strengthens the general validity of this conclusion.

In contrast to the great number of adverse effects and conspicuous interactions occurring in the MOAEL group, only a few minor changes were found in the NOAEL group (in fact the group receiving the combination of chemicals at their individual NOAEL). These changes included decreased haemoglobin content in males, decreased ALP and ALAT activities in blood plasma in females, and increased relative kidney weights in males. The decreases in ALAT and ALP activities are considered to be chance findings unrelated to treatment; the former because higher dosed females in the MOAEL group showed normal ALAT activities, and the latter because a similarly low activity was also seen in the 1/10 NOAEL group, whereas the ALP value for the 1/3 NOAEL group was comparable with (in fact even slightly higher than) the value of the controls. This leaves two adverse effects (decreased haemoglobin content and increased relative kidney weight) in the NOAEL group which most probably are treatment-related, but are of a slight degree and occurred in one sex only. Obviously the simultaneous administration of eight chemicals at their NOAEL produced a MOAEL (the observed adverse effects indeed being only minimal) for the combination allowing the conclusion that the present study provided some, but no convincing, evidence of increased risk from exposure to a combination of chemicals when each chemical was administered at its individual NOAEL. The present study also demonstrated absence of a simple additive effect.

At the lower dose levels only three of the many parameters measured differed in a statistically significant manner from the respective control values, that is, blood glucose (increased in males), ALP activity (decreased in females) and relative kidney weight (increased in females); the changes in these parameters are considered isolated findings of no toxicological relevance. Clearly, at the dose levels below the NOAEL there was no indication of increased toxicity of the combination of chemicals in comparison with the toxicity of the individual compounds.

Although the number of combinations of chemicals is nearly infinite and generalizations may be unjustifiable from a purely scientific point of view, by far the most important practical lesson that can be learnt from the present study is that in comparison with exposure to single compounds, exposure to a combination of compounds does not constitute an evidently increased risk provided the exposure level of each individual compound of the combination is a NOAEL. Although there was some evidence of interactive effects, a distinct additive effect was not observed at the NOAEL, and certainly no synergistic effect. Since in practice the levels of (combinations of) compounds to which humans are exposed are generally much lower than the experimentally obtained NOAELs, the possibility of interactive effects of combinations seems to be low (Groupement International des Associations Nationales de Fabricants de Produits Agrochimiques, 1988). On the other hand, it should be realized that what is valid for one combination of chemicals may not necessarily be true for other combinations. Exposure to a combination of compounds with the same target organ and the same or a very similar mechanism of action may cause additive (Könemann, 1980 and 1981) or synergistic (Reuzel *et al.*, 1990) effects. However, also with combinations of chemicals with the same target organ or similar mechanism of action the chance of additive or synergistic effects will most probably decrease with decreasing exposure levels. To examine possible interactions with this type of combination, studies with a combination of nephrotoxic compounds, similar in design and conduct to those presently reported, have

recently been completed and will be the subject of a subsequent paper.

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Chapter 3

Subacute (4-wk) oral toxicity of a combination of four nephrotoxins in rats: comparison with the toxicity of the individual compounds

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SUBACUTE (4-WK) ORAL TOXICITY OF A COMBINATION OF FOUR NEPHROTOXINS IN RATS: COMPARISON WITH THE TOXICITY OF THE INDIVIDUAL COMPOUNDS

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Abstract—In a 4-wk study, 10-wk-old Wistar rats were fed the nephrotoxins hexachloro-1,3-butadiene (HCBD), mercuric chloride, *d*-limonene and lysinoalanine either alone or in combination. These nephrotoxins damage epithelial cells of the proximal tubules, but by different mechanisms. Each chemical was given alone at a Minimum-Nephrotoxic-Effect Level (MNEL), and at a No-Nephrotoxic-Effect Level (NNEL). The combination was given at the MNEL, the NNEL and one-quarter of the NNEL of the individual chemicals. The individual nephrotoxins caused slight growth depression in males at the MNEL, but not at the NNEL, whereas the combination depressed growth slightly at the NNEL and severely at the MNEL. In females at the MNEL, only HCBD retarded growth; in contrast to the effect in males this was not aggravated by combined treatment. Nephrotoxicity was more severe in males fed the combination than in males given the nephrotoxins alone. The former showed decreased renal concentrating ability and moderate histopathological changes in the kidneys at the MNEL, and a dose-dependent increase in kidney weight and number of epithelial cells in the urine at the NNEL and the MNEL. The males treated with a single agent showed slightly increased kidney weights, and/or slight histopathological changes in the kidneys at the MNEL, and (with *d*-limonene only) epithelial cells in the urine at the NNEL and MNEL. In females, renal changes induced by the combination were not more severe than those observed with individual compounds. No adverse changes attributable to treatment were observed in rats fed the combination at one-quarter of the NNEL. In the present study, combined exposure to four nephrotoxins at their individual NNEL did not constitute an obviously increased hazard, indicating absence of synergistic interaction, whereas at the MNEL clearly enhanced (renal) toxicity occurred in males, although not in females.

INTRODUCTION

In evaluating health hazards from exposure to chemicals, compounds have traditionally been considered on an individual basis (Henderson and Schlesinger, 1989). However, in the human real world most chemical exposures are to mixtures rather than single agents, raising the possibility of toxicological interactions. Concern about such interactions is evidenced by, for example, the report of the National Research Council (1988) on this topic, and by the extensive research programme that has been in progress since 1987 at the National Toxicology Program in the National Institute of Environmental Health Sciences (Yang *et al.*, 1989; Yang and Rauckman, 1987). Reported toxicological studies on combinations, for example of drugs (Szmigielski, 1983), metals (Chmielnicka, 1983), solvents (Ikeda, 1983 and 1988) and pesticides (Kaloyanova, 1983; Kommission für Pflanzenschutz, Pflanzenbehandlungs- und Vorratsschutzmittel, 1975), mostly involved only two or three compounds, in general with the emphasis on acute toxicity (Ikeda, 1988; Yang and Rauckman, 1987), or related to carcinogenicity. There is clearly a dearth of experimental information on prolonged, repeated exposure to combinations of more than three chemicals. Moreover, emphasis should be placed on interactions at concentrations that are non-toxic individually. The importance of research into this type of combined exposure, which closely reflects the actual human exposure situation, is stressed by the well known lethal interaction of chlordecone and carbon tetrachloride at non-toxic doses (Mehendale, 1989).

In our laboratory, research regarding the toxicology of combinations was started with a 4-wk oral toxicity study in which Wistar rats received a combination of eight chemicals that exert their toxic effects on various organ systems (Jonker *et al.*, 1990). The chemicals were incorporated in the feed and drinking water at four levels, namely at the 'Minimum-Observed-Adverse-Effect Level' (MOAEL), the 'No-Observed-Adverse-Effect Level' (NOAEL) and at one-third and one-tenth of the NOAEL of the individual compounds. The results of this study provided some, but no convincing, evidence

of increased toxicity upon combined exposure at the individual NOAELs, and demonstrated the absence of an increased toxicity at lower levels. Furthermore, clear additivity was not observed at the NOAEL, let alone synergism.

As a sequel to this study with chemicals acting on different target organs, 4-wk feeding studies were conducted to examine the toxicity of a combination of chemicals with the same target organ. As humans may be exposed to nephrotoxic agents, for example during drug treatment or in industrial or domestic settings where heavy metals or organic solvents are used, it was decided to investigate a combination of nephrotoxins. Suitable nephrotoxins should damage cells of the proximal tubule, the part of the nephron that is the target of a majority of the known nephrotoxins (Commandeur and Vermeulen, 1990), in a 4-wk feeding study, without concurrently producing severe depression of food intake or effects on other organs that might obscure the renal effects. Additionally, in this study compounds were chosen which, although all acting on tubular epithelial cells, do so by way of different mechanisms. Nephrotoxicity induced by hexachloro-1,3-butadiene (HCBD) has been shown to result from initial conjugation to glutathione (e.g. Dekant *et al.*, 1990). The glutathione conjugate is converted into a cysteine conjugate, catalysed by γ-glutamyl transpeptidase, which in turn is bioactivated by the cysteine conjugate β-lyase present in cells of the renal proximal tubules. *d*-Limonene and, more specifically, its metabolite *d*-limonene-1,2-dioxide, associate, with the male rat-specific protein $\alpha_{2\mu}$-globulin, and this interaction may be responsible for the excessive accumulation of $\alpha_{2\mu}$-globulin in kidneys of male rats given *d*-limonene (Lehman-McKeeman *et al.*, 1989). When exposure to *d*-limonene continues, this primary response is followed by degeneration and necrosis of individual cells lining the P_2 segment of the proximal tubule, the formation of granular casts at the junction of the thin loop of Henle and the P_3 segment of the proximal tubule, and regeneration of proximal tubule epithelium (Swenberg *et al.*, 1989; Webb *et al.*, 1989). The processes involved in renal toxicity induced by mercuric chloride are still poorly understood. Proposed mechanisms are mitochondrial dysfunctioning due to ischaemia mediated by angiotensin–renin or inhibition of enzymes by direct binding to sulphhydryl groups (Commandeur and Vermeulen, 1990). In addition, the mechanisms underlying the nephrocytomegaly induced by lysinoalanine have yet to be elucidated, although it has been suggested that lysinoalanine may act as a metal ion chelator and, through this property, inactivate metalloenzymes (Pearce and Friedman, 1988). Other factors in the nephrotoxicity of lysinoalanine may be its incorporation into protein, which may result in the formation of proteins with compromised function, or its action as an inhibitor of lysyl-tRNA-synthetase and thereby of the synthesis of normal proteins (Lifsey *et al.*, 1988).

In a subsequent study the effect of a combination of chemicals with the same mechanism of action will be investigated.

The purpose of the study reported in this paper was to find out whether simultaneous administration of four nephrotoxic chemicals at a concentration equal to their individual 'No-Nephrotoxic-Effect Level' (NNEL) would result in an effect level for the combination. Should it transpire that combined NNELs do, indeed, induce (nephro)toxic effects, it would be highly relevant to know whether such interactive effects occur also at lower dose levels; the one-quarter NNEL group was therefore included in this study. Finally, a group fed the four toxins at their individual 'Minimum-Nephrotoxic-Effect Level' (MNEL) was included to ensure that interactive toxic effects could be studied; such information was considered necessary for the interpretation of the results obtained at the lower dose levels.

MATERIALS AND METHODS

Test compounds

Hexachloro-1,3-butadiene (HCBD, at least 98% pure) and mercuric chloride ($HgCl_2$, at least 99.5% pure) were obtained from E. Merck (Darmstadt, Germany), and *d*-limonene from Sigma Chemical Co. (St Louis, MO, USA). Free synthetic lysinoalanine·2HCl was synthesized in the former Organic Synthesis section of our Institute, following the directions of Okuda and Zahn (1965).

Animals and maintenance

Weanling or adult Wistar-derived SPF-bred rats (Bor:WISW or Crl:WI(WU)BR) were obtained from F. Winkelmann (Institute for the Breeding of Laboratory Animals GmbH & Co. KG, Borchen, Germany) (range-finding studies) or from Charles River Wiga GmbH (Sulzfeld, Germany) (main study). They were housed conventionally, in stainless-steel cages with wire-mesh floor and front, five rats per cage, in a room maintained at 22 ± 2°C, a relative humidity of at least 40%, and a 12-hr light/dark cycle. Feed and tap water were available *ad lib.*, except during urine collection when they were deprived of water for 24 hr and of food for 16 hr. During the last 16 hr of deprivation, the animals were kept individually in stainless-steel metabolism cages for urine collection.

Diets

The percentage composition of the Institute's cereal-based open-formula diet, used as basal diet in this study, was as follows: soya bean meal 11, fish meal 7, meat and bone scraps 4, wheat 36, maize 29.7, brewer's yeast 3, alfalfa meal 3, soyabean oil 3, whey powder 2, defatted bone meal 0.4, trace mineral salt

0.5, vitamin B mixture 0.1, and vitamin ADEK mixture 0.3. Test diets were prepared by blending the test compounds and basal diet in a Stephen cutter, and were then stored at 4°C until use. The prepared amounts were sufficient for 2 wk (diets containing HCBD or *d*-limonene) or for 4 wk. During the main study, the feed in the animal room was refreshed daily.

Experiments

Range-finding studies. Following a 6-day acclimatization period, the 4-wk-old rats were allocated randomly to groups of 10 (controls) or 5 rats/sex, in such a way that the mean body weights were about the same in all groups. Controls were kept on the basal diet and the other groups received diets containing the individual nephrotoxins for 4 wk. Each nephrotoxin was administered at three levels (Table 1). The lowest dose level should be without evidence of kidney damage production, whereas one or both of the higher doses should produce renal adverse effects within 4 wk without seriously depressing food intake or growth. In addition, 10-wk-old males were distributed over two groups of five rats each, comprising one control group kept on the basal diet and one group kept on a diet containing 4000 ppm *d*-limonene for 4 wk. Lysinoalanine was not included in the range-finding studies described here because information on this compound was available from studies conducted previously in our laboratory.

Main study. In the range-finding study *d*-limonene appeared to induce obvious renal changes in adult male rats but not in young rats; adult rats, about 10 wk old at the start of treatment, were used, therefore, in the main study. The animals were randomly assigned to one of 12 groups, each containing 10 (controls and groups given the combination) or five (groups given the individual nephrotoxins) rats/sex. After an acclimatization period of 10 days (males) or 13 days (females), the rats were placed on the test diets or the control (basal) diet for 4 wk. The dose levels used are given in Table 2. Each compound was given alone at two different dose levels, selected on the basis of the results of the range-finding studies, namely at a level intended to be a NNEL and at a MNEL. Three additional groups received the four nephrotoxins simultaneously, at their individual MNEL, NNEL or one-quarter of the NNEL, respectively.

Table 1. Dose levels used in 4-wk range-finding studies with various nephrotoxins in rats

	Dose (ppm in the diet)		
Nephrotoxin	Low	Mid	Top
Mercuric chloride	75	150	300
Hexachloro-1,3-butadiene	25	100	400
d-Limonene	250	1000	4000

Table 2. Dose levels used in a 4-wk feeding study to examine the effects of combined exposure to four nephrotoxins in rats

Nephrotoxin	NNEL/4	No-Nephrotoxic-Effect Level (NNEL)	Minimum-Nephrotoxic-Effect Level (MNEL)
		ppm in the diet	
Lysinoalanine	7.5	30	240
Mercuric chloride	3.75	15	120
Hexachloro-1,3-butadiene	5	20	100
d-Limonene	125	500	4000

Observations and analyses

The rats were weighed weekly and observed daily for condition and behaviour. Food intake was measured weekly over 1-wk periods and water intake daily, on a cage basis, by weighing the feeders or drinking bottles, respectively.

Haematology and clinical chemistry. Blood samples were collected from the tip of the tail of all animals in the fourth week of treatment, and examined for haemoglobin concentration, packed cell volume, and counts of red blood cells and total white blood cells (Coulter Counter model ZF, Coulter Electronics Ltd, UK, used only in the range-finding study with HCBD, or Sysmex K-1000 Haematology Analyzer, Toa Medical Electronics Co. Ltd, Japan). Blood samples collected from the abdominal aorta at the time of killing were centrifuged at 1250 *g* for 15 min and then analysed for alkaline phosphatase (ALP), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), total protein, albumin, total bilirubin, urea, creatinine, inorganic phosphate and calcium (Cobas-Bio Centrifugal Analyzer), chloride (Chloro Counter), and sodium and potassium (Electrolyte-2-Analyzer).

Urinalysis. In wk 1 (after 3 days of treatment) and wk 4, rats were deprived of water for 24 hr and of food for 16 hr. Urine was collected during the last 16 hr of deprivation and its volume (calibrated tubes) and density (Bellingham and Stanley refractometer) were determined. Semi-quantitative observations in the individual urine samples included appearance (visual inspection), pH protein, glucose, ketones, occult blood, urobilinogen and bilirubin (Combur-7-Test strips; Boehringer Mannheim GmbH, Mannheim, Germany). After centrifugation (5 min at 500 *g*), the sediment of individual samples was examined microscopically for red and white blood cells, epithelial cells, amorphous material, crystals, casts, bacteria, worm eggs and spermatozoa.

Pathology. At the end of the 4-wk treatment period, the rats were killed by exsanguination from the abdominal aorta under light ether anaesthesia, and a thorough autopsy was performed. The weights of the kidneys, adrenals and liver were recorded and the ratios of organ weight to body weight were calculated. After fixation in a 4% neutral buffered solution of formaldehyde, the kidneys were processed, embedded in paraffin, sectioned at 5 μm,

stained with haematoxylin and eosin and examined by light microscopy.

Statistical analysis

Body weights were evaluated by one-way analysis of covariance (covariate: body weight at the start of treatment) followed by Dunnett's multiple comparison tests. Haematology and clinical chemistry values, organ weights and urinary volume and density were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. The results of urinanalysis, except for volume and density, were analysed by the Mann–Whitney *U*-test, and the histopathological changes by Fisher's exact probability test. All analyses were two-sided.

RESULTS

Range-finding studies

Results of the range-finding studies with HCBD and mercuric chloride are summarized in Tables 3 and 4, respectively.

HCBD. This induced signs of toxicity at 100 and 400 ppm in both sexes. These signs included growth retardation, decreased intake of food and water, increased volume and decreased density of the urine (males receiving 100 ppm only), increased level of ketones (at 400 ppm only) and number of epithelial cells in the urine, increased plasma levels of ASAT and bilirubin, decreased plasma concentrations of total protein, albumin and calcium (males receiving 400 ppm only), decreased plasma urea levels (in males receiving 400 ppm and all treated groups of females), increased relative kidney weight, decreased absolute weight of the adrenals and/or liver, and increased relative weight of the adrenals (males receiving 400 ppm) or liver (males receiving 100 ppm). Females fed 400 ppm HCBD also showed clinical signs of poor health and one of them died on the fifth day of treatment. Microscopy (no table presented) revealed diffuse tubular cytomegaly in the inner cortex of kidneys of males and females fed 400 ppm HCBD, and of females fed 100 ppm HCBD. In addition, males of the 400 ppm group showed focal nephrosis. The dietary concentrations of HCBD selected for the combination study were 20 and 100 ppm for the NNEL and MNEL, respectively.

Mercuric chloride. With mercuric chloride, treatment-related changes were seen at 75, 150 and 300 ppm. The changes at 300 ppm consisted of growth retardation, decreased intake of food and water, decreased urinary density (males only), the presence of ketones in the urine (males only), increased plasma ASAT and ALP activity, increased plasma concentration of sodium and inorganic phosphate (females only), increased relative kidney weight, decreased absolute weight of the adrenals and/or liver, and increased relative weight of the adrenals (males only). Most of these changes were also observed in one or both sexes at 150 ppm, although to a lesser degree. The treatment-related changes at 75 ppm comprised slightly decreased water intake, the presence of ketones in the urine (males only), and increased relative kidney weight. In the absence of a dose–response relationship, the increased number of epithelial cells in the urine of males at 75 ppm cannot be ascribed with certainty to treatment. Histopathological findings were nephrosis and proteinaceous casts in the kidneys, which were observed in all groups fed mercuric chloride (no table presented), the changes being most clear in the 75 ppm group of males. Because the lowest level tested in this study was not without treatment-related effects, the results of a previously conducted range-finding study with 4, 16 and 64 ppm mercuric chloride (no data presented) were taken into account in the selection of the dose levels for the combination study. Thus, 15 and 120 ppm mercuric chloride were selected as the NNEL and MNEL, respectively, for the combination study.

d-Limonene. The effects of *d*-limonene (no table presented) in the 4-wk-old rats were limited to increases in the relative kidney weight and volume of the urine collected in wk 1 in males fed 4000 ppm (mean relative kidney weight was 8.04 g/kg at 4000 ppm *v.* 7.42 g/kg in controls; mean urinary volume was 2.4 ml/16 hr at 4000 ppm *v.* 1.4 ml/16 hr in controls). The 10-wk-old males fed 4000 ppm *d*-limonene showed an increased number of epithelial cells in the urine collected in wk 4 (mean score was 3 at 4000 ppm *v.* 1 in controls) and microscopic renal changes consisting of nephrosis and accumulation of proteinaceous droplets in tubular epithelial cells. For the combination study, 500 and 4000 ppm were selected as the NNEL and MNEL, respectively. Because only 4000 ppm was tested in adult males in the range-finding study, the choice of 500 ppm (providing about 30 mg/kg body weight/day) as a NNEL was based on data reported by Kanerva *et al.* (1987), who found histopathological effects in the kidneys of adult male Fischer-344 rats given *d*-limonene at 75, 150 or 300 mg/kg body weight by gavage (5 days/wk) over a 26-day period.

Lysinoalanine. The levels of lysinoalanine for the combination study, namely a NNEL of 30 ppm and a MNEL of 240 ppm, were selected on the basis of results obtained from earlier studies conducted in our laboratory (De Groot *et al.*, 1976; Feron *et al.*, 1978). In those studies lysinoalanine induced typical renal changes, termed nephrocytomegaly, when fed at dietary levels of 100 ppm and above.

Combination study

All rats survived to scheduled autopsy. Clinical signs of ill-health consisted of emaciation, seen in two of five females fed the MNEL of HCBD and in six of 10 females fed the MNEL of the combination, and rough fur or weakness seen in one female of the latter group. Data on body weights, food and water intake,

Table 3. Effects on selected parameters in rats fed diets containing different concentrations of hexachloro-1,3-butadiene (HCBD) for 4 wk (data from a range-finding study)

Parameter	Values for rats receiving HCBD at dietary concentrations (ppm) of:							
	0 (Control)	25	100	400	0 (Control)	25	100	400
	Males				**Females**			
Body weight (g)								
on day 0	61.8 ± 1.5	62.6 ± 2.2	62.6 ± 2.3	62.9 ± 1.7	57.9 ± 1.5	58.0 ± 2.2	58.5 ± 2.0	57.4 ± 2.4
28	203.3 ± 5.1	200.3 ± 5.1	183.5 ± 6.8*	134.1 ± 2.9**	147.8 ± 3.3	147.7 ± 3.8	125.8 ± 5.7**	97.3 ± 5.4**
Food intake (g/rat/day)								
over 4 wk	15.6	15.3	14.2	9.2	12.6	12.6	9.4	7.2
Water intake (g/rat/day)								
over 4 wk	17.6	17.6	17.8	13.4	15.3	15.6	12.6	10.5
Urinalysis wk 4								
volume (ml/16 hr)	2.0 ± 0.1	2.1 ± 0.2	2.6 ± 0.2*	2.3 ± 0.2	1.8 ± 0.1	2.4 ± 0.7	1.4 ± 0.1	1.4 ± 0.3
density (kg/litre)	1.066 ± 0.003	1.062 ± 0.003	1.049 ± 0.005*	1.053 ± 0.003	1.060 ± 0.003	1.055 ± 0.007	1.064 ± 0.003	1.061 ± 0.002
ketones (range 0–3)	0	0	0	1***	0	0	0	2*
epithelial cells (range 0–5)	1	1	3**	4***	1	2	3***	4**
Clinical chemistry								
ASAT (U/litre)	59.9 ± 2.1	61.6 ± 2.9	58.8 ± 2.6	86.0 ± 4.5**	57.6 ± 1.8	55.5 ± 2.8	59.3 ± 2.0	70.2 ± 2.7**
total protein (g/litre)	57.8 ± 0.4	57.4 ± 0.6	58.0 ± 0.8	55.4 ± 0.2**	58.8 ± 0.9	57.4 ± 0.4	57.4 ± 0.4	57.7 ± 0.7
albumin (g/litre)	34.2 ± 0.1	33.9 ± 0.9	35.1 ± 0.2	32.4 ± 0.7*	35.4 ± 0.6	35.1 ± 0.3	35.5 ± 0.5	34.7 ± 0.7
urea (mmol/litre)	6.45 ± 0.33	5.89 ± 0.23	6.44 ± 0.60	4.84 ± 0.29*	8.40 ± 0.46	6.26 ± 0.31**	6.27 ± 0.46**	5.63 ± 0.30**
creatinine (μmol/litre)	61.5 ± 2.5	58.6 ± 2.1	55.2 ± 3.9	50.1 ± 2.4	56.8 ± 1.5	53.6 ± 0.9	50.2 ± 1.2**	50.6 ± 1.2*
total bilirubin (μmol/litre)	0.31 ± 0.11	1.02 ± 0.91	0.46 ± 0.12	2.10 ± 0.20**	0.53 ± 0.13	0.47 ± 0.14	0.80 ± 0.16	1.30 ± 0.11**
calcium (mmol/litre)	2.74 ± 0.03	2.71 ± 0.04	2.67 ± 0.05	2.52 ± 0.10*	2.59 ± 0.04	2.56 ± 0.04	2.54 ± 0.02	2.56 ± 0.06
Kidney weight								
absolute (g)	1.59 ± 0.04	1.69 ± 0.05	1.62 ± 0.07	1.38 ± 0.07*	1.34 ± 0.05	1.35 ± 0.06	1.36 ± 0.09	1.24 ± 0.08
relative (g/kg body weight)	7.83 ± 0.09	8.42 ± 0.21	8.80 ± 0.13**	10.29 ± 0.39**	8.80 ± 0.23	8.93 ± 0.41	10.64 ± 0.51**	12.28 ± 0.79**
Adrenal weight								
absolute (g)	0.039 ± 0.001	0.041 ± 0.003	0.037 ± 0.005	0.035 ± 0.002	0.046 ± 0.002	0.045 ± 0.002	0.038 ± 0.002*	0.035 ± 0.002**
relative (g/kg body weight)	0.189 ± 0.005	0.202 ± 0.010	0.198 ± 0.020	0.259 ± 0.013**	0.302 ± 0.014	0.229 ± 0.012	0.298 ± 0.012	0.347 ± 0.031
Liver weight								
absolute (g)	9.24 ± 0.34	9.49 ± 0.47	9.38 ± 0.52	6.53 ± 0.15**	6.64 ± 0.19	6.48 ± 0.19	5.46 ± 0.35**	4.75 ± 0.31**
relative (g/kg body weight)	45.4 ± 0.8	47.4 ± 2.1	51.0 ± 1.5**	48.7 ± 0.4	43.5 ± 0.8	43.0 ± 1.0	42.5 ± 1.1	46.7 ± 0.9

Only the parameters showing treatment-related changes are included in this table. Values are means ± SEM for groups of 10 (controls) or five rats. Food and water intake are cage means (five rats/cage). Although food and water intake were recorded weekly and daily, respectively, only the mean over 4 wk is presented in the table. The values marked with asterisks differ significantly from controls (body weight: analysis of covariance + Dunnett's test; urinary volume and density, clinical chemistry, organ weights: ANOVA + Dunnett's test: $*P < 0.05$; $**P < 0.01$; semi-quantitative urinary findings: Mann–Whitney U-test: $*P < 0.05$; $**P < 0.02$; $***P < 0.002$).

Table 4. Effects on selected parameters in rats fed diets containing different concentrations of mercuric chloride for 4 wk (data from a range-finding study)

Parameter	Values for rats receiving mercuric chloride at dietary concentrations (ppm) of:							
	0 (Control)	75	150	300	0 (Control)	75	150	300
	Males				**Females**			
Body weight (g)								
on day 0	64.0 ± 1.9	64.0 ± 1.5	63.1 ± 2.7	64.1 ± 2.6	64.3 ± 0.9	63.6 ± 1.2	64.6 ± 2.4	64.3 ± 2.6
28	205.3 ± 5.7	190.6 ± 2.5	162.3 ± 8.4**	133.5 ± 7.8**	154.0 ± 3.0	146.1 ± 4.4	143.5 ± 4.4	112.8 ± 8.2**
Food intake (g/rat/day)								
over 4 wk	14.9	13.2	11.6	9.3	12.2	11.5	11.2	9.4
Water intake (g/rat/day)								
over 4 wk	18.4	16.9	14.8	13.1	17.0	14.8	13.4	11.5
Urinalysis wk 4								
volume (ml/16 hr)	1.3 ± 0.1	1.8 ± 0.1	1.6 ± 0.3	1.6 ± 0.2	1.3 ± 0.1	1.7 ± 0.2	1.7 ± 0.1	1.7 ± 0.1
density (kg/litre)	1.078 ± 0.002	1.067 ± 0.001	1.069 ± 0.008	1.058 ± 0.005**	1.077 ± 0.005	1.065 ± 0.005	1.064 ± 0.004	1.064 ± 0.004
ketones (range 0–3)	0	2**	1**	2**	0	0	0	0
epithelial cells (range 0–5)	1	2***	1	1	1	1	1	2
Clinical chemistry								
ASAT (U/litre)	63.4 ± 1.4	64.1 ± 2.3	63.7 ± 3.0	73.5 ± 3.2*	62.6 ± 1.4	59.1 ± 1.2	60.1 ± 2.1	73.9 ± 2.3**
ALP (U/litre)	366 ± 21	386 ± 25	427 ± 33	468 ± 17*	261 ± 11	283 ± 9	315 ± 19*	319 ± 24*
sodium (mmol/litre)	143.3 ± 0.5	143.9 ± 0.4	144.1 ± 0.3	145.3 ± 1.3	143.6 ± 0.4	143.8 ± 1.2	144.1 ± 0.4	146.2 ± 0.6*
inorganic phosphate (mmol/litre)	2.69 ± 0.06	2.80 ± 0.08	2.88 ± 0.10	2.81 ± 0.18	2.25 ± 0.04	2.47 ± 0.14	2.48 ± 0.08	2.67 ± 0.12**
Kidney weight								
absolute (g)	1.52 ± 0.04	1.60 ± 0.06	1.41 ± 0.10	1.23 ± 0.05**	1.25 ± 0.03	1.38 ± 0.04	1.41 ± 0.07	1.13 ± 0.09
relative (g/kg body weight)	7.42 ± 0.08	8.38 ± 0.23*	8.66 ± 0.29**	9.27 ± 0.42**	8.14 ± 0.21	9.47 ± 0.51*	9.85 ± 0.38**	9.95 ± 0.16**
Adrenal weight								
absolute (g)	0.037 ± 0.002	0.037 ± 0.002	0.032 ± 0.001	0.032 ± 0.002	0.046 ± 0.001	0.043 ± 0.002	0.042 ± 0.001	0.032 ± 0.004**
relative (g/kg body weight)	0.179 ± 0.005	0.193 ± 0.009	0.201 ± 0.010	0.239 ± 0.018**	0.301 ± 0.009	0.295 ± 0.018	0.298 ± 0.018	0.281 ± 0.016
Liver weight								
absolute (g)	9.22 ± 0.34	8.34 ± 0.17	7.02 ± 0.60**	5.74 ± 0.41**	6.60 ± 0.18	6.39 ± 0.24	5.88 ± 0.22	4.63 ± 0.48**
relative (g/kg body weight)	44.9 ± 0.9	43.8 ± 0.8	42.9 ± 1.8	43.0 ± 1.7	42.8 ± 0.8	43.8 ± 1.2	41.0 ± 1.0	40.8 ± 1.8

Only the parameters showing treatment-related changes are included in this table. Values are means ± SEM for groups of 10 (controls) or five rats. Food and water intake are cage means (five rats/cage). Although food and water intake were recorded weekly and daily, respectively, only the mean over 4 wk is presented in the table. The values marked with asterisks differ significantly from controls (body weight: analysis of covariance + Dunnett's test; urinary volume and density, clinical chemistry, organ weights: ANOVA + Dunnett's test: $*P < 0.05$; $**P < 0.01$; semi-quantitative urinary findings: Mann–Whitney U-test: $*P < 0.05$; $**P < 0.02$; $***P < 0.002$).

Table 5. Effects on selected parameters in male rats fed four nephrotoxins either concurrently or individually for 4 wk at one-quarter of the No-Nephrotoxic-Effect Level ($\frac{1}{4}$NNEL), the NNEL or the Minimum-Nephrotoxic Effect Level (MNEL)

	Values for rats of the various groups											
		Combination			Hexachloro-1,3-butadiene		Mercuric chloride		*d*-Limonene		Lysinoalanine	
Parameter	Control	$\frac{1}{4}$NNEL	NNEL	MNEL	NNEL	MNEL	NNEL	MNEL	NNEL	MNEL	NNEL	MNEL
Body weight (g)												
on day 0	302.7 ± 5.0	304.0 ± 4.5	302.4 ± 6.8	303.6 ± 5.2	304.4 ± 6.6	303.0 ± 6.5	303.0 ± 6.4	303.5 ± 9.7	305.5 ± 4.1	302.9 ± 3.9	303.6 ± 7.9	303.4 ± 8.7
28	372.1 ± 9.5	364.7 ± 4.8	355.0 ± 8.3**	313.3 ± 5.9**	364.8 ± 7.3	354.6 ± 8.7*	364.5 ± 7.3	351.6 ± 9.0**	368.9 ± 8.8	352.8 ± 8.4	377.5 ± 9.5	347.8 ± 11.4**
Food intake (g/rat/day) over 4 wk	19.2	19.0	18.2	15.3	18.4	17.9	18.3	18.9	19.1	17.2	20.2	16.8
Water intake (g/rat/day) over 4 wk	26.0	25.2	24.1	22.6	22.5	22.7	22.6	23.8	25.6	25.7	24.2	24.3
Urinalysis wk 1												
volume (ml/16 hr)	3.0 ± 0.3	2.7 ± 0.2	3.0 ± 0.2	5.3 ± 0.5**	2.7 ± 0.2	2.5 ± 0.3	2.8 ± 0.4	3.2 ± 0.4	3.6 ± 0.8	3.2 ± 0.2	2.8 ± 0.3	3.4 ± 0.1
density (kg/litre)	1.056 ± 0.003	1.056 ± 0.003	1.051 ± 0.003	1.041 ± 0.002**	1.060 ± 0.004	1.063 ± 0.003	1.061 ± 0.005	1.055 ± 0.005	1.049 ± 0.003	1.051 ± 0.001	1.059 ± 0.003	1.053 ± 0.003
epithelial cells (range 0–5)	1	1	1	1	1	1	1	1	1	2	1	1
Urinalysis wk 4												
volume (ml/16 hr)	3.8 ± 0.4	3.8 ± 0.3	4.0 ± 0.3	3.3 ± 0.3	3.4 ± 0.3	3.4 ± 0.4	3.7 ± 0.3	4.1 ± 0.5	4.3 ± 0.5	4.1 ± 0.3	2.7 ± 0.3	2.8 ± 0.4
density (kg/litre)	1.053 ± 0.003	1.046 ± 0.003	1.045 ± 0.003	1.045 ± 0.003	1.057 ± 0.004	1.046 ± 0.004	1.054 ± 0.005	1.047 ± 0.005	1.038 ± 0.003**	1.047 ± 0.003	1.063 ± 0.002	1.059 ± 0.006
ketones (range 0–3)	0	0	0	0	0	0	0	1**	0	0	0	0
epithelial cells (range 0–5)	1	1	2*	4***	1	2	1	1	3**	4***	1	1
Clinical chemistry												
ALP (U/litre)	166 ± 6	182 ± 10	154 ± 6	140 ± 5*	156 ± 8	156 ± 9	164 ± 9	175 ± 8	178 ± 12	151 ± 10	151 ± 14	146 ± 7
bilirubin (mmol/litre)	1.06 ± 0.07	1.23 ± 0.03	1.23 ± 0.07	1.36 ± 0.05**	1.07 ± 0.06	1.10 ± 0.10	0.74 ± 0.17	1.20 ± 0.11	1.13 ± 0.08	0.95 ± 0.17	0.97 ± 0.15	1.02 ± 0.14
Kidney weight												
absolute (g)	2.25 ± 0.07	2.25 ± 0.06	2.42 ± 0.08	2.53 ± 0.10*	2.37 ± 0.08	2.37 ± 0.09	2.38 ± 0.03	2.48 ± 0.05	2.31 ± 0.11	2.33 ± 0.18	2.17 ± 0.06	2.22 ± 0.07
relative (g/kg body weight)	6.05 ± 0.14	6.18 ± 0.10	6.82 ± 0.12*	8.07 ± 0.28**	6.49 ± 0.09	6.67 ± 0.11*	6.53 ± 0.16	7.07 ± 0.19**	6.25 ± 0.22	6.59 ± 0.36	5.74 ± 0.08	6.41 ± 0.22
Adrenal weight												
absolute (g)	0.046 ± 0.001	0.042 ± 0.001	0.047 ± 0.001	0.044 ± 0.001	0.046 ± 0.002	0.044 ± 0.002	0.048 ± 0.003	0.045 ± 0.004	0.041 ± 0.002	0.045 ± 0.002	0.049 ± 0.003	0.040 ± 0.002
relative (g/kg body weight	0.124 ± 0.003	0.116 ± 0.003	0.132 ± 0.004	0.142 ± 0.005*	0.125 ± 0.005	0.123 ± 0.006	0.131 ± 0.009	0.128 ± 0.011	0.111 ± 0.003*	0.128 ± 0.005	0.130 ± 0.007	0.116 ± 0.003
Liver weight												
absolute (g)	13.13 ± 0.48	12.61 ± 0.28	12.54 ± 0.54	11.25 ± 0.34**	12.50 ± 0.49	13.12 ± 0.48	12.76 ± 0.56	11.88 ± 0.43	13.51 ± 0.80	12.88 ± 0.68	13.26 ± 0.54	11.52 ± 0.61
relative (g/kg body weight)	35.2 ± 0.6	34.6 ± 0.6	35.2 ± 0.9	35.9 ± 1.0	34.2 ± 1.0	37.0 ± 0.5	34.9 ± 0.9	33.8 ± 0.5	36.5 ± 1.3	36.4 ± 1.1	35.1 ± 0.9	33.1 ± 1.2

Values are means ± SEM for groups of 10 (controls) or five rats. Food and water intake are cage means (five rats/cage). Although food and water intake were recorded weekly and daily, respectively, only the mean over 4 wk is presented in the table. The values marked with asterisks differ significantly from controls (body weight: analysis of covariance + Dunnett's test; urinary volume and density, clinical chemistry, organ weights: ANOVA + Dunnett's test: *$P < 0.05$; **$P < 0.01$; semi-quantitative urinary findings: Mann–Whitney U-test: *$P < 0.05$; **$P < 0.02$; ***$P < 0.002$).

Table 6. Effects on selected parameters in female rats fed four nephrotoxins either concurrently or individually for 4 wk at one-quarter of the NNEL, the NNEL or the MNEL

	Values for rats of the various groups											
		Combination			Hexachloro-1,3-butadiene		Mercuric chloride		*d*-Limonene		Lysinoalanine	
Parameter	Control	$\frac{1}{4}$ NNEL	NNEL	MNEL	NNEL	MNEL	NNEL	MNEL	NNEL	MNEL	NNEL	MNEL
Body weight (g)												
on day 0	185.3 ± 2.5	186.0 ± 2.2	185.2 ± 1.4	185.3 ± 3.4	185.3 ± 4.1	185.1 ± 5.1	185.2 ± 3.4	185.3 ± 5.2	185.3 ± 4.7	185.3 ± 2.6	184.9 ± 3.6	185.5 ± 3.9
28	209.2 ± 2.1	211.4 ± 2.5	204.4 ± 1.8	186.4 ± 4.2**	205.1 ± 3.3	184.8 ± 4.5**	206.1 ± 5.9	201.7 ± 4.3	210.0 ± 4.9	214.2 ± 3.1	210.2 ± 3.7	204.1 ± 6.6
Food intake (g/rat/day)												
over 4 wk	12.5	13.2	11.9	9.8	12.2	10.0	13.4	11.9	12.1	13.3	13.7	12.4
Water intake (g/rat/day)												
over 4 wk	20.4	19.0	19.9	20.0	18.9	17.5	17.7	16.6	20.5	20.1	18.6	20.3
Urinalysis wk 1												
volume (ml/16 hr)	1.9 ± 0.1	2.0 ± 0.1	1.9 ± 0.1	2.0 ± 0.2	1.8 ± 0.2	1.7 ± 0.1	1.6 ± 0.3	2.1 ± 0.1	1.9 ± 0.2	2.0 ± 0.2	1.8 ± 0.2	2.2 ± 0.1
density (kg/litre)	1.066 ± 0.003	1.067 ± 0.002	1.067 ± 0.002	1.058 ± 0.003	1.070 ± 0.004	1.067 ± 0.001	1.073 ± 0.005	1.064 ± 0.003	1.069 ± 0.003	1.068 ± 0.003	1.062 ± 0.003	1.058 ± 0.004
epithelial cells (range 0–5)	1	1	1	2**	1	3***	1	1	1	1	1	1
Urinalysis wk 4												
volume (ml/16 hr)	2.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	2.6 ± 0.3	1.9 ± 0.1	3.0 ± 0.5*	1.5 ± 0.1	2.3 ± 0.2	2.1 ± 0.2	2.2 ± 0.2	1.9 ± 0.2	2.7 ± 0.3*
density (kg/litre)	1.064 ± 0.002	1.063 ± 0.002	1.064 ± 0.002	1.050 ± 0.003**	1.069 ± 0.003	1.054 ± 0.006	1.073 ± 0.003	1.054 ± 0.004*	1.065 ± 0.003	1.065 ± 0.003	1.068 ± 0.001	1.051 ± 0.002**
epithelial cells (range 0–5)	1	1	1	3***	1	2**	1	1	1	1	1	1
Clinical chemistry												
ALP (U/litre)	144 ± 9	152 ± 9	121 ± 6	116 ± 6*	117 ± 12	118 ± 9	126 ± 11	127 ± 11	142 ± 10	141 ± 8	143 ± 9	141 ± 15
ASAT (U/litre)	60.3 ± 2.1	59.2 ± 1.2	59.3 ± 2.2	65.6 ± 2.5	64.5 ± 3.3	76.9 ± 4.9**	60.9 ± 2.4	56.2 ± 0.6	64.6 ± 4.3	59.9 ± 1.1	57.0 ± 2.5	63.0 ± 5.2
ALAT (U/litre)	36.8 ± 2.2	34.4 ± 1.6	34.2 ± 2.3	39.8 ± 2.8	38.7 ± 3.6	54.9 ± 4.8**	31.4 ± 2.7	32.4 ± 1.5	42.3 ± 5.1	34.3 ± 1.1	33.1 ± 2.3	42.1 ± 3.8
albumin (g/litre)	36.2 ± 0.3	35.2 ± 0.4	35.5 ± 0.4	34.0 ± 0.5**	35.4 ± 0.6	34.2 ± 0.3**	36.2 ± 0.7	35.1 ± 0.7	37.1 ± 0.9	35.6 ± 0.6	36.0 ± 0.8	35.2 ± 0.4
total protein (g/litre)	59.8 ± 0.3	58.2 ± 0.6	59.4 ± 0.5	57.6 ± 0.8*	59.2 ± 1.2	55.1 ± 0.6**	59.9 ± 1.2	58.7 ± 1.3	61.6 ± 1.3	58.7 ± 0.5	59.2 ± 0.9	56.3 ± 1.0**
calcium (mmol/litre)	2.43 ± 0.02	2.42 ± 0.02	2.46 ± 0.02	2.36 ± 0.02*	2.46 ± 0.01	2.28 ± 0.02**	2.45 ± 0.04	2.42 ± 0.03	2.55 ± 0.03**	2.49 ± 0.03	2.42 ± 0.05	2.36 ± 0.02
bilirubin (mmol/litre)	1.23 ± 0.03	1.11 ± 0.06	1.16 ± 0.04	1.19 ± 0.14	1.05 ± 0.27	1.26 ± 1.10	1.21 ± 0.10	1.20 ± 0.10	1.10 ± 0.08	1.28 ± 0.07	1.15 ± 0.04	1.15 ± 0.05
Kidney weight												
absolute (g)	1.45 ± 0.02	1.58 ± 0.03*	1.51 ± 0.03	1.41 ± 0.04	1.49 ± 0.02	1.32 ± 0.01*	1.61 ± 0.03*	1.68 ± 0.08**	1.38 ± 0.05	1.45 ± 0.06	1.45 ± 0.04	1.50 ± 0.06
relative (g/kg body weight)	6.94 ± 0.10	7.47 ± 0.21	7.37 ± 0.13	7.55 ± 0.19	7.27 ± 0.17	7.15 ± 0.13	7.81 ± 0.20**	8.30 ± 0.29**	6.59 ± 0.24	6.78 ± 0.24	6.93 ± 0.24	7.37 ± 0.22
Adrenal weight												
absolute (g)	0.058 ± 0.003	0.057 ± 0.001	0.058 ± 0.001	0.047 ± 0.002**	0.054 ± 0.002	0.045 ± 0.002**	0.061 ± 0.002	0.055 ± 0.002	0.055 ± 0.003	0.059 ± 0.004	0.057 ± 0.001	0.055 ± 0.002
relative (g/kg body weight)	0.276 ± 0.013	0.269 ± 0.009	0.283 ± 0.006	0.253 ± 0.008	0.262 ± 0.011	0.244 ± 0.018	0.296 ± 0.020	0.276 ± 0.015	0.264 ± 0.014	0.275 ± 0.022	0.270 ± 0.005	0.270 ± 0.008
Liver weight												
absolute (g)	6.70 ± 0.14	6.86 ± 0.16	6.83 ± 0.14	6.05 ± 0.26*	6.85 ± 0.17	5.55 ± 0.22**	6.64 ± 0.32	6.49 ± 0.17	7.05 ± 0.37	7.09 ± 0.21	6.67 ± 0.19	6.44 ± 0.45
relative (g/kg body weight)	32.0 ± 0.5	32.5 ± 0.7	33.4 ± 0.5	32.4 ± 0.8	33.4 ± 0.9	30.0 ± 1.1	32.2 ± 1.0	32.2 ± 0.6	33.5 ± 1.2	33.1 ± 0.9	31.8 ± 1.2	31.4 ± 1.2

Values are means ± SEM for groups of 10 (controls) or five rats. Food and water intake are cage means (five rats/cage). Although food and water intake were recorded weekly and daily, respectively, only the mean over 4 wk is presented in the table. The values marked with asterisks differ significantly from controls (body weight: analysis of covariance + Dunnett's test; urinary volume and density, clinical chemistry, organ weights: ANOVA + Dunnett's test: *$P < 0.05$; **$P < 0.01$; semi-quantitative urinary findings: Mann–Whitney U-test: *$P < 0.05$; **$P < 0.02$; ***$P < 0.002$).

Table 7. Histopathological changes in kidneys of rats fed four nephrotoxins either concurrently or individually for 4 wk at one-quarter of the NNEL, the NNEL or the MNEL

	Group								
		Combination			HCBD	Mercuric chloride	*d*-Limonene		Lysinoalanine
Observation	Control	$\frac{1}{4}$ NNEL	NNEL	MNEL	MNEL	MNEL	NNEL	MNEL	MNEL
Males									
Basophilic tubules in outer cortex	3/10	6/10	7/10	10/10**	4/5	5/5**	3/5	5/5**	0/5
single to a few	3	6	7	5	4	5	2	1	0
several	0	0	0	5	0	0	1	1	0
many	0	0	0	0	0	0	0	3	0
Proteinaceous casts	0/10	1/10	1/10	2/10	0/5	0/5	0/5	1/5	1/5
Dilated tubules	0/10	0/10	0/10	2/10	0/5	0/5	0/5	0/5	0/5
Cortical mononuclear cell infiltrate	1/10	0/10	1/10	0/10	1/5	0/5	0/5	2/5	1/5
Inner cortex:									
Karyomegaly	0/10	0/10	0/10	10/10**	0/5	0/5	0/5	0/5	5/5**
Hypercellularity	0/10	0/10	0/10	10/10**	0/5	0/5	0/5	0/5	0/5
Variable nuclear size	0/10	0/10	0/10	10/10**	0/5	0/5	0/5	0/5	0/5
Increased accumulation of proteinaceous droplets in tubular epithelial cells	0/10	2/10	4/10	8/10**	1/5	0/5	5/5**	5/5**	0/5
Females									
Basophilic tubules in outer cortex, single to a few	0/10	0/10	3/10	1/10	0/5	1/5	1/5	0/5	2/5
Cortical mononuclear cell infiltrate	1/10	1/10	0/10	0/10	0/5	0/5	0/5	0/5	0/5
Corticomedullary mineralization	0/10	0/10	0/10	0/10	0/5	0/5	1/5	0/5	1/5
Inner cortex:									
Necrosis	0/10	0/10	0/10	10/10**	5/5**	0/5	0/5	0/5	5/5**
Karyomegaly	0/10	0/10	0/10	10/10**	5/5**	0/5	0/5	0/5	5/5**
Hypercellularity	0/10	0/10	0/10	10/10**	5/5**	0/5	0/5	0/5	5/5**
Variable nuclear size	0/10	0/10	0/10	10/10**	5/5**	0/5	0/5	0/5	5/5**

Values are numbers of rats with the indicated observation, based on microscopic assessment of sections stained with haematoxylin and eosin. The groups fed hexachloro-1,3-butadiene (HCBD), mercuric chloride or lysinoalanine individually at their NNEL did not show significant changes and are therefore not presented in this table. Values marked with asterisks differ significantly (Fisher's exact test) from the controls: $*P < 0.05$; $**P < 0.01$.

selected results of urinanalysis and clinical chemistry, and organ weights, are presented in Tables 5 and 6, with histopathological findings in Table 7. Apart from pale or soft kidneys in two of five males fed the MNEL of *d*-limonene and in three of 10 males fed the MNEL of the combination, no significant gross abnormalities were observed at autopsy. Haematology data and part of the urinary and clinical chemistry parameters measured did not show any abnormality attributable to treatment and therefore are not presented.

At the NNEL, combined exposure induced slight growth retardation and increases in the relative kidney weight and number of epithelial cells in the urine of males, whereas no alterations were seen in co-exposed females. Contrary to expectation, some statistically significant differences from the controls were seen in two single-agent NNEL groups: with mercuric chloride a higher kidney weight was noted in females; with *d*-limonene there was a higher plasma concentration of calcium in females, a lower urinary density and relative adrenal weight in males, a higher number of epithelial cells in the urine in males, and increased accumulation of proteinaceous droplets in tubular epithelial cells in males. The changes in calcium, urinary density and adrenal weight are not considered to be compound related, because higher-dosed animals from both the range-finding and the combination study did not show such changes. In the one-quarter NNEL combination group, only a slight (although statistically significant) increase in the absolute kidney weight of females was observed; however, as the relative kidney weight in this group was not significantly increased and, moreover, females of the higher-dosed groups showed normal kidney weights, this increased absolute kidney weight in the one-quarter NNEL group is judged to be a chance finding unrelated to treatment.

As anticipated, several changes indicative of renal or general toxicity were observed at the MNEL of the individual compounds and the combination. Each of the four compounds induced slight growth retardation in male rats when administered alone at its MNEL. This effect appeared to be significantly enhanced when the compounds were given simultaneously, as indicated by the nearly complete cessation of growth in males of the combination MNEL group. Female rats exhibited markedly depressed weight gain on administration of the MNEL of the combination or of HCBD only. However, in contrast to males, there was no evidence of aggravation of the effect by combined treatment.

In male rats of the combination MNEL group, changes indicative of nephrotoxicity comprised increased volume and decreased density of the urine collected in the first week of treatment (pointing to

impaired concentrating ability of the kidneys), increased number of epithelial cells in the urine collected towards the end of treatment, increased absolute and relative kidney weight, and histopathological alterations. The histopathological changes consisted of an increased number of basophilic tubules in the outer cortex of the kidney, increased accumulation of proteinaceous droplets in tubular epithelial cells, and diffuse signs of regeneration in the inner cortex characterized by karyomegaly, hypercellularity and variable nuclear size. Males of the single-agent MNEL groups showed only slightly increased relative kidney weights (with HCBD and mercuric chloride), more epithelial cells in the urine (with *d*-limonene), an increase in the number of basophilic tubules in the outer cortex of the kidney (with mercuric chloride and *d*-limonene), karyomegaly (with lysinoalanine), or increased accumulation of proteinaceous droplets in tubular epithelial cells (with *d*-limonene). Clearly, at the MNEL in males, nephrotoxicity of the combination was more severe than that of the individual compounds.

The slight, but statistically significant increase in relative kidney weight in males of the combination NNEL group might suggest increased nephrotoxicity on combined exposure at the NNEL as well. It should be realized, however, that relative kidney weights in two single-agent NNEL groups (HCBD and mercuric chloride) were also higher than those of the controls, although the differences did not reach the level of statistical significance. For this reason, the increased kidney weight in the combination NNEL group is not convincing proof of increased renal toxicity. Furthermore, the only other significant renal change observed in males of the combination NNEL group (an increase in epithelial cells in the urine) is not considered to be a reflection of increased toxicity, either, because a similar change occurred in males of the *d*-limonene NNEL group.

Renal toxicity in females co-exposed at the MNEL was evident from the presence of epithelial cells in the urine, slightly decreased urinary density, and severe renal necrosis. The necrosis was diffuse and characterized by degeneration of tubular cells in the cortex, and accompanied by signs of regeneration (hypercellularity, karyomegaly and variable nuclear size). However, the renal effects in females given the combination were not more severe than those seen with the individual nephrotoxins. At the NNEL or one-quarter NNEL, females of the combination group did not exhibit any renal change attributable to treatment.

The increased plasma ALAT and ASAT activities seen in females given the effective dose of HCBD are suggestive of hepatotoxicity. HCBD-induced liver changes, consisting of increased liver weight and cytoplasmic basophilia of hepatocytes, were also observed by Harleman and Seinen (1979) in a 13-wk gavage study. In that study, plasma ASAT activity was unaffected and the hepatic changes were more marked in males than in females, which is contrary to our findings. Remarkably, females of the combination MNEL group did not show an increase in ASAT or ALAT activity, suggesting some kind of antagonistic interaction with respect to these endpoints.

A number of other changes observed at the MNEL of HCBD or the combination (decreased absolute organ weights and decreased plasma levels of ALP, albumin, total protein and calcium) were considered to be secondary effects related to the reduced growth and food intake in these groups, rather than primary effects of the compound(s) (Feron *et al.*, 1973; Oishi *et al.*, 1979; Falke and Til, unpublished observations, 1984–85).

DISCUSSION

The results of the study reported here demonstrated that combined exposure to four nephrotoxins at effective, minimally nephrotoxic levels resulted in increased general toxicity (growth depression) and increased renal toxicity (reflected in renal weight, morphology and urine-concentrating ability) in male but not in female rats. This sex difference may be related to the facts that *d*-limonene is nephrotoxic in male rats only (Kanerva and Alden, 1987; Lehman-McKeeman *et al.*, 1989; Webb *et al.*, 1989) and that female rats are less susceptible to mercuric chloride toxicity than male rats (Harber and Jennings, 1965). On the other hand, female rats are more susceptible than male rats to HCBD nephrotoxicity (Harleman and Seinen, 1979; Hook *et al.*, 1983); our study, also, showed that HCBD was more toxic to females than to males. In fact, the effects of HCBD on body weight and kidneys of females fed HCBD alone at its MNEL were already so severe, that this might have precluded the manifestation of a possible enhancement of toxicity by the combination.

In contrast to the obviously increased toxicity at the MNEL, simultaneous administration of the four nephrotoxins at their NNEL produced only weak indications of increased toxicity, again in males only (slightly retarded growth and, even less convincing, increased kidney weight). Combined exposure below the NNEL produced no signs of toxicity at all. As in our previous study of eight compounds with different target organs (Jonker *et al.*, 1990), the study, reported here, of compounds with the same target organ, clearly demonstrated absence of synergistic effects upon combined exposure when each compound is present at a sub-toxic level. A similar result was obtained in a 24-hr single-dose experiment in which a combination of the nephrotoxins mercuric chloride, potassium dichromate, *d*-limonene and HCBD was examined (D. Jonker *et al.*, 1993). Our findings are consistent with the results reported in a majority of the papers on combined exposure reviewed by Ikeda (1988), who detected no case to indicate that assumption of additive effects is unsafe. Similarly a committee of the National Research Council found that, in

most cases, the additive approach for dealing with the components of mixtures was appropriate (Henderson and Schlesinger, 1989). Contrary to these reassuring findings, we are faced with the recently reported synergism in induction of preneoplastic foci on combined exposure to five heterocyclic amines at levels as low as 1/25 of the carcinogenic doses of the individual compounds (Ito *et al.*, 1991). Furthermore, results from National Toxicology Program studies with a chemically defined mixture of 25 groundwater contaminants, administered at environmental levels, raised the possibility of synergistic interaction between a background long-term, low-level exposure to a chemical mixture and a subsequent acute dose resulting from accidental exposure or drug intake, including alcohol abuse (Yang *et al.*, 1989). Clearly, these examples call for further research in the area of the toxicology of chemical mixtures in order to detect, from the infinite number of possible combinations, those that present an increased hazard to health due to additive, synergistic or potentiating effects.

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Chapter 4

Acute (24 hr) toxicity of a combination of four nephrotoxicants in rats compared with the toxicity of the individual compounds

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ACUTE (24 HR) TOXICITY OF A COMBINATION OF FOUR NEPHROTOXICANTS IN RATS COMPARED WITH THE TOXICITY OF THE INDIVIDUAL COMPOUNDS

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Abstract—To identify possible hazards of combined exposure to chemicals with the same target organ, a 24-hr single dose experiment was carried out in which the renal toxicity of mercuric chloride, potassium dichromate, *d*-limonene and hexachloro-1:3-butadiene administered simultaneously was compared with the nephrotoxicity of the individual compounds, using a total of 11 groups each consisting of five 12-wk-old male Wistar rats. The dose levels used were based on the results of a range-finding study with the individual compounds in the same strain of rats kept under similar experimental conditions, and comprised the 'Minimum-Nephrotoxic-Effect Level' (MNEL) and the 'No-Nephrotoxic-Effect Level' (NNEL) of each of the four compounds alone and in combination. A group of vehicle-treated rats served as controls. At the MNEL of the combination, antagonism of effects was encountered, seen for example as less severely increased activity of γ-glutamyl transferase in the urine. Synergism of effects was also observed, for example increased severity of renal tubular necrosis, and more markedly increased activity of urinary lysozyme, lactate dehydrogenase, alkaline phosphatase and *N*-acetyl-β-glucosaminidase. More importantly, however, at the NNEL of the combination no signs of impaired renal function or renal damage were observed, suggesting absence of both dose additivity and potentiating interaction at the tested subeffective levels of the individual nephrotoxicants.

INTRODUCTION

Although exposure to combinations of compounds is the human 'real world' exposure situation, the great majority of toxicological studies still involve exposure to single compounds (Henderson and Schlesinger, 1989). On the other hand, the number of papers dealing with the toxicology of mixtures has gradually increased, indicating the interest of toxicologists in that branch of toxicology designated the 'toxicology of the 1990s and beyond' (Yang *et al.*, 1989).

Recently we have published a subacute oral study in rats in which the toxicity of a combination of eight chemicals was compared with that of the individual compounds, using dose levels equal to the 'Minimum-Observed-Adverse-Effect Level' (MOAEL), the 'No-Observed-Adverse-Effect Level' (NOAEL) and one-third and one-tenth of the NOAEL (Jonker *et al.*, 1990). The choice of the compounds was fully arbitrary with regard to type of action, possible interaction and target organ. In contrast to a great number of adverse effects and conspicuous interactions found at the MOAEL, there was no convincing evidence of increased toxicity of the combination of compounds at and below the NOAEL of the individual chemicals. For a combination of compounds with the same target organ and a similar mechanism of action, it is common practice to assume additivity of effects (Ikeda, 1988). To examine the possible additivity and/or interactions of chemicals with the same target organ, acute and subacute toxicity studies in rats were carried out with a combination of nephrotoxic compounds. The nephrotoxicants used affect the same part of the kidney, namely the proximal tubule, although presumably by different modes of action. The nephrotoxicity of hexachloro-1:3-butadiene (HCBD) has been shown to result from initial conjugation to glutathione (e.g. Dekant *et al.*, 1990). In a reaction catalysed by γ-glutamyl transpeptidase, the glutathione conjugate is converted into a cysteine conjugate, which is bioactivated by cysteine conjugate β-lyase present in cells of the renal proximal tubules. *d*-Limonene is one of a diverse group of chemicals that produce nephrotoxicity, specific to male rats, manifested acutely as an exacerbation of hyaline (protein) droplets in renal proximal tubular cells (Swenberg *et al.*, 1989; Webb *et al.*, 1989). *d*-Limonene and, more specifically, its metabolite *d*-limonene-1,2-oxide, associates with the

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Abbreviations: ALP = alkaline phosphatase; GGT = γ-glutamyl transferase; HCBD = hexachloro-1:3-butadiene; LDH = lactate dehydrogenase; MNEL = minimum-nephrotoxic-effect level; MOAEL = minimum-observed-adverse-effect level; NAG = *N*-acetyl-β-glucosaminidase; NNEL = no-nephrotoxic-effect level; NOAEL = no-observed-adverse-effect level.

male rat-specific protein $\alpha_{2\mu}$-globulin, and this interaction may be responsible for excessive accumulation of $\alpha_{2\mu}$-globulin in kidneys of male rats exposed to *d*-limonene (Lehman-McKeeman *et al.*, 1989). The mechanisms underlying the renal toxicity of the heavy metal salts mercuric chloride and potassium dichromate are still poorly understood. Proposed mechanisms involved in mercury-induced tubular toxicity are mitochondrial dysfunctioning due to angiotensin–renin-mediated ischaemia or inhibition of enzymes by direct binding to sulphhydryl groups (Commandeur and Vermeulen, 1990). The toxicity of hexavalent chromium is thought to be related to its intracellular reduction to trivalent chromium (Ryberg and Alexander, 1984); this process was suggested as a possible explanation for the impaired mitochondrial respiration observed soon after injection of dichromate (Kim and Na, 1990).

This paper describes the acute (24 hr) toxicity studies, comprising a range-finding study conducted to establish a 'No-Nephrotoxic-Effect Level' (NNEL) and a 'Minimum-Nephrotoxic-Effect Level' (MNEL), for each of the individual nephrotoxicants, and a main study. The goal of the main study was to compare, at a subtoxic and a toxic level, the toxicity of the four toxicants when given in combination, with the toxicity of the individual compounds. Because our intention was to identify possible hazards of combined exposure rather than to investigate the mechanism underlying such hazards, the studies are of a descriptive rather than a mechanistic nature.

MATERIALS AND METHODS

Test substances. Hexachloro-1:3-butadiene (HCBD, at least 98% pure) and mercuric chloride ($HgCl_2$, at least 99.5% pure) were obtained from E. Merck (Darmstadt, Germany), *d*-limonene from Sigma Chemical Co. (St Louis, MO, USA) and potassium dichromate puriss.cryst. ($K_2Cr_2O_7$) from De Onderlinge Pharmaceutische Groothandel G. A. (Utrecht, The Netherlands).

Animals and maintenance. 9-wk-old male Wistar rats [Crl:WI(WU)BR] were purchased from Charles River Wiga GmbH (Sulzfeld, Germany). They were housed conventionally, in a room controlled to maintain a temperature of 22–25°C, a relative humidity of 50–80% and a 12-hr light/dark cycle. Throughout the study the Institute's cereal-based stock diet and tap water were provided *ad lib.* On arrival, the rats were housed in groups of five in suspended stainless-steel cages with wire-mesh floor and front, and acclimatized for at least 6 days. Two or three days before dosing, the rats were weighed, distributed randomly and proportionately according to body weight over the various groups (five rats/group), and placed in stainless-steel metabolism cages (one rat/cage) until they were killed. The rats were dosed at the age of 10 wk (range-finding study) or 12 wk (main study), and were killed 24 hr after dosing by exsanguination from the abdominal aorta while under light ether anaesthesia.

Treatments. HCBD and *d*-limonene were dissolved in corn oil (Remia, Den Dolder, The Netherlands) and administered by oral gavage in a volume of 10 ml/kg. $HgCl_2$ and $K_2Cr_2O_7$ were dissolved in demineralized water and injected sc (neck region) in a volume of 1 ml/kg. For simultaneous administration of the four chemicals, two solutions were prepared: these were water containing both $HgCl_2$ and $K_2Cr_2O_7$, and corn oil containing both HCBD and *d*-limonene. The aqueous solutions were neutralized, using a 0.5 N-KOH solution, to minimize the possibility of tissue damage at the site of injection. The animals received a single dose of the nephrotoxicants or the vehicles at about 09.00 hr and were killed 24 hr thereafter.

Range-finding study. Three different doses of each nephrotoxicant were administered (Table 1). The lowest dose should be without evidence of kidney damage, whereas one or both of the higher doses should produce renal adverse effects within 24 hr. Two concurrent control groups received demineralized water (sc, 1 ml/kg) or corn oil only (orally, 10 ml/kg). The dose levels and routes of administration tested in the range-finding study were selected on the basis of literature data on the acute renal toxicity of the individual nephrotoxicants.

Main study. The doses used in the main study are shown in Table 2. Each nephrotoxicant was given alone at two different dose levels, selected on the basis of the results of the range-finding study, namely at a dose intended to be an NNEL and at an MNEL. Two additional groups received the four nephrotoxicants simultaneously at their individual NNEL and MNEL, respectively. Controls were dosed sc with water and orally with corn oil.

Collection of urine and blood

Two portions of urine were collected from each rat: the first portion was collected during the first 6 hr after dosing (09.00–15.00 hr), the second portion during the next 18 hr (15.00–09.00 hr). During collection all samples were kept cold in insulated containers filled with ice. At the time of killing, blood was sampled from the abdominal aorta in heparinized plastic tubes.

Table 1. Dose levels used in a 24-hr single dose range-finding study with four nephrotoxins in rats

	Dose level (mg/kg body weight)		
Nephrotoxin	low	mid	top
Potassium dichromate*	2.5	10	20
Mercuric chloride*	0.25	0.5	1.0
Hexachloro-1:3-butadiene†	10	100	200
d-Limonene†	10	50	200

*Injected sc as an aqueous solution, neutralized with 0.5 N-KOH, in a volume of 1 ml/kg.

†Administered by oral gavage as a solution in corn oil in a volume of 10 ml/kg.

Observations and analyses

The rats were weighed shortly before and 24 hr after dosing. Food and water intake over this 24-hr period were measured for each rat individually. Abnormalities in appearance or behaviour were recorded.

Urinalysis. At the end of each collection period, each sample was characterized with respect to volume (calibrated tubes), density (Bellingham and Stanley refractometer), appearance (visual inspection), and pH, protein, glucose, occult blood, ketones, uribilinogen and bilirubin (Combur-7-Test strips; Boehringer Mannheim GmbH, Mannheim, Germany). After centrifugation (5 min at 500 ***g***), the sediment was examined microscopically for red and white blood cells, epithelial cells, amorphous material, crystals, casts, bacteria, worm eggs and sperms. In the centrifuged urine samples, the following parameters were determined quantitatively: creatinine, alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), lactate dehydrogenase (LDH), total protein and glucose (Cobas-Bio Centrifugal Analyzer), *N*-acetyl-β-glucosaminidase (NAG; Boehringer reagent kit no. 815406, Boehringer Mannheim GmbH, Mannheim, Germany), potassium and sodium (Electrolyte-2-Analyzer), and lysozyme (as described by Harrison *et al.*, 1968, except that extinctions were measured at 37°C instead of 25°C). The quantitative parameters were expressed as units excreted per 6 hr (first portion) or 18 hr (second portion) by multiplying the concentrations measured by the volume of the urine excreted in these periods.

Plasma analyses. The blood samples were centrifuged at 1250 ***g*** for about 15 min, using Sure-sep II dispensers from General Diagnostics. In the plasma, urea and creatinine concentrations were measured with a Cobas-Bio Centrifugal Analyzer.

Pathology. The kidneys of all rats were weighed and the ratios of kidney weight to body weight were calculated. After fixation in 10% neutral buffered formalin, samples of the kidneys were embedded in paraffin, sectioned at 5 μm, stained with haematoxylin and eosin, and examined by light microscopy. In addition, kidney sections from control rats and rats treated with *d*-limonene were stained with Mallory trichrome (Azan) to facilitate detection of hyalin droplets in tubular epithelial cells.

Statistical analyses. Body weights were evaluated by one-way analysis of covariance (covariable: body weight before dosing), followed by Dunnett's multiple comparison tests. Food and water intake, organ weights, plasma urea and creatinine, urinary volume and density, and the results of quantitative measurements in the urine were evaluated by one-way analysis of variance, followed by Dunnett's multiple comparison tests.

Semi-quantitative observations in the urine were analysed by the Kruskal–Wallis nonparametric analysis of variance followed by the Mann–Whitney *U*-test. The histopathological changes were examined by Fisher's exact probability test. All analyses were two-sided.

RESULTS

Range-finding study

Results of the range-finding study are summarized in Tables 3 and 4. There were no deaths.

Mercuric chloride. Treatment-related effects were seen in animals receiving the mid and top doses. Effects in the top dose group (1 mg/kg) comprised reduced weight gain, decreased food and water intake, increased urea and creatinine blood plasma levels, increased number of epithelial cells in the urine, increased glucose excretion in the urine, decreased sodium excretion in the urine, strongly increased GGT, ALP and LDH activities in the urine, and extensive tubular necrosis in the outer strip of the outer renal medulla. At the mid-dose level (0.5 mg/kg), which was only a factor of two lower than the top dose level, the only adverse effects found were relatively low sodium excretion and slight renal tubular necrosis in two of five rats. The levels selected for the combination study were 0.25 and 1.0 mg $HgCl_2$/kg for the NNEL and MNEL, respectively.

Potassium dichromate. This induced fairly severe (renal) changes in the groups receiving the mid (10 mg/kg) and top (20 mg/kg) dose groups. The effects consisted of reduced weight gain and food intake, increased urea and creatinine levels in blood plasma (top dose only), decreased urinary pH (top dose only), the presence of occult blood and fewer crystals in the urine (top dose only), increased urinary excretion of protein and glucose, decreased sodium excretion in the urine, increased lysozyme, GGT, ALP, LDH and NAG activity in the urine, and moderate focal necrosis in the outer renal cortex. In

Table 2. Dose levels used in a 24-hr single dose experiment to study the effects of combined exposure to four nephrotoxins

Nephrotoxin	No-Nephrotoxic-Effect Level (NNEL) (mg/kg body weight)	Minimum-Nephrotoxic-Effect Level (MNEL) (mg/kg body weight)
Potassium dichromate*	1	10
Mercuric chloride*	0.25	1.0
Hexachloro-1:3-butadiene†	10	100
d-Limonene†	10	200

*Injected sc as an aqueous solution, neutralized with 0.5 N-KOH, in a volume of 1 ml/kg.
†Administered by oral gavage as a solution in corn oil in a volume of 10 ml/kg.

the low dose group (2.5 mg/kg) there was a tendency towards decreased sodium excretion and increased GGT and ALP activity in the 0–6-hr urine. The levels chosen for the combination study were 1.0 and 10 mg $K_2Cr_2O_7$/kg for the NNEL and the MNEL, respectively.

d-Limonene. The effect was restricted to exacerbated accumulation of protein (hyalin) droplets in epithelial cells of the renal proximal tubules ($\alpha_{2\mu}$-globulin nephropathy) of rats of the two highest dose groups (200 and 50 mg/kg). No significant changes were observed at 10 mg/kg. The levels selected for the combination study were 10 and 200 mg *d*-limonene/kg for the NNEL and the MNEL, respectively.

Hexachloro-1:3-butadiene. HCBD induced a variety of adverse effects at the two highest dose levels. In the top dose group (200 mg/kg), weight gain, food intake and density of the urine were decreased, whereas kidney weight, urea and creatinine blood plasma levels, urinary pH, occult blood and number of epithelial cells in the urine, urinary volume, excretion of protein, glucose and potassium, together with activities of GGT, ALP, LDH and NAG were increased. In the mid-dose group (100 mg/kg), kidney weight, blood plasma creatinine level, urinary pH, protein and glucose excretion, occult blood, number of epithelial cells, and activities of GGT, ALP, LDH and NAG were increased. These changes did not always reach the level of statistical significance, owing to the sometimes large variation in responses of individual animals. Microscopic examination of the kidneys revealed extensive tubular necrosis in rats given the top dose and limited, focal necrosis in those in the mid-dose group. No compound-related effects were found in the low dose group (10 mg/kg). For the combination study, 10 and 100 mg HCBD/kg were selected for the NNEL and MNEL, respectively.

Combination study

No deaths occurred. Animals given the MNEL of the combination showed a swollen nose and lethargy

Table 3. Effects on selected parameters in rats administered different doses of mercuric chloride or potassium dichromate; data from a 24-hr single dose range-finding study†

	Treatment						
		Mercuric chloride (mg/kg)			Potassium dichromate (mg/kg)		
Parameter	Control (water, 1 ml/kg)	0.25	0.5	1.0	2.5	10	20
Body weight, g	309.2 ± 3.4	308.4 ± 5.4	310.8 ± 5.1	301.7 ± 4.3*	311.6 ± 5.6	295.0 ± 6.5*	295.1 ± 1.5*
Food intake, g/24 hr	18.7 ± 0.2	17.4 ± 0.4	17.1 ± 0.8	10.8 ± 1.1*	20.3 ± 1.1	10.1 ± 1.3*	6.3 ± 0.8*
Water intake, g/24 hr	23.2 ± 1.6	20.8 ± 1.4	22.7 ± 1.2	15.5 ± 1.2*	23.2 ± 1.3	17.5 ± 2.9	23.7 ± 4.8
Kidney weight, g/kg	6.42 ± 0.16	6.64 ± 0.21	6.34 ± 0.06	6.94 ± 0.11	6.46 ± 0.32	7.09 ± 0.25	6.94 ± 0.24
Urea, mmol/litre	5.51 ± 0.36	5.63 ± 0.28	5.72 ± 0.19	7.98 ± 0.49*	5.58 ± 0.28	6.01 ± 0.35	12.05 ± 0.85*
Creatinine, μmol/litre	65.9 ± 2.4	65.6 ± 1.0	69.2 ± 2.7	85.4 ± 7.6*	65.9 ± 2.1	78.5 ± 2.7	135.3 ± 10.0*
Urinalysis 0–6 hr							
pH	7.1	6.7	6.9	7.6	6.8	6.6	5.6*
Occult blood (0–3)	1	1	0	0	1	1	2
Volume, ml/6 hr	2.6 ± 0.5	2.3 ± 0.3	2.4 ± 0.5	1.8 ± 0.3	2.0 ± 0.3	2.9 ± 0.7	2.6 ± 0.3
Density, kg/litre	1.032 ± 0.002	1.038 ± 0.004	1.033 ± 0.006	1.032 ± 0.003	1.031 ± 0.003	1.037 ± 0.006	1.038 ± 0.003
Protein, mg/6 hr	5.22 ± 0.76	5.17 ± 1.28	4.49 ± 0.51	3.73 ± 0.58	4.84 ± 0.77	10.77 ± 1.72*	15.08 ± 1.16*
Glucose, mM/6 hr	1.90 ± 0.29	1.74 ± 0.22	1.55 ± 0.20	1.24 ± 0.25	1.63 ± 0.34	3.37 ± 0.58	90.17 ± 48.43*
Potassium, μM/6 hr	453 ± 62	400 ± 39	367 ± 19	348 ± 65	375 ± 83	649 ± 77	620 ± 33
Sodium, μM/6 hr	426 ± 85	329 ± 54	287 ± 40	223 ± 42*	224 ± 73	206 ± 36	153 ± 16*
GGT, U/6 hr	8.6 ± 2.0	10.2 ± 2.2	9.2 ± 1.8	6.3 ± 1.3	12.7 ± 1.9	24.5 ± 5.4*	34.3 ± 3.0*
ALP, U/6 hr	0.47 ± 0.15	0.53 ± 0.11	0.46 ± 0.06	0.26 ± 0.08	0.62 ± 0.08	1.46 ± 0.29*	2.42 ± 0.23*
LDH, mU/6 hr	84.8 ± 28.3	55.7 ± 8.4	54.8 ± 5.9	39.1 ± 3.0	78.4 ± 9.4	253.8 ± 53.7	563.2 ± 110.1*
NAG, mU/6 hr	41.7 ± 8.4	36.1 ± 8.1	28.2 ± 4.5	20.6 ± 5.8	35.4 ± 7.7	84.7 ± 14.6	254.7 ± 53.5*
Lysozyme (0–4)	0	0	0	0	0	1	4
Urinalysis 6–24 hr							
Occult blood (0–3)	1	1	0	1	1	1	3
Epithelial cells (0–5)	1	1	1	3	1	2	2
Crystals (0–5)	4	4	4	4	3	3	1*
Volume, ml/18 hr	6.3 ± 0.8	6.5 ± 0.3	5.9 ± 0.8	5.3 ± 0.3	6.5 ± 0.7	9.6 ± 1.7	15.3 ± 3.2*
Density, kg/litre	1.051 ± 0.001	1.052 ± 0.003	1.054 ± 0.004	1.059 ± 0.002	1.055 ± 0.002	1.039 ± 0.004	1.030 ± 0.006*
Protein, mg/18 hr	18.82 ± 2.86	21.28 ± 1.51	17.17 ± 2.44	22.80 ± 3.30	21.62 ± 2.34	49.90 ± 4.01*	67.41 ± 3.67*
Glucose, mM/18 hr	10.61 ± 1.13	11.49 ± 1.21	9.81 ± 1.36	52.88 ± 23.67	11.22 ± 0.39	10.44 ± 1.02	18.23 ± 4.5
Potassium, μM/18 hr	2205 ± 268	2423 ± 201	2194 ± 265	2252 ± 132	2425 ± 192	2169 ± 206	2199 ± 116
Sodium, μM/18 hr	1590 ± 183	1427 ± 148	1171 ± 93	863 ± 72*	1642 ± 163	1608 ± 168	1406 ± 171
GGT, U/18 hr	17.4 ± 3.0	19.0 ± 3.0	16.7 ± 4.0	70.9 ± 22.1*	24.1 ± 2.4	25.3 ± 1.7	23.5 ± 1.2
ALP, U/18 hr	1.15 ± 0.14	1.35 ± 0.16	0.93 ± 0.21	4.97 ± 1.67*	1.33 ± 0.19	2.35 ± 0.34*	3.10 ± 0.11*
LDH, mU/18 hr	378 ± 75	467 ± 78	422 ± 86	3125 ± 1107*	336 ± 31	3095 ± 657*	5465 ± 901*
NAG, mU/18 hr	185 ± 35	172 ± 16	122 ± 14	166 ± 17	207 ± 19	877 ± 43*	1707 ± 139*
Lysozyme (0–4)	0	0	0	0	0	3	4
Renal microscopy							
Tubular necrosis (number of rats)	0	0	2	5*	1	5*	5*

GGT = γ-glutamyl transferase ALP = alkaline phosphatase LDH = lactate dehydrogenase NAG = *N*-acetyl-β-glucosaminidase

†Rats received a single sc dose of the individual nephrotoxins and were killed 24 hr later.

Values are means ± SE (n = 5); for parameters measured semi-quantitatively, only the means are given.

*Statistically significantly different from control ($P < 0.05$).

Table 4. Effects on selected parameters in rats administered different doses of *d*-limonene or hexachloro-1:3-butadiene, data from a 24-hr single dose range-finding study†

Parameter	Control (corn oil, 10 ml/kg)	*d*-Limonene (mg/kg)			Hexachloro-1:3-butadiene (mg/kg)		
		10	50	200	10	100	200
Body weight, g	302.8 ± 5.0	296.3 ± 4.6	299.1 ± 6.8	297.6 ± 6.7	297.0 ± 3.9	295.7 ± 4.3	295.5 ± 4.3*
Food intake, g/24 hr	11.7 ± 0.7	11.9 ± 0.5	12.2 ± 1.0	11.7 ± 1.1	10.0 ± 0.6	8.9 ± 1.3	6.9 ± 0.9*
Water intake, g/24 hr	21.6 ± 1.7	21.2 ± 0.7	20.3 ± 0.8	17.6 ± 0.8	19.2 ± 1.4	19.4 ± 3.3	36.4 ± 8.8
Kidney weight, g/kg	6.36 ± 0.06	6.17 ± 0.15	6.60 ± 0.12	6.31 ± 0.11	6.54 ± 0.17	7.15 ± 0.25*	7.64 ± 0.10*
Urea, mmol/litre	5.17 ± 0.19	5.27 ± 0.26	5.31 ± 0.22	5.37 ± 0.39	5.02 ± 0.14	5.46 ± 0.27	8.73 ± 0.40*
Creatinine, μmol/litre	64.7 ± 2.3	65.9 ± 2.5	61.4 ± 2.3	68.5 ± 3.0	63.3 ± 1.7	80.6 ± 5.5*	125.9 ± 3.1*
Urinalysis 0–6 hr							
No treatment-related changes							
Urinalysis 6–24 hr							
pH	5.6	5.6	5.4	5.3	5.4	6.9*	7.2*
Occult blood (0–3)	0	1	0	0	0	2*	2*
Epithelial cells (0–5)	1	1	1	1	1	4*	4*
Volume, ml/18 hr	4.7 ± 0.4	5.8 ± 0.6	4.2 ± 0.7	4.6 ± 0.2	4.5 ± 0.7	8.5 ± 3.8	26.6 ± 7.4*
Density, kg/litre	1.052 ± 0.001	1.053 ± 0.002	1.058 ± 0.003	1.058 ± 0.001	1.057 ± 0.001	1.046 ± 0.007	1.021 ± 0.003*
Protein, mg/18 hr	17.42 ± 1.52	20.31 ± 1.14	14.42 ± 2.05	13.68 ± 2.19	15.87 ± 2.89	24.38 ± 4.59	48.31 ± 3.79*
Glucose, mM/18 hr	8.58 ± 0.76	9.60 ± 1.06	9.07 ± 1.53	8.73 ± 0.52	7.98 ± 1.30	25.14 ± 13.90	699.9 ± 148.5*
Potassium, μM/18 hr	1494 ± 138	1775 ± 173	1500 ± 245	1411 ± 121	1472 ± 245	1713 ± 133	2371 ± 101*
Sodium, μM/18 hr	890 ± 92	1038 ± 54	795 ± 170	795 ± 56	685 ± 119	897 ± 168	1110 ± 172
GGT, U/18 hr	24.0 ± 1.3	31.1 ± 4.4	26.4 ± 4.7	20.3 ± 2.3	25.8 ± 5.3	62.2 ± 19.7	176.9 ± 14.3*
ALP, U/18 hr	0.88 ± 0.13	1.29 ± 0.18	0.94 ± 0.16	0.72 ± 0.12	0.70 ± 0.09	3.40 ± 0.90	13.77 ± 2.31*
LDH, mU/18 hr	275 ± 40	437 ± 75	309 ± 60	339 ± 48	318 ± 54	3453 ± 1084*	12655 ± 583*
NAG, mU/18 hr	115 ± 11	154 ± 7	143 ± 24	140 ± 19	134 ± 27	303 ± 58*	582 ± 69*
Renal microscopy number of rats with:							
Tubular necrosis	0	0	0	0	0	5*	5*
Accumulation of protein droplets	0	1	5*	5*	0	0	0

Values are means ± SE (n = 5); for parameters measured semi-quantitatively, only the means are given.
†Rats received a single oral dose of the individual nephrotoxins and were killed 24 hr later.
*Statistically significantly different from control ($P < 0.05$).

shortly after dosing, and encrustations around the nostrils when killed. Lethargy and swollen noses were also observed in the group given the effective dose of chromate. Tables 5 and 6 show that many of the parameters measured were adversely affected at the MNEL of both the combination and the individual compounds, with the exception of *d*-limonene that, as expected, only induced excessive accumulation of protein droplets in renal proximal tubular epithelial cells. At the NNEL, *d*-limonene (contrary to expectation) and the combination appeared to have exacerbated protein droplet accumulation; otherwise, no treatment-related changes were observed following combined or single exposure at the NNEL.

DISCUSSION AND CONCLUSION

The results of the study described here suggest that simultaneous administration of four nephrotoxicants in effective (toxic) doses results in a myriad of (renal) adverse effects that, in comparison with the effects of the individual compounds, are similar or different in type, and range from less to (much) more severe.

The marked increases in the urinary activities of LDH, NAG and lysozyme in the combination MNEL group were much greater than those induced by the individual nephrotoxicants, indicating synergistic interaction (Fig. 1). Enhancement of effects on combined exposure, although less pronounced, was also seen with blood plasma creatinine and urea. When evaluating the effect on plasma urea, the decrease in urea concentration in the HCBD MNEL group (Table 5) was considered to be a casual finding unrelated to HCBD, because in the range-finding study plasma urea either was not affected or was increased in the HCBD groups (Table 4). On the other hand, the increase in urinary GGT excretion (6–24 hr) in the combination MNEL group was smaller than that in the mercury and HCBD MNEL groups, suggesting antagonism of effects for this endpoint.

Approximately additive responses were observed for the excretion of glucose and protein in the urine (6–24 hr). Combined exposure appeared to accelerate the onset of the effect on protein excretion, as only the combination group showed a significantly increased protein excretion between 0 and 6 hr after dosing. Urinary ALP (6–24 hr) showed partially additive responses, the response in the combination group being larger than that induced by the individual nephrotoxicants, but smaller than the sum of the individual responses.

The findings for urinary sodium and potassium excretion are difficult to interpret. The urinary sodium excretion in the combination MNEL group was similar to that in controls, despite a strikingly decreased urinary sodium excretion in the mercury MNEL group. On the other hand, urinary potassium excretion was increased in the combination group but not in any of the single agent groups. The increased

Table 5. Selected parameters influenced by the individual nephrotoxins or their combination at the Minimum-Nephrotoxic-Effect Level of the individual compounds†

Parameter	Group				
	Control	Combination	Mercuric chloride	Potassium dichromate	Hexachloro-1:3-butadiene
Food intake, g/24 hr	9.9 ± 0.2	2.8 ± 0.6*	4.4 ± 1.2*	3.7 ± 1.1*	8.6 ± 1.4‡
Water intake, g/24 hr	15.1 ± 0.9	23.9 ± 5.8	10.6 ± 1.2*	15.5 ± 4.6	13.1 ± 1.9
Kidney weight, g/kg	6.23 ± 0.08	7.91 ± 0.24*	7.43 ± 0.38*	6.43 ± 0.15‡	7.39 ± 0.13*
Urea, mmol/litre	6.00 ± 0.21	13.89 ± 1.52*	8.64 ± 0.45*	5.26 ± 0.20‡	4.95 ± 0.23*,‡
Creatinine, μmol/litre	59.2 ± 2.1	179.9 ± 21.0*	95.8 ± 9.6*,‡	71.6 ± 3.3*,‡	67.1 ± 3.0‡
Urinalysis 0–6 hr					
pH	7.2	6.4*	6.9	6.2*	7.7
Occult blood (0–3)	0	2*	1	1	1
Volume, ml/6 hr	2.9 ± 0.2	3.9 ± 1.4	1.8 ± 0.3*	4.2 ± 1.9	1.6 ± 0.4*
Density, kg/litre	1.033 ± 0.003	1.039 ± 0.009	1.043 ± 0.006	1.033 ± 0.010	1.032 ± 0.001
Protein, mg/6 hr	6.91 ± 1.06	12.12 ± 1.59*	5.20 ± 0.99	9.78 ± 0.60	3.96 ± 1.32
Glucose, mM/6 hr	2.01 ± 0.15	5.70 ± 2.14	1.76 ± 0.31	10.01 ± 2.75*	1.19 ± 0.34*
Potassium, μM/6 hr	398 ± 38	674 ± 64*	350 ± 64	394 ± 28	287 ± 270
Sodium, μM/6 hr	258 ± 39	228 ± 37	182 ± 37	141 ± 28*	143 ± 40
GGT, U/6 hr	14.9 ± 2.0	19.4 ± 1.9	14.9 ± 2.9	26.1 ± 1.2*	7.8 ± 2.2*
ALP, U/6 hr	0.60 ± 0.07	1.06 ± 0.18*	0.51 ± 0.11	1.98 ± 0.23*,‡	0.24 ± 0.11*,‡
LDH, mU/6 hr	73.8 ± 13.5	204.3 ± 50.8*	50.7 ± 11.9	374.6 ± 78.2*	37.7 ± 13.7
NAG, mU/6 hr	53.8 ± 4.8	85.5 ± 9.1*	38.8 ± 7.0‡	88.5 ± 3.6*	22.9 ± 9.6*,‡
Lysozyme, μg/6 hr	0 ± 0	6 ± 3	0 ± 0	1 ± 1	0 ± 0
Urinalysis 6–24 hr					
pH	5.2	6.7*	5.8*,‡	6.4*	6.8*
Occult blood (0–3)	1	2*	2	2	2*
Epithelial cells (0–5)	1	3*	4*	1‡	4*
Crystals (0–5)	3	0*	2	2	4
Volume, ml/18 hr	4.5 ± 0.3	18.6 ± 2.8*	3.7 ± 0.3‡	10.5 ± 1.9*,‡	6.4 ± 0.4*,‡
Density, kg/litre	1.065 ± 0.003	1.023 ± 0.003*	1.062 ± 0.003‡	1.033 ± 0.004*	1.052 ± 0.005‡
Protein, mg/18 hr	19.44 ± 1.71	73.87 ± 5.86*	18.25 ± 1.22‡	45.46 ± 3.12*,‡	29.01 ± 2.22*,‡
Glucose, mM/18 hr	10.47 ± 0.62	63.13 ± 27.95	60.27 ± 22.23*	8.56 ± 0.97	17.17 ± 2.60*
Potassium, μM/18 hr	1637 ± 57	2148 ± 176*	1300 ± 118	1608 ± 172	1792 ± 134
Sodium, μM/18 hr	796 ± 56	728 ± 76	243 ± 88*	856 ± 93	768 ± 101
GGT, U/18 hr	27.5 ± 2.6	43.8 ± 6.0*	69.4 ± 8.2*,‡	35.0 ± 2.4	50.3 ± 5.9*
ALP, U/18 hr	0.83 ± 0.09	4.61 ± 0.74*	3.52 ± 0.51*	2.88 ± 0.25*,†	2.18 ± 0.20*,‡
LDH, mU/18 hr	318 ± 78	32269 ± 6888*	3576 ± 671*,‡	968 ± 127*,‡	2565 ± 253*,‡
NAG, mU/18 hr	175 ± 20	1812 ± 460*	150 ± 20‡	502 ± 56*,‡	301 ± 19*,‡
Lysozyme, μg/18 hr	0 ± 0	1140 ± 163*	1 ± 0	478 ± 251	0 ± 0

†Rats received a single dose of the individual nephrotoxins or their combination, at the no-nephrotoxic (NNEL) or the nephrotoxic (MNEL) level of the individual toxins, and were killed 24 hr later. Values at the NNEL were unremarkable and are therefore not presented. *d*-Limonene was not included in the Table because the only renal effect seen was protein droplet accumulation in proximal tubular epithelium.

*Statistically significantly different from control ($P < 0.05$).

‡Statistically significantly different from the MNEL of the combination ($P < 0.05$).

Differences between the combination and the individual toxins were evaluated statistically only where both the combination and one or more of the individual toxins differed statistically significantly from controls.

Values are mean ± SE (n = 5); for parameters measured semi-quantitatively only the means are given.

potassium excretion may have resulted from extrarenal tissue damage induced by the combination but not by the individual compounds.

This study focused exclusively on establishing the presence or absence of interactive effects, and elucidating their nature if present. Explanatory studies

Table 6. Histopathological changes in kidneys of rats administered four different nephrotoxins either alone or in combination†

Observation	Group (five rats/group)										
	Control	Combination		Mercuric chloride		Potassium dichromate		Hexachloro-1:3-butadiene		*d*-Limonene	
		NNEL	MNEL	NNEL	MNEL	NNEL	MNEL	NNEL	MNEL	NNEL	MNEL
Necrosis											
minimal	0	0	0	0	0	0	0	0	2	0	0
slight	0	0	0	0	0	0	5*	0	0	0	0
moderate	0	0	0	0	5*	0	0	0	1	0	0
severe	0	0	5*	0	0	0	0	0	0	0	0
Protein droplet accumulation											
+ +	0	5*	0	0	0	0	0	0	0	5*	0
+ + +	0	0	0	0	0	0	0	0	0	0	5*

†Rats received a single dose of the individual nephrotoxins or their combination, at the no-nephrotoxic (NNEL) or the nephrotoxic (MNEL) level of the individual toxins, and were killed 24 hr later.

*Statistically significantly different from control ($P < 0.05$).

Values are the number of rats with the indicated observation, based on microscopic assessment of sections stained with haematoxylin and eosin or with Mallory trichrome.

should include combinations of two compounds, more specific endpoints and pharmacokinetic/dynamic analyses. In this regard it would be of interest, for example to determine why the specific

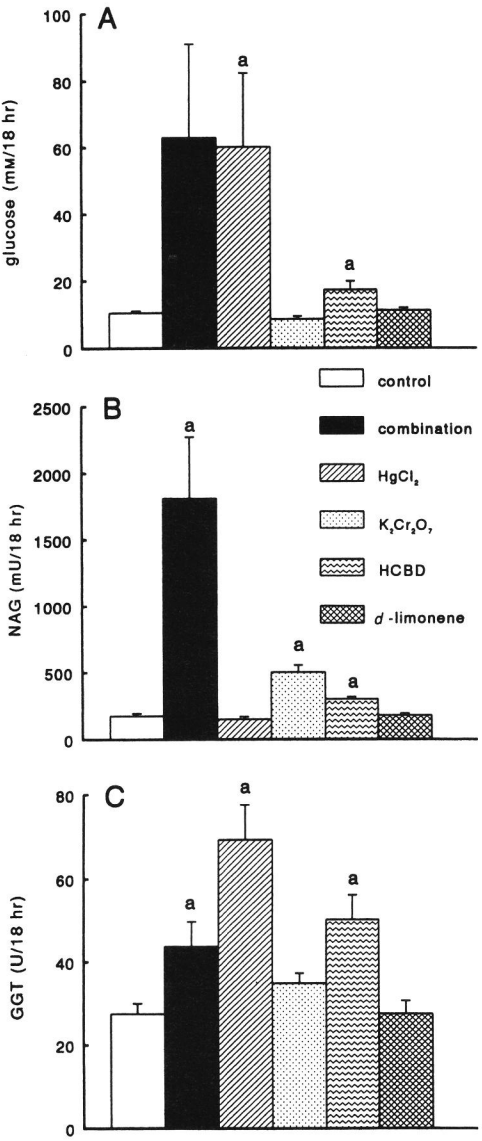

Fig. 1. Diagrams illustrating (A) additivity, (B) synergistic interaction and (C) antagonistic interaction. The excretion of glucose, *N*-acetyl-β-glucosaminidase (NAG) and γ-glutamyl transferase (GGT) in the urine was measured between 6 and 24 hr after administration of the individual nephrotoxins or their combination at the nephrotoxic (MNEL) level of the individual compounds. Bars represent means $\pm$ SE (n = 5). [a]Significantly different from the control group ($P < 0.05$).

d-limonene effect (accumulation of hyaline droplets in renal tubular cells; Webb *et al.*, 1989) was absent after the combined treatment at the MNEL. Further, it is challenging to detect the mechanisms underlying the high excretion of potassium, LDH, NAG and lysozyme in the urine on combined exposure.

No signs of impaired renal function or renal damage were observed at the NNEL of the combination, suggesting absence of both dose additivity and potentiating interaction at the subtoxic effect levels of the individual substances. Clearly, the simultaneous treatment of rats with subtoxic doses of the four nephrotoxicants did not lead to any serious renal toxicity, despite the fact that these compounds have the same critical target organ. An obvious explanation for the absence of additivity is that the mode of action including the critical renal target cells of the various agents was sufficiently different to avoid additivity. Another (trivial) explanation could be that the gap between those NNELs tested and the (unknown) true minimum adverse effect levels was too large to detect possible additivity. However, narrowing down further the gap between the NNELs and MNELs tested would have increased the chance of using one or more NNELs that, because of interstudy variability, for example, in the combination study turned out to be an effect level. The increased protein droplet accumulation at the NNEL of *d*-limonene (10 mg/kg), observed in the combination study but not in the range-finding study, illustrates that such an unwanted situation may occur easily.

The absence of potentiating interaction on combined exposure at the NNEL in this study is in contrast with the potentiating action of $K_2Cr_2O_7$ reported in the literature. Co-administration of a subtoxic dose of $K_2Cr_2O_7$ (10 mg/kg) with minimally toxic or ineffective doses of various, chemically unrelated, nephrotoxicants ($HgCl_2$, citrinin or maleic acid) invariably resulted in enhanced nephrotoxicity (Baggett and Berndt, 1984, 1985 and 1986; Christenson *et al.*, 1989; Haberman *et al.*, 1987). The difference in the subtoxic chromate doses used (1 *v.* 10 mg/kg) may explain this discrepancy.

A logical sequel to the study described in this paper would be a study in which the renal toxicity of a combination of a number of nephrotoxicants with very similar, if not identical, mechanisms of action would be compared with the renal toxicity of the individual nephrotoxicants. Such a study is currently being performed in our laboratory. In this respect it may be of interest to refer to a recent study by Ito *et al.* (1991), who demonstrated additive and synergistic effects on the rat liver of five heterocyclic amines administered simultaneously at concentrations of one-fifth or one-twenty-fifth of the effective (carcinogenic) levels of the individual amines.

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Chapter 5

The additivity assumption tested for combinations of similarly acting nephrotoxicants

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The additivity assumption tested for mixtures of similarly acting nephrotoxicants

Abstract

The present study was conducted to test the additivity assumption (dose addition) under conditions of concurrent, repeated exposure to similarly acting nephrotoxicants, at subtoxic levels of the individual chemicals. Tetrachloroethylene, trichloroethylene, hexachloro-1:3-butadiene and 1,1,2-trichloro-3,3,3-trifluoropropene were used as model compounds. Their nephrotoxicity results from initial conjugation to glutathione in the liver, followed by further biotransformation to cysteine conjugates and cysteine conjugate ß-lyase-mediated formation of reactive metabolites in the renal proximal tubular epithelial cells. The nephrotoxicants were given to female Wistar rats by daily oral gavage for 32 days either alone, both at a Lowest-Observed-Nephrotoxic-Effect Level (LONEL) and at a No-Observed-Nephrotoxic-Effect Level (NONEL, equal to 1/4 LONEL), or in combinations of four (at the NONEL and 1/2 LONEL) or three (at 1/3 LONEL). Relative kidney weight was increased following exposure to the individual nephrotoxicants at their LONEL and, to about the same extent, following combined exposure at the NONEL or 1/3 LONEL. The other endpoints used to assess renal toxicity (viz. renal morphology and concentrating ability, plasma levels of creatinine and urea, urinary excretion of glucose, protein and marker enzymes) were not or only scarcely affected upon combined exposure at the NONEL or 1/3 LONEL. Interpretation hereof is complicated because these endpoints, unlike kidney weight, were not affected at the LONEL of each of the individual chemicals. Combined exposure at 1/2 LONEL resulted in clear nephrotoxicity as indicated by the effects on most of the above endpoints. At this level the increase in kidney weight was clearly less than expected on the basis of dose additivity. It was concluded that the results of this study provided support for the assumption of dose additivity for mixtures of similarly acting systemic toxicants under conditions of concurrent, repeated exposure at dose levels below the toxicity thresholds of the individual chemicals.

Abbreviations

ALAT = alanine aminotransferase; ALP = alkaline phosphatase; ASAT = aspartate aminotransferase; GGT = γ-glutamyl transferase; HCBD = hexachloro-1,3-butadiene; LDH = lactate dehydrogenase; LONEL = Lowest-Observed-Nephrotoxic-Effect Level; NAG = N-acetyl-β-glucosaminidase; NOAEL = No-Observed-Adverse-Effect Level; NONEL = No-Observed-Nephrotoxic-Effect Level; TCTFP = 1,1,2-trichloro-3,3,3-trifluoropropene; TETRA = tetrachloroethylene; TRI = trichloroethylene.

Introduction

The assessment of health hazards from exposure to mixtures of chemicals, which is the usual human exposure situation, is complicated by a relative paucity of empirical data on the toxicity of mixtures, especially under conditions of prolonged exposure to low, non-toxic levels of the individual chemicals. Therefore, one of the major aims of our laboratory's research programme on mixture toxicity is to test the hypothesis that as a rule exposure to mixtures of chemicals at (low) non-toxic doses of the individual chemicals is of no health concern (Feron *et al*, 1995).

In previously conducted subacute toxicity studies in rats with mixtures of eight or nine chemicals with different primary target organs or mixtures of four chemicals with the same target organ (the kidney) but dissimilar modes of action we found that combined exposure at non-toxic levels of the individual chemicals did not constitute an obviously increased hazard (Groten *et al.*, 1997; Jonker *et al.*, 1990, 1993a,b). The highest non-toxic level tested in these studies was the No-Observed-Adverse-(Renal) Effect Level (NOAEL) of the individual chemicals, which was only 3-5 times lower than the individual chemicals' minimally toxic level in most cases. The absence of obvious toxic effects upon combined exposure at the NOAEL, therefore, allowed the conclusion that there were no marked synergistic effects at this level.

When assessing the health risk from exposure to chemical mixtures it is also important to know whether mixture components act in a dose-additive manner because this type of joint action implies that the summed dose can be high enough to elicit toxic effects even when the components are present at individually non-toxic levels (Bliss, 1939; Plackett and Hewlett, 1952; Finney, 1971). The assumption of dose addition is most clearly justified for chemicals which produce the same effect by a similar mode of action. In practice, however, a chemical's mode of action is generally unknown and, therefore, the justification of the assumption of dose addition will often be limited to similarities in toxic effects or target organs (EPA, 1986).

There is not much experimental evidence to support the validity of additivity assumptions in mammalian species, partly because most of the published studies on mixture toxicity, including our above mentioned studies with nephrotoxicants, did not use experimental designs or statistical methods suitable for (quantitative) evaluation of the type of joint action (Mumtaz and Hertzberg, 1993). The results of a few older studies, in which many pairs of industrial organic chemicals were administered to rats, demonstrated that dose additivity predicted reasonably well the acute toxicity (LD50 or LC50) of mixtures of randomly selected chemicals (Pozzani *et al.*, 1959; Smyth *et al.*, 1969, 1970). The assumption of dose addition was also supported by the results of an

in vitro study in which blood of sheep was exposed to binary or ternary mixtures of methaemoglobin-producing agents (chlorite, nitrite, copper) at minimally effective concentrations (Langlois and Calabrese, 1992). We were not aware of any studies examining the validity of dose addition under conditions of concurrent, repeated exposure to more than two similarly acting systemic toxicants at exposure levels slightly below the toxicity thresholds of the individual chemicals. Therefore, the aim of the present study was to test the additivity assumption (dose addition) under such conditions, using the same test system (rat), exposure route and duration (oral, about 4 weeks), and target organ (kidney) as in our previous study with dissimilarly acting nephrotoxicants (Jonker *et al*, 1993a).

Tetrachloroethylene (TETRA), trichloroethylene (TRI), hexachloro-1:3-butadiene (HCBD) and 1,1,2-trichloro-3,3,3-trifluoropropene (TCTFP) were selected as model compounds for this study on the basis of the availability of data indicating that these chemicals affect the same organ by a similar mode of action. Though occurrence of simultaneous exposure of humans to these chemicals was not a criterion for selection, TETRA and TRI are organic solvents of widespread industrial use and frequently occur together in contaminated media (soil, air, water) near hazardous waste sites (Johnson and DeRosa, 1995). HCBD is also a widely distributed environmental contaminant. It is a by-product of the manufacture of chlorinated hydrocarbons such as TETRA, TRI and carbon tetrachloride and has a variety of industrial and commercial applications (IARC, 1979). Consequently, human occupational or environmental exposure to mixtures including these chemicals is likely to occur. TCTFP has no widespread uses. Its nephrotoxicity was examined to verify predictions for metabolic pathways based on its chemical reactivity and structural similarity to TETRA (Vamvakas *et al.*, 1989).

The nephrotoxicity of the model compounds has been shown to result from initial conjugation to glutathione in the liver (Anthony *et al.*, 1994; Dekant *et al.*, 1986a,b and 1990; Goeptar *et al.*, 1995; Odum and Green, 1987; Vamvakas *et al.*, 1989). In several steps, catalysed by gamma-glutamyl transpeptidase and cysteinylglycine dipeptidase, the glutathione conjugate is converted into a cysteine conjugate. The cysteine conjugate, in turn, is bioactivated by the cysteine conjugate ß-lyase present in epithelial cells of renal proximal tubules. For TETRA and TRI, which are primarily metabolised by the cytochrome P450 mixed function oxidase system, the glutathione pathway is minor at low dose levels in the rat. However, it increases markedly at higher dose levels following saturation of the cytochrome P450 pathway (Dekant *et al.*, 1986c; Green *et al.*, 1990).

The four nephrotoxicants were given to the rats by daily oral gavage either alone, both at a Lowest-Observed-Nephrotoxic-Effect Level (LONEL) and at a No-Observed-

Nephrotoxic-Effect Level (NONEL), or as ternary or quaternary mixtures at fractions of the individual LONELs. Prior to the mixture study, the individual chemicals were tested in a range-finding study to establish their LONEL and NONEL under experimental conditions similar to those of the mixture study; the results are included in this chapter. For each nephrotoxicant the NONEL and LONEL were selected such that the NONEL was one fourth of the LONEL, to enable comparison of the effect of combined exposure to four different chemicals at their respective NONELs with the effect of exposure to four times the NONEL (= the LONEL) of a single chemical. The groups given a mixture of three chemicals received each chemical at a level equivalent to one third of its LONEL. When full additivity is assumed, the effects of these mixtures and those of the single chemicals at their LONEL are expected to be the same ('toxicity unit method'). Additionally, the study included a group given the four chemicals simultaneously at one half of their LONEL to enable evaluation of the predictive value of mixture results obtained at minimally toxic levels for what would occur at non-toxic levels of the individual chemicals.

Materials and methods

Materials

Hexachloro-1,3-butadiene (98%) was obtained from Aldrich-Chemie (Steinheim, Germany), tetrachloroethylene (99.9+%, HPLC grade) from Aldrich Chemical Co., Ltd. (Dorset, England), trichloroethylene (99+%, spectrophotometric grade) from Aldrich Chemical Company, Inc. (Milwaukee, USA), and 1,1,2-trichloro-3,3,3-trifluoro-1-propene from ABCR GmbH & Co (Kalsruhe, Germany). Corn oil was obtained from Remia (Den Dolder, The Netherlands).

Animals and maintenance

Weanling female SPF-bred Wistar rats (Crl:WI(WU) BR) were obtained from Charles River Wiga GmbH (Sulzfeld, Germany). Male rats were considered less suitable for this study. Firstly because male rats are burdened with an extra load of physiological proteins in the urine which might obscure possible treatment-related changes in urinary protein excretion. Secondly, TETRA induces the male-rat specific $\alpha_{2\mu}$-globulin nephropathy by a mechanism that differs from the mechanism of concern in this study (Goldsworthy *et al.*, 1988). At initiation of treatment, the rats were about five weeks old (except for the range-finding study with TCTFP in which they were eight weeks old). The rats were housed conventionally, in suspended stainless-steel cages with wire-mesh floor and front, five rats per cage, in a room maintained at

22±2°C with a relative humidity of 40-70% and a 12-hr light/dark cycle (lighting by fluorescent tubes between 7:30 and 19:30 hr). For collection of urine, the animals were placed individually in stainless-steel metabolism cages. Feed (our routinely used cereal-based rodent diet, described in Rutten and De Groot, 1992) and tap water were freely available except during the urine concentration test.

Administration route and duration

The nephrotoxicants were dissolved in corn oil, either alone or in combination, and administered by oral gavage in a volume of 10 ml per kg body weight, once daily for 32 consecutive days (exceptions: in the range-finding study TCTFP and HCBD were given for 27 and 33 days, respectively, and in the second range-finding study with TETRA and TRI treatment lasted 29 days). Concurrent controls were dosed with corn oil only. The dose volumes were adjusted twice per week for changes in body weight. Fresh dosing solutions were prepared once (mixture study, second range-finding study with TETRA and TRI) or twice, divided over portions for one week (second range-finding study with TETRA and TRI) or half a week, and stored in a refrigerator (2-10°C) until use. Stability of the nephrotoxicants under simulated experimental conditions was confirmed by analysis.

Experiments

Range-finding studies. Range-finding studies were conducted to obtain for each nephrotoxicant a NONEL and a LONEL under experimental conditions similar to those of the mixture study. Following an acclimatization period of at least 6 days, the rats were allocated randomly to groups of ten (controls) or five, in such a way that the mean body weights were about the same in all groups. In the first range-finding study, each nephrotoxicant was given at four levels (Table 1). Since this study did not yield a clear LONEL for TETRA and TRI these chemicals were examined at higher dose levels in a second study (Table 1).

Mixture study. On arrival the animals were randomly allocated to one of 15 groups of ten (vehicle controls) or five rats each. After an acclimatization period of 16 days treatment was started. The dose levels are given in Table 2. The four nephrotoxicants were given either alone at their NONEL or LONEL, or in combinations of four at their NONEL or 1/2 LONEL, or in the four possible combinations of three at 1/3 LONEL. For each chemical the NONEL and LONEL were selected such that the NONEL was one fourth of the LONEL, to enable comparison of the effect of combined exposure to four different compounds at their respective NONELs with the effect of exposure to

Table 1. Dose levels used in the range-finding studies

	Dose level (mg/kg bw/day)			
	TETRA	TRI	TCTFP	HCBD
First study				
low-dose	225	200	0.2	0.4
mid-dose 1	450	400	0.6	1.3
mid-dose 2	900	800	2.0	4.0
high-dose	1800	1600	6.0	12.0
Second study				
high-dose 1	2500	2000		
high-dose 2	3200[1]/2800[2]	2800[1]/2400[2]		

[1] Administered on the first two days of the treatment period.
[2] Administered from the third day of the treatment period.

Table 2. Treatments and dose levels in the mixture study

Treatment	Total dose in toxicity units
Control: corn oil 10 ml/kg	0
Individual chemicals at NONEL	
TETRA 600 mg/kg bw/day	1/4
TRI 500 mg/kg bw/day	1/4
TCTFP 1.5 mg/kg bw/day	1/4
HCBD 1 mg/kg bw/day	1/4
Individual chemicals at LONEL	
TETRA 2400 mg/kg bw/day	1
TRI 2000 mg/kg bw/day	1
TCTFP 6 mg/kg bw/day	1
HCBD 4 mg/kg bw/day	1
Mixtures of all 4 chemicals	
each chemical at NONEL	1
each chemical at LONEL/2	2
Mixtures of 3 chemicals at 1/3 LONEL	
TETRA + TRI + TCTFP	1
TETRA + TRI + HCBD	1
TETRA + TCTFP + HCBD	1
TRI + TCTFP + HCBD	1

NONEL = No-Observed-Nephrotoxic-Effect Level (= 1/ 4 LONEL)
LONEL = Lowest-Observed-Nephrotoxic-Effect Level

four times the NONEL (= the LONEL) of a single chemical. Under full dose additivity, the effects of the combinations at the NONEL or 1/3 LONEL and those of the single chemicals at their LONEL are expected to be the same. In addition, the study initially included a group given the four chemicals simultaneously at their LONEL to examine the toxicity of the combination at slightly nephrotoxic levels of the individual chemicals. However, due to the strong narcotic effect of TETRA and TRI (one rat died after the first dose) combined exposure at this level had to be discontinued. Instead, the group given the four chemicals simultaneously at 1/2 LONEL was included (this group was formed from reserve rats and dosed from the second day of the study).

Observations and analyses

Clinical observations were made daily before and after dosing. The rats were weighed twice per week and at final necropsy. Food consumption was measured each week, over 4- or 7-day periods, on a cage basis, by weighing the feeders. Water consumption was measured on three or four consecutive days each week, on a cage basis, by weighing the drinking bottles. In addition, water consumption of individual animals was measured during the collection of 24-hr urine samples in weeks one and four.

Urinalysis. Urine was collected towards the end of the first week (or halfway the second week in the range-finding study with TCTFP) and the fourth week of treatment. The rats were kept in metabolism cages (one rat/cage) for three consecutive days. After one day of acclimatization, 24-hr urine samples were collected, in ice-cooled containers, from unfasted rats (food and water freely available). Each sample was characterised with respect to appearance, volume, density (Bellingham and Stanley refractometer), glucose, total protein, creatinine, alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), lactate dehydrogenase (LDH) and N-acetyl-ß-glucosaminidase (NAG) (Automatic analyser model 911 from Hitachi, Japan). Glucose, protein, creatinine and the enzyme activities were expressed as units excreted per 24 hr by multiplying the concentrations measured by the volume of the urine produced in these 24 hr. On the third day, the rats were deprived of water for 24 hr and of food during the last 16 hr of this period. Urine was collected during the latter 16 hr and the volume and density of the samples were recorded to assess the renal concentrating ability (in the range-finding study with TCTFP no concentration test was carried out towards the end of the study).

Clinical chemistry. At final necropsy, blood samples were collected from the abdominal aorta in heparinized tubes. The samples were centrifuged and the plasma was analysed for aspartate aminotransferase (ASAT), alanine aminotransferase

(ALAT), ALP, GGT, LDH, total protein, albumin, glucose (non-fasting), urea and creatinine (Automatic analyser model 911 from Hitachi, Japan).

Pathology. At the end of the treatment period, the rats were killed by exsanguination from the abdominal aorta under light ether anaesthesia and examined grossly for pathological changes. The liver and kidneys were weighed. After fixation in a 4% neutral buffered solution of formaldehyde, the kidneys were processed, embedded in paraffin, sectioned at 5 μm, stained with haematoxylin and eosin, and examined by light microscopy.

Statistical analysis

Body weights were evaluated by one-way analysis of covariance followed by Dunnett's multiple comparison tests. Water consumption measured per cage was subjected to repeated measures analysis of variance followed by pairwise comparisons with vehicle controls using linear orthogonal components (α=0.01) (mixture study only). Water consumption measured per animal, the results of clinical chemistry and urinalysis (except for appearance), and organ weights were evaluated by one-way analysis of variance followed by Dunnett's multiple comparison tests. The histopathological changes were analysed by Fisher's exact probability test. All analyses were two-sided.

Results

Range-finding studies

There was no chemical-related mortality. Clinical observations revealed an increased incidence of focal alopecia in the groups given TETRA at 1800 mg/kg or more, TRI at all dose levels except for 1600 mg/kg (a dose-related response was seen only in the second range-finding study), HCBD at 12 mg/kg and, less severely, TCTFP at 6 mg/kg (no data presented). In addition, rats given the high dose levels of TETRA or TRI showed transient behavioural signs shortly after dosing (with TETRA sluggishness in the first week and hyperreactivity thereafter, with TRI sluggishness only), the severity of which decreased in the course of the study. Other results, summarized in the Appendix to this chapter, are described below per chemical.

TCTFP-induced changes were observed from 2 mg/kg bw. At 2 mg/kg, urinary LDH excretion was slightly increased (in week two only). At 6 mg/kg, treatment with TCTFP resulted in a decreased urinary density (statistically significant in week four only), an increased urinary excretion of protein (in week four only) and LDH, increased relative kidney weight, increased plasma ALAT activity and microscopic renal changes

consisting of diffuse or multifocal vacuolation, slight focal degeneration, and multifocal or diffuse karyomegaly of tubular cells. For the mixture study 1.5 and 6 mg TCTFP/kg bw were selected as a NONEL and LONEL, respectively.

HCBD was toxic from 4 mg/kg bw. The effects included growth retardation, reduced food consumption, decreased urinary density, and increases in urinary volume, excretion of LDH, relative kidney weight and plasma ALAT activity. Microscopically, regeneration, hyperplasia, slight necrosis, and multifocal or diffuse karyomegaly of the renal tubular epithelium were observed. There was a clear dose-response relationship except for the effect on urinary LDH. For the mixture study 1 and 4 mg HCBD/kg bw were selected as a NONEL and LONEL, respectively.

The results of the first range-finding study with dose levels of TETRA up to 1800 mg/kg bw yielded no clear nephrotoxic effect level (no data presented). In the second study, rats given TETRA at 2500 mg/kg showed an increased water consumption, decreased urinary density, increased urinary volume, increased excretion of protein, GGT and ALP (the urinary changes occurred mainly in week one), increased kidney weight and histopathological changes consisting of focal tubular vacuolation and cyto/karyomegaly. The severity of these changes did not increase with the dose. On the contrary, at 2800 mg/kg the urinary changes were even smaller or absent. TETRA was also hepatotoxic, as shown by the increased liver weight (from 1800 mg/kg in the first range-finding study), the increased plasma levels of ALP, ASAT, ALAT and GGT, and the dark discoloration of the liver observed at necropsy. For the mixture study 600 and 2400 mg TETRA/kg bw were selected as a NONEL and LONEL, respectively.

In the first range-finding study with TRI, the only renal change observed consisted of slight focal tubular vacuolation at 1600 mg/kg bw. In the second study, urinary volume (concentration test) and kidney weight were increased dose-dependently, urinary excretion of GGT was decreased (in week four), and focal tubular vacuolation and cyto/karyomegaly occurred to about the same extent in kidneys of rats given 2000 or 2400 mg/kg. In addition, TRI induced signs of hepatotoxicity, viz. increased liver weight (from 200 mg/kg bw in the first range-finding study), increased plasma levels of ALP, ASAT, ALAT and GGT, and dark discoloration of the liver. The increase in the plasma level of albumin observed from 400 mg/kg bw in the first range-finding study (and in the mixture study) was not confirmed in the second range-finding study. For the mixture study 500 and 2000 mg TRI/kg bw were selected as a NONEL and LONEL, respectively.

Mixture study

Three rats died between the fourth and tenth day of the study: one given TETRA at its NONEL due to a dosing error, one given TETRA at its LONEL (this rat was replaced) and one given TETRA, TRI and TCTFP simultaneously. During the major part of the study, rats given TETRA or TRI individually at their LONEL, or the four chemicals simultaneously at 1/2 LONEL showed severe but transient signs of central nervous system depression within a few hours after dosing (no data presented). In weeks two and three such signs were also observed in the group given the combination of TETRA, TRI and TCTFP. Rats of the other groups showed these signs only once or twice or not at all.

Data on body weight gain and consumption of food and water are presented in Table 3. Growth or food consumption were not adversely affected by any of the treatments. Water consumption was increased nearly twofold in rats given TETRA alone at its LONEL or the four chemicals simultaneously at 1/2 LONEL.

Table 3. Body weight gain, food intake and water intake in the mixture study

Treatment	Weight gain (g/rat/32 days)	Food intake (g/rat/day)	Water intake (g/rat/day)		
			per cage in wk 1-4	per rat on day 6	per rat on day 27
Control	64 ± 2	13.0	17.2	16.2 ± 0.7	16.4 ± 1.6
Chemicals singly at NONEL					
TETRA	64 ± 5	11.8	18.4	15.9 ± 1.0	18.9 ± 2.5
TRI	68 ± 3	13.0	19.2	16.7 ± 0.4	17.6 ± 1.5
TCTFP	67 ± 5	13.5	18.4	16.8 ± 1.1	13.6 ± 1.4
HCBD	73 ± 5	12.5	17.8	16.5 ± 1.0	18.4 ± 3.7
Chemicals singly at LONEL					
TETRA	72 ± 4	12.7	29.9**	27.0 ± 2.9**	26.8 ± 2.5**
TRI	64 ± 4	12.5	22.1	19.5 ± 0.6	22.4 ± 3.3
TCTFP	64 ± 2	12.4	19.1	17.7 ± 1.4	17.5 ± 1.4
HCBD	61 ± 2	11.3	19.8	18.2 ± 2.4	18.1 ± 2.8
Mixtures of 4 chemicals					
at NONEL (1/4 LONEL)	66 ± 4	13.0	23.8	18.4 ± 0.2	18.4 ± 1.3
at 1/2 LONEL	75 ± 5	13.2	32.6**	28.5 ± 4.9**	27.9 ± 3.1**
Mixtures of 3 chemicals at 1/3 LONEL					
TETRA + TRI + TCTFP	74 ± 2	15.6	22.4	19.5 ± 2.2	17.1 ± 1.5
TETRA + TRI + HCBD	70 ± 5	14.3	20.0	18.4 ± 1.0	19.2 ± 1.8
TETRA + TCTFP + HCBD	72 ± 3	14.2	21.1	17.0 ± 0.9	18.7 ± 1.4
TRI + TCTFP + HCBD	60 ± 4	14.1	19.8	15.7 ± 0.5	17.9 ± 1.5

Values for body weight gain and water intake per rat are means ± sem for groups of 10 (controls) or five rats (exception: n=4 for TETRA at NONEL and for TETRA + TRI + TCTFP).
Values for the water intake per cage are means of 14 daily measurements. Food intake was measured per cage (five rats/cage) over 4 periods of 4 or 7 days; the values presented are the means of these periods.
Asterisks indicate significant differences from controls: ** $P<0.01$.

Table 4. Results of urinalysis in the first week of the mixture study

Treatment	Concentration test		excretion per 24 hours								
	Volume (ml)	density (kg/l)	volume (ml)	density (kg/l)	protein (mg)	glucose (mmol)	GGT (U)	ALP (U)	LDH (mU)	NAG (mU)	creatinine (µmol)
Control	1.6 ± 0.1	1.059 ± 0.003	5.3 ± 0.4	1.042 ± 0.002	2.6 ± 0.2	11.57 ± 1.51	1.5 ± 0.1	1.37 ± 0.32	354 ± 32	68.3 ± 5.6	26.1 ± 1.7
Chemicals singly at NONEL											
TETRA	1.6 ± 0.2	1.059 ± 0.006	3.9 ± 1.2	1.047 ± 0.003	1.8 ± 0.4	9.29 ± 1.80	1.1 ± 0.4	1.02 ± 0.47	225 ± 55	48.9 ± 11.2	19.8 ± 4.9
TRI	2.1 ± 0.2	1.053 ± 0.004	5.8 ± 0.3	1.044 ± 0.001	2.9 ± 0.2	13.54 ± 0.65	1.2 ± 0.1	1.06 ± 0.17	319 ± 38	77.1 ± 2.4	27.0 ± 1.0
TCTFP	1.7 ± 0.2	1.059 ± 0.005	7.9 ± 0.5	1.037 ± 0.002	3.0 ± 0.2	11.79 ± 0.87	1.6 ± 0.2	1.14 ± 0.27	453 ± 100	84.7 ± 6.5	36.1 ± 0.9*
HCBD	1.9 ± 0.2	1.049 ± 0.004	5.6 ± 0.2	1.040 ± 0.002	2.2 ± 0.2	8.80 ± 1.04	1.7 ± 0.2	0.68 ± 0.12	390 ± 62	61.0 ± 5.3	27.5 ± 2.0
Chemicals singly at LONEL											
TETRA	1.7 ± 0.2	1.048 ± 0.004	14.1 ± 2.2**	1.020 ± 0.002**	3.0 ± 0.4	15.29 ± 2.64	3.0 ± 0.3**	2.78 ± 0.71*	557 ± 101	89.1 ± 6.3	25.5 ± 2.0
TRI	1.7 ± 0.3	1.055 ± 0.006	8.6 ± 1.7	1.035 ± 0.005	2.6 ± 0.3	13.18 ± 2.55	1.4 ± 0.2	1.34 ± 0.36	274 ± 42	70.7 ± 8.1	27.1 ± 2.3
TCTFP	1.9 ± 0.2	1.054 ± 0.004	7.1 ± 0.9	1.041 ± 0.005	2.9 ± 0.2	13.02 ± 0.79	1.2 ± 0.1	1.29 ± 0.23	524 ± 85	83.4 ± 3.4	30.9 ± 1.7
HCBD	1.5 ± 0.2	1.056 ± 0.002	4.9 ± 0.5	1.040 ± 0.003	2.2 ± 0.3	9.45 ± 1.51	1.6 ± 0.3	0.78 ± 0.14	329 ± 52	61.2 ± 7.2	22.8 ± 2.7
Mixtures of 4 chemicals											
At NONEL (1/4 LONEL)	1.5 ± 0.2	1.065 ± 0.008	5.0 ± 0.8	1.046 ± 0.002	2.4 ± 0.1	11.89 ± 1.49	1.2 ± 0.2	1.03 ± 0.29	402 ± 69	65.7 ± 4.9	23.9 ± 3.4
At 1/2 LONEL	2.2 ± 0.2	1.046 ± 0.002	17.7 ± 4.5**	1.024 ± 0.007**	3.2 ± 0.4	14.11 ± 1.78	1.4 ± 0.1	1.64 ± 0.11	519 ± 70	95.5 ± 6.6*	29.4 ± 1.5
Mixtures of 3 chemicals at 1/3 LONEL											
TETRA + TRI + TCTFP	1.9 ± 0.1	1.051 ± 0.002	8.2 ± 1.7	1.038 ± 0.006	2.8 ± 0.3	16.21 ± 2.01	1.2 ± 0.2	3.00 ± 0.81*	508 ± 51	83.5 ± 4.5	29.0 ± 2.4
TETRA + TRI + HCBD	1.9 ± 0.2	1.059 ± 0.004	4.7 ± 0.5	1.048 ± 0.002	2.5 ± 0.3	14.11 ± 1.11	1.4 ± 0.2	1.89 ± 0.39	434 ± 73	65.1 ± 6.6	22.5 ± 2.8
TETRA + TCTFP + HCBD	2.0 ± 0.2	1.051 ± 0.004	4.9 ± 0.7	1.043 ± 0.002	2.0 ± 0.3	10.64 ± 1.46	1.0 ± 0.1	0.84 ± 0.24	333 ± 48	59.0 ± 8.1	21.8 ± 2.4
TRI + TCTFP + HCBD	1.8 ± 0.3	1.062 ± 0.003	4.6 ± 0.4	1.046 ± 0.002	2.2 ± 0.2	13.41 ± 1.28	1.0 ± 0.1	0.96 ± 0.18	252 ± 37	63.9 ± 6.3	22.8 ± 1.9

Values are means ± sem for groups of 10 (controls) or five rats (exception: n=4 for TETRA at NONEL and for TETRA + TRI + TCTFP).
Asterisks indicate significant differences from controls: *P<0.05 ** P<0.01.

Table 5. Results of urinalysis in the fourth week of the mixture study

Treatment	concentration test		excretion per 24 hours								
	volume (ml)	density (kg/l)	volume (ml)	density (kg/l)	protein (mg)	glucose (mmol)	GGT (U)	ALP (U)	LDH (mU)	NAG (mU)	creatinine (µmol)
Control	1.5 ± 0.1	1.065 ± 0.004	5.3 ± 0.4	1.043 ± 0.002	2.5 ± 0.2	11.37 ± 0.68	1.9 ± 0.1	0.71 ± 0.11	305 ± 26	67.9 ± 4.2	38.0 ± 1.8
Chemicals singly at NONEL											
TETRA	1.9 ± 0.2	1.064 ± 0.006	6.1 ± 0.9	1.037 ± 0.006	2.3 ± 0.4	10.47 ± 1.78	1.7 ± 0.2	0.76 ± 0.15	316 ± 55	64.0 ± 8.7	32.0 ± 6.0
TRI	1.6 ± 0.1	1.067 ± 0.003	6.8 ± 0.7	1.039 ± 0.003	3.0 ± 0.4	15.32 ± 1.70	1.5 ± 0.2	1.07 ± 0.21	222 ± 27	88.9 ± 10.5	35.9 ± 4.5
TCTFP	1.7 ± 0.2	1.066 ± 0.002	3.6 ± 0.5	1.050 ± 0.004	2.2 ± 0.2	10.04 ± 1.23	1.2 ± 0.2*	0.72 ± 0.15	207 ± 25	58.1 ± 7.2	28.2 ± 3.2
HCBD	1.4 ± 0.2	1.073 ± 0.005	5.2 ± 0.9	1.047 ± 0.005	2.8 ± 0.3	11.62 ± 0.55	2.8 ± 0.3**	1.11 ± 0.12	343 ± 98	73.8 ± 6.0	37.0 ± 2.5
Chemicals singly at LONEL											
TETRA	2.3 ± 0.2*	1.058 ± 0.005	13.8 ± 2.9**	1.031 ± 0.004	4.1 ± 0.5**	16.39 ± 1.90	3.1 ± 0.2**	2.34 ± 0.25**	683 ± 50**	136.1 ± 9.4**	43.9 ± 2.3
TRI	2.5 ± 0.3*	1.059 ± 0.001	10.8 ± 1.0**	1.032 ± 0.003	3.3 ± 0.3	17.53 ± 2.34*	1.9 ± 0.1	2.17 ± 0.54**	362 ± 70	114.0 ± 6.7**	38.7 ± 0.9
TCTFP	2.0 ± 0.1	1.053 ± 0.002	5.3 ± 0.4	1.041 ± 0.003	3.0 ± 0.4	11.43 ± 2.05	1.1 ± 0.2**	0.48 ± 0.08	302 ± 55	77.4 ± 12.2	31.5 ± 3.5
HCBD	1.2 ± 0.1	1.068 ± 0.003	7.4 ± 1.4	1.037 ± 0.005	3.0 ± 0.2	13.43 ± 2.21	3.4 ± 0.1**	1.31 ± 0.09	307 ± 35	88.7 ± 4.8	38.9 ± 1.8
Mixtures of 4 chemicals											
at NONEL (1/4 LONEL)	2.2 ± 0.3	1.057 ± 0.006	5.6 ± 0.8	1.037 ± 0.001	1.9 ± 0.2	8.88 ± 1.18	1.2 ± 0.2*	0.62 ± 0.12	297 ± 74	71.2 ± 10.0	27.7 ± 3.8*
at 1/2 LONEL	4.0 ± 0.5**	1.047 ± 0.002**	15.6 ± 2.3**	1.030 ± 0.004	3.8 ± 0.1**	20.01 ± 0.78**	2.0 ± 0.0	2.85 ± 0.53**	549 ± 29**	140.2 ± 7.3**	48.4 ± 2.0*
Mixtures of 3 chemicals at 1/3 LONEL											
TETRA + TRI + TCTFP	2.1 ± 0.4	1.058 ± 0.004	7.5 ± 0.5	1.044 ± 0.003	3.4 ± 0.3	17.28 ± 2.23*	1.7 ± 0.1	1.71 ± 0.39*	432 ± 26	100.7 ± 5.0*	39.9 ± 2.6
TETRA + TRI + HCBD	2.1 ± 0.1	1.066 ± 0.002	6.6 ± 0.9	1.041 ± 0.003	2.7 ± 0.3	13.13 ± 1.33	2.3 ± 0.3	1.31 ± 0.23	310 ± 35	82.6 ± 7.9	33.3 ± 2.6
TETRA + TCTFP + HCBD	2.4 ± 0.3*	1.055 ± 0.004	7.2 ± 0.3	1.039 ± 0.003	3.0 ± 0.1	14.31 ± 0.90	2.0 ± 0.2	1.79 ± 0.30*	386 ± 40	94.5 ± 4.9	39.7 ± 2.1
TRI + TCTFP + HCBD	2.0 ± 0.1	1.058 ± 0.003	7.3 ± 0.4	1.041 ± 0.001	3.4 ± 0.1	14.96 ± 1.60	2.0 ± 0.1	0.99 ± 0.13	381 ± 91	99.8 ± 4.7*	40.8 ± 1.6

Values are means ± sem for groups of 10 (controls) or five rats (exception: n=4 for TETRA at NONEL and for TETRA + TRI + TCTFP).
Asterisks indicate significant differences from controls: *P<0.05 ** P<0.01.

Table 6. Results of clinical chemistry and relative organ weights in the mixture study

	albumin (g/l)	protein (g/l)	glucose (mmol/l)	creatinine (µmol/l)	urea (mmol/l)	ALP (U/l)	ASAT (U/l)	ALAT (U/l)	GGT (mU/l)	LDH (mU/l)	liver weight (g/kg bw)	kidney weight (g/kg bw)[1]
Control	35 ± 1	59 ± 1	7.82 ± 0.16	19 ± 0	5.8 ± 0.3	293 ± 16	65 ± 2	46 ± 2	0.1 ± 0.1	220 ± 72	34.4 ± 0.7	7.46 ± 0.08
Chemicals singly at NONEL												
TETRA	36 ± 0	59 ± 0	7.85 ± 0.16	17 ± 1	5.3 ± 0.5	328 ± 42	67 ± 3	51 ± 4	0.2 ± 0.0	163 ± 20	39.2 ± 0.8**	7.73 ± 0.09
TRI	36 ± 0*	58 ± 1	7.44 ± 0.16	17 ± 1	4.3 ± 0.4*	317 ± 22	73 ± 3	52 ± 5	0.0 ± 0.0	159 ± 14	40.7 ± 0.7**	7.93 ± 0.15
TCTFP	34 ± 0	57 ± 1	7.63 ± 0.18	17 ± 1	4.6 ± 0.4	342 ± 27	71 ± 2	45 ± 4	0.2 ± 0.1	181 ± 15	33.2 ± 0.6	7.53 ± 0.13
HCBD	34 ± 0	57 ± 1	8.13 ± 0.24	20 ± 1	6.0 ± 0.3	382 ± 16*	67 ± 1	45 ± 2	0.4 ± 0.1	156 ± 10	33.4 ± 1.3	7.56 ± 0.30
Chemicals singly at LONEL												
TETRA	35 ± 0	61 ± 1	8.53 ± 0.24	17 ± 1	6.2 ± 0.2	304 ± 16	88 ± 1**	72 ± 4**	0.2 ± 0.1	169 ± 10	50.4 ± 0.9**	8.36 ± 0.14**
TRI	37 ± 0**	62 ± 1	7.83 ± 0.09	17 ± 1	5.7 ± 0.4	372 ± 34	73 ± 3	56 ± 2	0.3 ± 0.2	138 ± 13	51.6 ± 1.8**	8.26 ± 0.32**
TCTFP	35 ± 0	58 ± 0	8.09 ± 0.38	15 ± 0*	4.8 ± 0.4	363 ± 28	68 ± 3	46 ± 4	0.2 ± 0.1	155 ± 16	36.5 ± 1.2	8.03 ± 0.20
HCBD	35 ± 0	58 ± 0	7.34 ± 0.23	16 ± 0	5.0 ± 0.3	323 ± 29	64 ± 2	39 ± 2	0.1 ± 0.0	148 ± 5	32.7 ± 0.7	8.40 ± 0.23**
Mixtures of 4 chemicals												
at NONEL (1/4 LONEL)	35 ± 0	58 ± 0	7.76 ± 0.34	15 ± 1**	4.7 ± 0.3	343 ± 22	73 ± 1	54 ± 1	0.3 ± 0.1	139 ± 11	43.8 ± 0.6**	8.41 ± 0.08**
at 1/2 LONEL	35 ± 0	60 ± 0	8.82 ± 0.40*	16 ± 1	5.5 ± 0.4	404 ± 30**	81 ± 5**	71 ± 5**	0.4 ± 0.2	192 ± 48	54.5 ± 0.9**	8.36 ± 0.17**
Mixtures of 3 chemicals at 1/3 LONEL												
TETRA + TRI + TCTFP	36 ± 0	60 ± 0	7.53 ± 0.21	18 ± 0	5.5 ± 0.2	368 ± 16	77 ± 3*	62 ± 3**	0.2 ± 0.1	207 ± 71	48.4 ± 0.9**	8.19 ± 0.14*
TETRA + TRI + HCBD	36 ± 0*	61 ± 1	7.91 ± 0.15	18 ± 1	5.9 ± 0.5	319 ± 35	67 ± 2	54 ± 2	0.2 ± 0.1	145 ± 17	48.0 ± 1.1**	8.21 ± 0.08**
TETRA + TCTFP + HCBD	35 ± 1	59 ± 1	8.14 ± 0.27	17 ± 1	5.4 ± 0.1	306 ± 9	72 ± 3	55 ± 3	0.2 ± 0.0	142 ± 11	40.4 ± 0.4**	8.06 ± 0.18
TRI + TCTFP + HCBD	37 ± 0**	60 ± 1	7.51 ± 0.31	16 ± 1	5.4 ± 0.3	320 ± 21	72 ± 1	51 ± 2	0.4 ± 0.2	211 ± 37	41.8 ± 0.9**	8.39 ± 0.13**

Values are means ± sem for groups of 10 (controls) or five rats (exception: n=4 for TETRA at NONEL and for TETRA + TRI + TCTFP).
Asterisks indicate significant differences from controls: *P<0.05 ** P<0.01.

[1] One-way analysis of variance revealed no statistically significant differences in relative kidney weight among the groups treated with 'one toxicity unit'.

Table 7. Renal histopathological changes in the mixture study

Treatment	number of animals examined	focal tubular vacuolation				multifocal tubular vacuolation				karyomegaly			
		total	very slight	slight	moderate	**total**	slight	moderate	severe	**total**	very slight	slight	moderate
Control	10	**2**	2	0	0	**0**	0	0	0	**0**	0	0	0
Chemicals singly at NONEL													
TETRA	5	**0**	0	0	0	**0**	0	0	0	**0**	0	0	0
TRI	5	**5****	4	1	0	**0**	0	0	0	**0**	0	0	0
TCTFP	5	**1**	1	0	0	**0**	0	0	0	**0**	0	0	0
HCBD	5	**2**	1	1	0	**0**	0	0	0	**0**	0	0	0
Chemicals singly at LONEL													
TETRA	5	**1**	0	0	1	**4****	2	2	0	**4****	3	1	0
TRI	5	**2**	0	1	1	**3***	2	1	0	**5****	2	3	0
TCTFP	5	**4**	1	2	1	**1**	0	1	0	**4****	2	1	1
HCBD	5	**4**	1	3	0	**0**	0	0	0	**1**	0	1	0
Mixtures of 4 chemicals													
at NONEL (1/4 LONEL)	5	**3**	1	2	0	**0**	0	0	0	**0**	0	0	0
at 1/2 LONEL	5	**0**	0	0	0	**5****	0	2	3	**2**	0	2	0
Mixtures of 3 chemicals at 1/3 LONEL													
TETRA + TRI + TCTFP	5	**3**	1	2	0	**2**	1	1	0	**3***	0	3	0
TETRA + TRI + HCBD	5	**0**	0	0	0	**4****	4	0	0	**5****	3	1	1
TETRA + TCTFP + HCBD	5	**4**	3	1	0	**1**	0	1	0	**0**	0	0	0
TRI + TCTFP + HCBD	5	**3**	2	1	0	**1**	1	0	0	**1**	1	0	0

Values are numbers of rats with the indicated observation, based on microscopic assessment of sections stained with haematoxylin and eosin.
Asterisks indicate significant differences from controls: *P<0.05 **P<0.01 (Fisher's exact test on total incidences).

Urinalysis results are given in Table 4 (week one) and Table 5 (week four). The urine concentration test conducted after one week of treatment did not reveal statistically significant intergroup differences, though urinary density tended to be decreased in the groups given TETRA alone at its LONEL or the four chemicals simultaneously at 1/2 LONEL. After four weeks of treatment, the concentration test revealed a significantly increased urinary volume and decreased density in the latter group, and an increased urinary volume in the groups given TETRA or TRI alone at their LONEL or the combination of TETRA, TCTFP and HCBD at 1/3 LONEL.

Analysis of the 24-hr urine samples showed that administration of the nephrotoxicants at their NONEL, either alone or in combination, did not result in treatment-related changes indicative of renal toxicity. Though a few statistically significant differences between single-chemical NONEL-groups and controls were observed, these were not considered convincing evidence of chemical-induced nephrotoxicity for the following reasons: no dose-related response (increased creatinine excretion with TCTFP in week one); the change was not indicative of renal damage (decreased GGT excretion with TCTFP in week four); no similar change in the range-finding study (increased GGT excretion with HCBD in week four; in the range-finding study, HCBD did not affect GGT excretion despite the more severe renal damage compared with the mixture study). The individual chemicals at their LONEL had different effect profiles for the urinary endpoints. TCTFP-treated animals showed only a decrease in GGT excretion in week four, a change considered of doubtful toxicological significance (in the range-finding study, urinary GGT excretion was also decreased in animals given TCTFP but not dose-dependently). The only statistically significant effect of HCBD at its LONEL was an increase in GGT excretion in week four. Administration of TRI at its LONEL resulted in significant increases in urinary volume and excretion of glucose, ALP and NAG, and slight, statistically insignificant changes in urinary density and protein excretion (in week four). Rats given TETRA at its LONEL showed an increased urinary volume, decreased density, and increased excretion of protein, glucose (not statistically significantly), GGT, ALP, LDH and NAG in weeks one and/or four; the effects on protein, ALP, LDH and NAG were most pronounced in week four. Simultaneous administration of the four chemicals at 1/2 LONEL induced changes in almost all urinary endpoints in week four (exception: GGT excretion), and in urinary volume, density and excretion of NAG in week one. On the other hand, the groups given three chemicals simultaneously at 1/3 LONEL showed no urinary changes at all (TETRA, TRI plus HCBD), or changes in one (TCTFP, HCBD plus TETRA/TRI) or three (TETRA, TRI plus TCTFP) endpoints only.

Clinical chemistry values in plasma collected terminally are given in Table 6. A few statistically significant changes occurred at the NONEL of the individual chemicals, viz. increased albumin and decreased urea in the TRI-group and increased ALP in the HCBD-group. Except for the increased albumin, these changes were considered fortuitous findings unrelated to treatment because they did not occur at the LONEL of the individual chemicals. The only treatment-related changes following exposure to the individual chemicals at their LONEL consisted of increased levels of albumin (TRI-group) or ASAT and ALAT (TETRA-group). The decrease in creatinine at the LONEL of TCTFP was considered of no toxicological relevance because renal damage would have resulted in an increase rather than a decrease in plasma creatinine. For the same reason, no toxicological significance was attached to the only change (viz. decreased creatinine) in the group given the four chemicals simultaneously at the NONEL. Co-exposure to the four chemicals at 1/2 LONEL resulted in increased plasma levels of glucose, ALP, ASAT and ALAT. Significant changes following combined exposure to three chemicals at 1/3 LONEL were limited to increased levels of albumin (groups given TRI, HCBD plus TCTFP/TRI) or ASAT and ALAT (group given TETRA, TRI plus TCTFP).

Relative kidney weight (Table 6) was increased following exposure to the individual chemicals at their LONEL and, to about the same extent, following combined exposure at the NONEL or 1/2 LONEL (quaternary mixtures) or 1/3 LONEL (ternary mixtures). Relative liver weight was increased dose-dependently in the single-chemical groups given TETRA or TRI and in all mixture groups.

Gross examination at necropsy revealed no clear treatment-related changes (no data presented). The only renal abnormalities observed (viz. soft and/or enlarged kidney, unilateral) occurred in one rat given the four chemicals simultaneously at 1/2 LONEL and in two rats given HCBD at its LONEL.

The results of microscopic examination of the kidneys are presented in Table 7. Except for an increased incidence of (very) slight focal tubular vacuolation at the NONEL of TRI, there were no histopathological changes in the groups given the nephrotoxicants at their NOEL, either alone or in combination. When given alone at their LONEL, TETRA and TRI induced karyomegaly (very slight to slight) and multifocal tubular vacuolation (slight to moderate), and TCTFP induced karyomegaly only. Unexpectedly, no significant renal changes were seen in the group given HCBD at its LONEL. Rats given the four chemicals simultaneously at 1/2 LONEL showed moderate to severe multifocal tubular vacuolation. The renal changes observed in the groups given the ternary mixtures ranged from no abnormalities (groups given TCTFP, HCBD plus TETRA/TRI) to karyomegaly only (group given TETRA, TRI plus

TCTFP) or karyomegaly and slight multifocal vacuolation (group given TETRA, TRI plus HCBD).

Discussion

The present study was conducted to test the additivity assumption (dose addition), under conditions of concurrent, repeated exposure to similarly acting nephrotoxicants at exposure levels slightly below the individual chemicals' thresholds for renal toxicity.

The results showed that relative kidney weight was increased following simultaneous exposure to four chemicals at their NONEL (=1/4 LONEL) or to three chemicals at 1/3 LONEL, and, to about the same extent, following exposure to each of the individual nephrotoxicants at their LONEL. The finding that the magnitude of the increase in kidney weight was comparable in all groups treated with 'one toxicity unit' supports the validity of the assumption of dose addition for these similarly acting nephrotoxicants.

The other endpoints measured as indices of renal toxicity (viz. renal morphology and concentrating ability, plasma levels of creatinine and urea, urinary excretion of glucose, protein and marker enzymes) were not affected at all upon combined exposure to the four chemicals at the NONEL, and showed only a few slight changes upon combined exposure to three chemicals at 1/3 LONEL. Interpretation of the mixture results for these endpoints in terms of presence or absence of dose additivity is complicated because these endpoints, unlike kidney weight, were not affected at the LONEL of each of the individual chemicals. Consequently, these endpoints could not be evaluated by the 'toxicity unit method' for testing the validity of dose additivity. Yet, from the absence of significant effects on these endpoints in the mixture groups treated with 'one toxicity unit' it can be inferred that there was no marked synergistic interaction upon combined exposure at the NONEL or 1/3 LONEL.

At a slightly higher exposure level (viz. 1/2 LONEL, corresponding to 'two toxicity units'), simultaneous exposure to the nephrotoxicants resulted in clear renal toxicity, as indicated by the increased renal weight, decreased renal concentrating ability, increased urinary excretion of protein, glucose, ALP, LDH and NAG, and histopathological alterations. Because the study did not include single-chemical groups dosed with 'two toxicity units', the comparison between observed and 'predicted' effects could not be made for this exposure level. Nevertheless, it was obvious that the increase in renal weight upon combined exposure to 'two toxicity units' was less than expected on the basis of dose additivity, since the magnitude of the increase (viz. about 10%) was similar to that seen after exposure to 'one toxicity unit' of a single chemical. This less

than additive effect on kidney weight at 1/2 LONEL could not be explained by a 'maximum effect size phenomenon' because kidney weight was increased by up to 35% in the range-finding studies. The effects on other endpoints in the mixture group dosed with 'two toxicity units' were also comparable to those observed in single-chemical groups dosed with 'one toxicity unit', with the exception of the impairment of renal concentrating ability and multifocal tubular vacuolation which were most severe in the mixture group.

In the absence of data on tissue concentrations of the nephrotoxicants, their metabolites or endogenous factors involved in their nephrotoxicity, such as glutathione, the less than additive effects of the mixture at 1/2 LONEL cannot be explained. Given the complexity of the metabolic pathway leading to nephrotoxicity and the existence of other, major metabolic pathways for at least two of the mixture components, there are many steps at which interactions may occur. For example, on the one hand it is plausible that the four nephrotoxicants compete for glutathione and thus mutually reduce the levels of their nephrotoxic (conjugative) metabolites. On the other hand, at high exposure levels TETRA and TRI may inhibit each other's oxidative metabolism via the saturable, cytochrome P450-mediated pathway. This would result in higher availability of the parent compounds for glutathione conjugation and, consequently, a higher level of their nephrotoxic metabolites. The occurrence of the latter metabolic interaction has recently been demonstrated in rats exposed simultaneously to TRI, TETRA and/or 1,1,1-trichloroethane (Dobrev *et al*, 2001).

In conclusion, the results of this study provided support for the assumption of dose additivity for mixtures of similarly acting systemic toxicants under conditions of concurrent, repeated exposure at dose levels below the toxicity thresholds of the individual chemicals. In addition, it was shown that mixture results obtained at a slightly higher level of the individual chemicals (1/2 LONEL), which probably was minimally nephrotoxic, were not predictive of the mixture's effect at lower, non-toxic levels of the individual chemicals.

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Chapter 5

Appendix Results of the range-finding studies

Endpoint	Control	**Range-finding study: TCTFP** (mg/kg bw/day)			
		0.2	0.6	2.0	6.0
Terminal body weight (g)	207 ± 7	203 ± 8	205 ± 4	200 ± 9	194 ± 2
Food intake wk 1-4 (g/rat/day)	10.1	9.7	9.5	9.4	8.6
Water intake (g/rat/day)					
per cage in wk 1-2	18.3	18.7	18.8	17.9	18.7
per rat in wk 2 (=day 27)	15.4 ± 1.8	16.4 ± 1.0	17.1 ± 1.9	18.4 ± 0.6	19.3 ± 2.4
per rat in wk 4 (=day 41)	17.5 ± 1.5	15.9 ± 1.3	18.3 ± 2.1	15.1 ± 1.0	22.2 ± 1.3
Urinalysis wk 2					
conc. test volume (ml/16h)	2.4 ± 0.3	3.0 ± 0.2	2.6 ± 0.3	2.1 ± 0.1	3.2± 0.4
conc. test density (kg/l)	1.056 ± 0.006	1.044 ± 0.002	1.044 ± 0.004	1.055 ± 0.001	1.046 ± 0.008
volume (ml/24h)	6.6 ± 1.0	7.0 ± 0.5	7.0 ± 1.1	6.2 ± 0.6	10.2 ± 2.7
density (kg/l)	1.040 ± 0.005	1.037 ± 0.002	1.036 ± 0.003	1.036 ± 0.003	1.027 ± 0.005
protein (mg/24h)	1.7 ± 0.2	2.2 ± 0.2	1.9 ± 0.2	2.1 ± 0.3	2.2 ± 0.2
glucose (mmol/24h)	7.77 ± 0.78	8.73 ± 0.69	8.84 ± 0.76	8.11 ± 1.35	7.81 ± 0.76
GGT (U/24h)	6.1 ± 0.8	5.3 ± 0.4	5.4 ± 0.6	2.6 ± 0.4**	3.3 ± 0.4*
ALP (U/24h)	1.10 ± 0.19	1.25 ± 0.24	1.18 ± 0.14	0.69 ± 0.13	0.51 ± 0.07
LDH (mU/24h)	194 ± 23	277 ± 36	288 ± 17	334 ± 42*	389 ± 69**
NAG (mU/24h)	122.0 ± 13.4	135.3 ± 7.4	160.1 ± 41.9	129.0 ± 17.5	143.7 ± 17.1
Creatinine (µmol/24h)	48.7 ± 4.7	52.9 ± 1.6	55.1 ± 4.1	50.5 ± 6.1	47.9 ± 4.4
Urinalysis wk 4					
conc. test not performed					
volume (ml/24)	8.3 ± 1.4	5.8 ± 1.3	6.9 ± 2.2	6.6 ± 0.7	12.0 ± 0.9
density (kg/l)	1.036 ± 0.003	1.036 ± 0.001	1.036 ± 0.004	1.034 ± 0.003	1.023 ± 0.002*
protein (mg/24h)	2.2 ± 0.2	1.8 ± 0.3	1.7 ± 0.2	2.2 ± 0.2	3.2 ± 0.2*
glucose (mmol/24h)	9.03 ± 0.84	6.73 ± 1.92	6.70 ± 0.84	8.51 ± 0.53	8.81 ± 0.90
GGT (U/24U)	6.4 ± 1.1	3.3 ± 0.9*	4.3 ± 0.9	2.2 ± 0.2**	2.2 ± 0.3**
ALP (U/24h)	1.49 ± 0.19	1.12 ± 0.28	1.12 ± 0.23	0.80 ± 0.17	0.90 ± 0.12
LDH (mU/24h)	278 ± 38	299 ± 108	200 ± 31	297 ± 29	555 ± 41*
NAG (mU/24h)	69.3 ± 7.8	54.1 ± 12.8	52.3 ± 9.4	62.9 ± 4.9	73.6 ± 5.3
Creatinine (µmol/24h)	56.4 ± 5.4	45.3 ± 8.4	46.4 ± 6.0	52.5 ± 3.3	51.6 ± 3.7
Clinical chemistry					
albumin (g/l)	36 ± 0	35 ± 1	37 ± 0	35 ± 1	35 ± 0
protein (g/l)	61 ± 0	59 ± 1	61 ± 1	59 ± 1	58 ± 1
glucose (mmol/l)	9.49 ± 0.35	9.53 ± 0.33	9.68 ± 0.38	9.44 ± 0.47	8.86 ± 0.54
creatinine (µmol/l)	40 ± 4	41 ± 4	40 ± 3	41 ± 2	34 ± 1
urea (mmol/l)	9.4 ± 1.9	8.3 ± 1.6	7.2 ± 1.2	9.7 ± 1.3	7.0 ± 0.6
ALP (U/l)	209 ± 19	224 ± 30	190 ± 18	214 ± 19	255 ± 26
ASAT (U/l)	66 ± 3	61 ± 3	67 ± 3	57 ± 2	72 ± 3
ALAT (U/l)	24 ± 1	28 ± 1	22 ± 2	26 ± 1	34 ± 4*
GGT (U/l)	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.1
LDH (U/l)	200 ± 26	206 ± 15	211 ± 17	198 ± 21	213 ± 21
Relative organ weights					
kidney weight (g/kg bw)	6.19 ± 0.19	6.37 ± 0.10	6.19 ± 0.26	6.43 ± 0.18	7.21 ± 0.20**
liver weight (g/kg bw)	34.1 ± 1.1	34.2 ± 0.7	33.8 ± 1.2	33.3 ± 0.8	35.6 ± 0.6
Renal histopathology					
vacuolation, (multi)focal	2	0	0	4	1
vacuolation, diffuse	0	0	0	0	4**
degeneration, focal	0	0	0	0	4**
karyomegaly	0	0	0	0	3*

Values are means ± sem (histopathology: number of affected animals) for groups of 10 (water intake per rat day 27 and urinalysis wk 2 in controls only) or five rats (exception: n=4 for clinical chemistry in controls).
Asterisks indicate significant differences from controls: *P<0.05 ** P<0.01.

Endpoint	Control	**Range-finding study: HCBD** (mg/kg bw/day)			
		0.4	1.3	4.0	12.0
Weight gain (g/rat/33 days)	67 ± 2	73 ± 7	71 ± 4	39 ± 5**	41 ± 4**
Food intake wk 1-4 (g/rat/day)	10.7	10.6	10.9	8.4	7.6
Water intake (g/rat/day)					
per cage in wk 1-4	19.3	19.8	22.6	17.2	17.6
per rat on day 6	16.0 ± 0.8	15.6 ± 1.7	20.1 ± 3.2	16.0 ± 2.4	18.8 ± 2.6
per rat on day 27	15.4 ± 1.8	20.0 ± 1.7	19.0 ± 1.0	15.7 ± 1.5	23.2 ± 4.8
Urinalysis wk 1					
conc. test volume (ml/16h)	2.4 ± 0.3	2.6 ± 0.5	2.7 ± 0.3	3.1 ± 0.8	2.4 ± 0.2
conc. test density (kg/l)	1.048 ± 0.004	1.045 ± 0.004	1.042 ± 0.002	1.038 ± 0.005	1.038 ± 0.002
volume (ml/24h)	5.9 ± 0.7	6.4 ± 0.9	7.0 ± 0.9	6.9 ± 1.6	9.1 ± 1.5
density (kg/l)	1.042 ± 0.003	1.038 ± 0.002	1.034 ± 0.002	1.029 ± 0.003**	1.022 ± 0.002**
protein (mg/24h)	1.9 ± 0.1	2.1 ± 0.2	2.2 ± 0.2	3.5 ± 1.2	3.2 ± 0.7
glucose (mmol/24h)	9.32 ± 0.74	9.00 ± 0.44	9.29 ± 0.55	6.85 ± 1.77	7.56 ± 1.45
GGT (U/24h)	3.9 ± 0.3	3.8 ± 0.8	5.7 ± 0.7	5.6 ± 0.9	3.6 ± 0.8
ALP (U/24h)	1.45 ± 0.18	1.31 ± 0.14	1.59 ± 0.13	1.60 ± 0.21	1.06 ± 0.20
LDH (mU/24h)	392 ± 128	660 ± 263	577 ± 175	1349 ± 394**	741 ± 129
NAG (mU/24h)	103.2 ± 9.3	119.1 ± 13.0	113.4 ± 15.1	126.3 ± 20.6	118.1 ± 12.6
Creatinine (µmol/24h)	31.9 ± 2.3	35.4 ± 1.2	33.3 ± 3.2	27.9 ± 2.9	28.0 ± 1.0
Urinalysis wk 4					
conc. test volume (ml/16h)	2.4 ± 0.3	3.2 ± 0.5	2.8 ± 0.4	3.1 ± 0.3	3.0 ± 0.2
conc. test density (kg/l)	1.056 ± 0.006	1.043 ± 0.003	1.043 ± 0.006	1.036 ± 0.004*	1.037 ± 0.003*
volume (ml/24)	6.6 ± 1.0	7.2 ± 1.1	6.6 ± 0.6	5.5 ± 0.9	12.9 ± 3.4*
density (kg/l)	1.040 ± 0.005	1.035 ± 0.003	1.036 ± 0.002	1.034 ± 0.005	1.022 ± 0.003
protein (mg/24h)	1.7 ± 0.2	2.1 ± 0.2	2.1 ± 0.2	2.3 ± 0.5	2.9 ± 0.5
glucose (mmol/24h)	7.77 ± 0.78	7.99 ± 1.20	8.27 ± 0.68	5.91 ± 0.86	7.40 ± 1.05
GGT (U/24U)	6.1 ± 0.8	4.9 ± 0.6	7.2 ± 1.0	4.2 ± 0.2	3.7 ± 0.3
ALP (U/24h)	1.10 ± 0.19	0.94 ± 0.17	1.43 ± 0.23	0.74 ± 0.06	0.56 ± 0.05
LDH (mU/24h)	194 ± 23	230 ± 20	269 ± 45	936 ± 543	447 ± 32
NAG (mU/24h)	122.0 ± 13.4	111.6 ± 14.4	127.2 ± 20.7	115.3 ± 11.8	145.2 ± 11.8
Creatinine (µmol/24h)	48.7 ± 4.7	46.6 ± 4.4	51.0 ± 3.7	38.1 ± 1.7	44.1 ± 3.4
Clinical chemistry					
albumin (g/l)	37 ± 0	36 ± 1	35 ± 1	35 ± 1	35 ± 0
protein (g/l)	60 ± 1	59 ± 1	58 ± 0	58 ± 1	56 ± 0
glucose (mmol/l)	8.32 ± 0.41	8.85 ± 0.18	9.11 ± 0.31	9.11 ± 0.24	9.28 ± 0.17
creatinine (µmol/l)	41 ± 2	44 ± 3	40 ± 3	41 ± 3	38 ± 2
urea (mmol/l)	9.8 ± 0.9	10.0 ± 0.9	8.7 ± 0.6	6.9 ± 0.6	7.3 ± 0.9
ALP (U/l)	212 ± 23	258 ± 27	279 ± 25	253 ± 28	263 ± 27
ASAT (U/l)	70 ± 3	67 ± 2	69 ± 1	81 ± 7	86 ± 7
ALAT (U/l)	29 ± 3	29 ± 3	30 ± 3	44 ± 5*	49 ± 5**
GGT (U/l)	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
LDH (U/l)	336 ± 62	294 ± 19	233 ± 23	224 ± 14	302 ± 41
Relative organ weights					
kidney weight (g/kg bw)	6.57 ± 0.25	6.47 ± 0.18	6.99 ± 0.10	7.62 ± 0.28*	8.87 ± 0.35**
liver weight (g/kg bw)	37.7 ± 0.8	36.1 ± 0.9	38.0 ± 0.7	35.3 ± 0.7	39.8 ± 0.6
Renal histopathology					
regeneration	0	0	0	5**	5**
hyperplasia	0	0	0	2	5**
necrosis, focal/multifocal	0	0	0	2	5**
karyomegaly	0	1	0	5**	5**

Values are means ± sem (histopathology: number of affected animals) for groups of 10 (controls, except for clinical chemistry and organ weights) or five rats. Asterisks indicate significant differences from controls: *P<0.05 ** P<0.01.

Endpoint	Control	**Range-finding study: TETRA** (mg/kg bw/day)		**Range-finding stdudy: TRI** (mg/kg bw/day)	
		2500	2800	2000	2400
Weight gain (g/rat/29 days)	59 ± 2	56 ± 9	45 ± 5	53 ± 4	52 ± 2
Food intake (g/rat/day)	11.3	11.2	10.4	11.7	10.9
Water intake (g/rat/day)					
per cage in wk 1-4	23.1	35.8	36.7	24.4	30.1
per rat on day 6	18.2 ± 2.4	36.0 ± 5.8*	29.3 ± 6.4	22.5 ± 2.2	25.7 ± 2.7
per rat on day 26	22.8 ± 3.4	33.0 ± 4.0	35.9 ± 4.0	24.3 ± 2.7	27.3 ± 4.9
Urinalysis wk 1					
conc. test volume (ml/16h)	2.5 ± 0.2	4.8 ± 0.7**	3.5 ± 0.2	3.7 ± 0.1**	5.1 ± 0.3**
conc. test density (kg/l)	1.047 ± 0.002	1.037 ± 0.003*	1.041 ± 0.003	1.047 ± 0.001	1.041 ± 0.003
volume (ml/24h)	8.6 ± 1.2	23.6 ± 5.0**	19.8 ± 5.2*	11.2 ± 1.8	13.5 ± 2.2
density (kg/l)	1.033 ± 0.003	1.018 ± 0.003**	1.019 ± 0.005*	1.032 ± 0.004	1.028 ± 0.003
protein (mg/24h)	2.2 ± 0.2	4.2 ± 0.8**	2.6 ± 0.3	3.0 ± 0.5	2.8 ± 0.2
glucose (mmol/24h)	6.99 ± 0.77	9.74 ± 1.20	8.05 ± 0.67	8.58 ± 0.75	8.10 ± 0.66
GGT (U/24h)	3.9 ± 0.4	6.1 ± 0.8*	5.2 ± 0.5	4.0 ± 0.6	2.6 ± 0.3
ALP (U/24h)	1.50 ± 0.14	3.13 ± 0.55**	2.22 ± 0.10	2.04 ± 0.43	1.27 ± 0.22
LDH (mU/24h)	412 ± 51	583 ± 68	519 ± 89	408 ± 88	611 ± 263
NAG (mU/24h)	105.7 ± 8.3	152.6 ± 22.5	133.3 ± 22.0	134.0 ± 16.6	139.1 ± 10.2
Creatinine (µmol/24h)	34.7 ± 2.0	32.1 ± 2.0	26.5 ± 1.5*	31.9 ± 1.9	32.8 ± 2.7
Urinalysis wk 4					
conc. test volume (ml/16h)	2.9 ± 0.3	5.7 ± 1.1**	3.2 ± 0.6	4.4 ± 0.5*	4.7 ± 0.6**
conc. test density (kg/l)	1.049 ± 0.003	1.039 ± 0.005	1.056 ± 0.008	1.045 ± 0.003	1.039 ± 0.002
volume (ml/24)	13.6 ± 2.6	16.5 ± 4.6	18.6 ± 3.7	14.3 ± 2.2	17.4 ± 2.7
density (kg/l)	1.030 ± 0.004	1.026 ± 0.006	1.026 ± 0.008	1.030 ± 0.004	1.031 ± 0.002
protein (mg/24h)	2.8 ± 0.3	3.4 ± 0.7	3.7 ± 0.2	3.0 ± 0.3	3.0 ± 0.2
glucose (mmol/24h)	8.85 ± 0.53	9.32 ± 1.62	11.23 ± 0.88	9.54 ± 0.52	9.77 ± 0.51
GGT (U/24U)	4.6 ± 0.3	5.7 ± 1.5	5.5 ± 0.5	2.5 ± 0.4**	2.8 ± 0.2**
ALP (U/24h)	1.41 ± 0.11	4.67 ± 1.37**	3.43 ± 0.14*	1.49 ± 0.28	1.63 ± 0.12
LDH (mU/24h)	396 ± 34	579 ± 164	627 ± 62	359 ± 48	539 ± 89
NAG (mU/24h)	77.1 ± 5.6	85.2 ± 17.2	99.7 ± 11.0	86.7 ± 7.3	85.9 ± 4.7
Creatinine (µmol/24h)	52.3 ± 2.0	39.8 ± 5.3*	43.3 ± 2.9	44.8 ± 3.0	45.7 ± 1.3
Clinical chemistry					
albumin (g/l)	36 ± 0	35 ± 1	34 ± 1	37 ± 1	35 ± 0
protein (g/l)	61 ± 1	59 ± 2	59 ± 2	61 ± 2	59 ± 0
glucose (mmol/l)	9.64 ± 0.32	9.81 ± 0.63	9.27 ± 0.63	8.69 ± 0.30	8.38 ± 0.23*
creatinine (µmol/l)	29 ± 2	26 ± 1	22 ± 1*	26 ± 1	23 ± 1*
urea (mmol/l)	10.0 ± 0.8	10.6 ± 0.9	11.2 ± 1.7	10.9 ± 0.7	9.4 ± 0.6
ALP (U/l)	233 ± 18	336 ± 54	376 ± 48*	477 ± 34**	478 ± 74**
ASAT (U/l)	60 ± 3	78 ± 6	94 ± 10**	77 ± 2**	72 ± 3*
ALAT (U/l)	39 ± 2	55 ± 4*	63 ± 7**	61 ± 3**	78 ± 9**
GGT (U/l)	0.1 ± 0.0	0.3 ± 0.2	0.7 ± 0.3*	0.6 ± 0.1**	0.5 ± 0.1**
LDH (U/l)	171 ± 17	156 ± 20	187 ± 19	177 ± 19	188 ± 24
Relative organ weights					
kidney weight (g/kg bw)	6.53 ± 0.10	7.67 ± 0.21**	7.70 ± 0.35**	7.48 ± 0.23**	7.85 ± 0.25**
liver weight (g/kg bw)	37.6 ± 0.6	50.6 ± 1.8**	48.8 ± 0.8**	50.2 ± 1.1**	51.5 ± 1.3**
Renal histopathology					
vacuolation, focal	2	4	5**	5**	2
cyto/karyomegaly	0	2	0	4**	5**

Values are means ± sem (histopathology: number of affected animals) for groups of 10 (controls) or five rats. Asterisks indicate significant differences from controls: *P<0.05 ** P<0.01 (TETRA and TRI were evaluated separately).

Chapter 6

General discussion and conclusions

6.1 Introduction

Given the reality of concurrent or sequential exposure of humans to multiple chemicals, it is important to assess health risks of exposure to chemical mixtures. However, exposure assessment, hazard identification and risk assessment of chemicals has traditionally focused on single chemicals. Consequently, there is a scarcity of scientific knowledge and understanding of mixture toxicity. Obviously, it is impossible to examine the toxicity of the infinite number of combinations of chemicals. Yet, we need more knowledge and better understanding of mixture toxicity to answer the question whether current approaches to risk assessment of chemicals afford adequate protection of public health (Sexton *et al.*, 1995). Recognizing this, the toxicity of chemical mixtures has received considerable attention from the international scientific and regulatory community (Feron *et al.*, 1998a, 1998b, 2002; Hansen *et al.*, 1998). This has yielded interesting experimental results, under which data on the relatively unexplored low dose domain, as well as new or refined approaches for risk assessment of chemical mixtures.

In the Netherlands, the Health Council addressed the issue of mixture toxicity in her advisory report on the setting of health-based standards for non-carcinogenic substances (Health Council of The Netherlands, 1985). The Health Council concluded that there was no immediate reason to take combination effects into account at concentrations well below the standards for individual substances. Further, after having established that there were no useful data on effects of prolonged exposure to chemical mixtures at concentrations around the experimental No-Observed-Adverse-Effect Levels (NOAELs) of the individual substances, the Health Council recommended research to fill this data gap. With this as a backdrop, a research programme on mixture toxicity was started at TNO Nutrition and Food Research. The studies described in the previous chapters were part of this programme. One of the major aims was to test the hypothesis that as a rule exposure to mixtures of chemicals at (low) non-toxic doses of the individual chemicals is of no health concern. For this purpose, we conducted a series of toxicity studies in rats with defined mixtures of chemicals with similar or dissimilar target organs and/or modes of action. As recommended by the Health Council, the chemicals were administered at levels at or around (viz. not more than about an order of magnitude below or above) their individual NOAEL.

The different studies are discussed below. This discussion is followed by a brief overview of approaches to risk assessment of chemical mixtures and the experimental support for underlying assumptions, a summary of (complementary) experimental work in the low dose region by other researchers, and finally conclusions.

6.2 Toxicity of mixtures of chemicals with similar or dissimilar target organs and/or modes of action

6.2.1 Chemicals with different target organs

In our first study (chapter 2), we examined the toxicity of a mixture of eight chemicals upon continuous exposure via the diet or drinking water for four weeks. The design of this study was similar to that routinely used to examine the sub-acute oral toxicity of single chemicals. The choice of chemicals was based on practical criteria (free availability of sub-acute oral toxicity data produced in our laboratory, no marked decrease in consumption of food or water due to poor palatability), rather than on target organ or mode of action. This yielded a selection of chemicals with different primary target organs. The main objective of this study was to determine whether simultaneous administration of the chemicals at dose levels equal to the NOAEL of each of the individual chemicals would result in a NOAEL or an adverse-effect level for the mixture. The mixture was also examined at two levels below the individual NOAELs (1/3 and 1/10 NOAEL) to approach more closely people's actual, low-level exposure scenarios. Though these experimental levels are still very high compared with most human environmental exposures (for example, standards are often set 100 times below the NOAEL from a life-span animal study), they may be realistic for accidental or occupational exposures. In addition, the mixture was given at the Minimum-Observed-Adverse-Effect Level (MOAEL) of the individual chemicals.

Animals given the mixture at the NOAEL of the individual chemicals showed minimal adverse effects on two of the nearly 70 endpoints examined (viz. decreased haemoglobin content and increased relative kidney weight). This suggested that combined exposure to the chemicals at their individual NOAEL represented an increased hazard compared with single exposure at the same level. However, the effects in the NOAEL mixture group were not considered convincing proof of such an increased hazard because they were small and seen in one sex only. Moreover, it cannot be ruled out that the presumed NOAELs of the individual chemicals actually were MOAELs due to inter-study variation (these NOAELs were derived from previous single chemical studies in which the experimental conditions resembled those of the mixture study as closely as possible; they were not verified in the mixture study). As evidenced by the absence of treatment-related changes in the 1/3 or 1/10 NOAEL mixture groups, simultaneous administration of the chemicals at dose levels below their individual NOAEL resulted in a clear NOAEL for the mixture. In contrast, animals of the MOAEL group showed a large number of all sorts of adverse effects.

Compared with the effects at the MOAEL in the single chemical studies, both more severe and less severe effects were seen upon combined exposure at the MOAEL, suggesting interactive joint action at the effective (toxic) levels of the individual chemicals. The occurrence of interaction at effective dose levels in our study is not surprising in view of the many published examples of interaction at effective dose levels (e.g. Calabrese, 1991; Krishnan and Brodeur, 1991,1994). Given the descriptive nature of our study, no attempt was made to further characterise the interactions at the MOAEL. From a public health point of view, however, it is important to characterise the type of joint action at non-toxic levels of the individual chemicals, since synergistic interaction or similar joint action (dose addition) can bring the net effect of the mixture above the threshold of toxicity even when each individual chemical is present at a level below its own toxicity threshold. Proper characterisation of the type of joint action requires establishing whether the observed mixture response deviates from the response calculated on the basis of an additivity assumption (dose or response addition). Our study was not designed to support such a quantitative evaluation. Yet, we can make some qualitative assessments. For most of the eight chemicals, the NOAEL was only a factor 3-5 lower than the MOAEL. Though this leaves room for slight increases in the toxicity of an individual chemical to occur unnoticed (assuming that effects smaller than those at the MOAEL are below the limit of detection), the absence of obvious toxic effects of the mixture at the NOAEL allows the conclusion that there were no marked synergistic effects at this level. Further, the absence of obvious toxicity of the mixture at the NOAEL indicates that the mixture response could not be predicted by full dose additivity. This becomes evident when the individual NOAELs are expressed as fraction of the pertaining MOAELs, and then summed. The sum approached 2. Under dose addition, a sum of unity or greater would predict that the mixture induces adverse effects. Clearly, dose addition overestimated the toxicity of the mixture of chemicals with different target organs. This confirms the common idea that application of dose additivity is inappropriate for such mixtures.

Following the above study with eight arbitrarily chosen chemicals, Groten conducted a similar type of study using a combination of nine compounds highly relevant to the general population in terms of use pattern and level and frequency of exposure (Groten *et al.*, 1997). In this study, male rats received seven chemicals via their diet (aspirin, di(2-ethylhexyl)phthalate, cadmium chloride, stannous chloride, butyl hydroxyanisol, loperamide, spermine) and two by inhalation (formaldehyde, dichloromethane), at dose levels equal to the chemicals' individual MOAEL, their individual NOAEL or 1/3 NOAEL. A few organs (liver, red blood) were targeted by more than one of these chemicals. As in the study with eight chemicals, combined

exposure at the MOAEL resulted in many adverse effects which were qualitatively and quantitatively similar or dissimilar to those of the individual chemicals. At the lower dose levels, treatment-related findings were limited to the following minor changes: histological changes of the nasal epithelium, hepatocellular hypertrophy, decreased plasma levels of triglycerides and alkaline phosphatase activity, and increased kidney weight at the NOAEL, and increased kidney weight at the 1/3 NOAEL.

Overall, an important practical lesson from these two studies was that simultaneous exposure to chemicals with different target organs did not constitute an evidently increased hazard compared to exposure to each of the chemicals separately, provided that the exposure level of each chemical did not exceed its own NOAEL.

6.2.2 Chemicals with a similar target organ but dissimilar modes of action

The objective of the studies described in chapters 3 and 4 was to examine whether concurrent administration of four chemicals selected to affect the same organ (the kidney) by different modes of action would result in an effect level when each chemical was given at its own No-Observed-Nephrotoxic-Effect Level (NONEL). In the 4-week study (chapter 3) the mixture of hexachloro-1,3-butadiene, mercuric chloride, *d*-limonene and lysinoalanine was given via the diet to male and female rats at three dose levels, namely the individual chemicals' Lowest-Observed-Nephrotoxic-Effect Level (LONEL), their NONEL and 1/4 NONEL. The mixture in the acute study (chapter 4) comprised hexachloro-1,3-butadiene, mercuric chloride, *d*-limonene and potassium dichromate, and was examined in male rats at the LONEL and NONEL only. To enable comparison with the toxicity of the individual chemicals, both studies included groups administered the chemicals alone at their NONEL or LONEL.

Combined exposure to the nephrotoxicants at a level slightly below their individual threshold for renal toxicity (1/4 NONEL) resulted in a clear no-effect level for the mixture. When the mixture was given at the individual NONELs, slight growth retardation (4-week study only) and a few minor signs of renal toxicity (including increased relative kidney weight) were observed. Since none of the individual chemicals induced growth retardation at its NONEL, exposure to the mixture constituted a slightly increased risk in terms of general toxicity compared with exposure to the chemicals separately. In terms of organ-specific toxicity, however, the slight effects on renal endpoints in the NONEL mixture groups were not considered to reflect increased toxicity upon combined exposure because similar effects were observed in one or two single compound groups at the NONEL. As expected, animals given the mixture at the individual LONELs showed all sorts of adverse effects. In

comparison with the effects of the single chemicals at their LONEL, the mixture effects ranged from less to more severe.

The design of these two studies with mixtures of chemicals with the same target organ but different modes of action does not allow quantitative characterisation of the type of joint action. Qualitatively, it can be concluded that the absence of increased renal toxicity upon combined exposure at the NONEL demonstrated absence of marked synergistic interaction when the chemicals were present at subtoxic levels. An assessment of the appropriateness of (dose) additivity for this type of mixture at subtoxic levels of the individual chemicals is hampered by the relatively large gap between the NONELs and LONELs tested. For both mixtures the sum of the NONEL/LONEL ratios was less than unity (when ignoring the finding that the NONEL for some chemicals turned out to be an effect level, the sum of these ratios was only 0.5 or 0.6). Moreover, the chemicals showed different effect profiles for the battery of renal endpoints examined, rendering the selection of a suitable endpoint for the assessment problematic.

Despite these limitations, Mumtaz and colleagues (1998) were able to use the data from the 4-week study for a quantitative evaluation of different joint toxicity assessment methods (viz. dose addition, response addition with a completely positive correlation of tolerances, and the weight-of-evidence method). Using the endpoint relative kidney weight (which was significantly increased by hexachloro-1,3-butadiene and mercuric chloride only), they compared the observed dose-response relationship with dose-response relationships predicted based on the various joint toxicity models. At the NONEL and 1/2 LONEL, the observed responses did not deviate significantly from the predicted responses, regardless of the model used. At the LONEL, however, differences became apparent: in female rats all models overestimated the response, in male rats response addition underestimated the response whereas dose addition and the weight-of-evidence model slightly overestimated it.

The toxicity of a mixture of chemicals with the same target organ was also addressed by Cassee who investigated the possible additive or interactive effects on the nose of formaldehyde, acetaldehyde and acrolein (Cassee *et al.*, 1996). These aldehydes targeted the same organ and exerted the same type of effect (irritation/cytotoxicity), but they affected different regions of the nasal mucosa. Rats were exposed by inhalation for three days to the individual chemicals or to three mixtures at non-toxic or slightly toxic levels. Each aldehyde was given at a low level (at which acrolein induced slight effects whereas formaldehyde and acetaldehyde induced no effects) and a high level (at which all three aldehydes were slightly toxic). The two mixtures containing the aldehydes at their low level (viz. the mixture of all three aldehydes, and the binary mixture of

formaldehyde plus acrolein) resulted in nasal histopathological changes which were very similar to those induced by the low level of acrolein alone. The third mixture, which included the three aldehydes at their high (toxic) level, induced nasal lesions which were more severe than those observed after exposure to the individual aldehydes at this dose level. Since the gap between the two levels of each aldehyde was small (a factor of 2-3), it could be concluded that neither dose addition nor potentiating interaction occurred when the exposure concentrations of the aldehydes were NOAELs.

In conclusion, the results of our studies with chemicals with a similar target organ but dissimilar modes or sites of action supported the hypothesis that adverse effects are unlikely when the components in a chemical mixture are at levels below their individual thresholds.

6.2.3 Chemicals with a similar target organ and similar mode of action

The study described in chapter 5 was conducted to test the additivity assumption (dose addition) under conditions of concurrent, repeated exposure to similarly acting nephrotoxicants at exposure levels slightly below the individual chemicals' thresholds for renal toxicity. Tetrachloroethylene, trichloroethylene, hexachloro-1,3-butadiene (HCBD) and 1,1,2-trichloro-3,3,3-trifluoropropene (TCTFP) were given to female rats by daily oral gavage for 32 days either alone at their individual LONEL and NONEL (set at 1/4 of the LONEL), or in combinations of four (at the NONEL and 1/2 LONEL) or three (at 1/3 LONEL). Under the assumption of full dose addition (and linear, parallel dose-response curves in the dose range studied), the response predicted for the ternary mixtures at 1/3 LONEL and the quaternary mixture at 1/4 LONEL would be equal to that for the single chemicals at their own LONEL. Thus, possible departures from additivity can be detected by comparing the responses observed for these mixtures with those observed for the chemicals separately at their LONEL.

Based on the endpoint relative kidney weight, which was increased to about the same extent in the groups given the mixture at 1/3 or 1/4 LONEL and all groups given the individual chemicals at their LONEL, dose addition was a valid assumption for these similarly acting nephrotoxicants. Unlike kidney weight, the other endpoints used to assess nephrotoxicity (renal morphology and concentrating ability, plasma levels of urea and creatinine, and excretion of protein, glucose and marker enzymes in the urine) were not affected by each of the individual chemicals at their LONEL. Consequently, these endpoints could not be analysed by the above 'toxicity unit method' for predicting mixture responses and testing of the validity of the additivity assumption. Nevertheless, from the finding that the observed mixture responses for these endpoints

were generally not significantly different from the background (vehicle control) response (except in the 1/2 LONEL mixture group) it can be inferred that there was no marked synergistic interaction upon combined exposure at the NONEL or 1/3 LONEL.

Results from this study were reanalysed by Mumtaz and colleagues (1998) who used dose-response modeling instead of the toxicity unit method. The dose-response models were based on assumptions of dose addition, response addition with a completely positive correlation of tolerances, or the weight-of-evidence method. The latter method was based on binary-weight-of-evidence assessments which suggested a less than additive interaction for renal toxicity for all possible binary combinations of hexachloro-1,3-butadiene and 1,1,2-trichloro-3,3,3-trifluoropropene (because competition for glutathione was anticipated to reduce the toxicity of these chemicals) and an additive renal toxicity for all possible binary combinations of tetra- and trichloroethylene. For the endpoints relative kidney weight and relative liver weight the analyses confirmed that the mixture responses in the subnephrotoxic dose region were consistent with dose additivity (note: tetra- and trichloroethylene were hepatotoxic at these dose levels). It should be added that the mixture responses predicted by the models based on response addition or the weight-of-evidence method were also close to the observed responses in this dose region. On the other hand, at the higher dose level (1/2 LONEL) dose addition overestimated the effect on kidney weight, response addition underestimated it and the weight-of-evidence method predicted it very closely. The effect on liver weight at the higher dose level was underestimated by all three models. The different outcomes of the weight-of-evidence method for these two organs clearly demonstrated that weight-of-evidence evaluations should be target-organ specific.

In another study evaluating the weight-of-evidence method, this method was applied on data obtained in an *in vitro* assay in which rat kidney slices were exposed to mixtures of metabolites (viz. the cysteine conjugates) of the four nephrotoxicants used in our *in vivo* study with similarly acting nephrotoxicant (El-Masri *et al*, 2001). The weight-of-evidence method predicted that dose addition would be the most likely form of joint action *in vitro*. The experimental results confirmed this prediction. Interestingly, the *in vivo* joint effect (on kidney weight) of the parent chemicals at their NONEL or 1/3 LONEL was also consistent with dose addition, despite the (many) possible interactions in toxicokinetic processes that precede the formation of the cysteine conjugates and their disposition in the kidneys.

6.3 Health risk assessment of chemical mixtures

6.3.1 Regulatory approaches

Despite the historical focus on single chemicals, approaches for health risk assessment of chemicals have been addressing the issue of multiple chemical exposure for many years. For example, the U.S. Food and Drug Administration mentioned the possibility of increased toxicity due to combination effects as one of the factors necessitating an at least 100-fold margin of safety for food additives (Lehman and Fitzhugh, 1954). In the 1960s a law of the U.S. Food and Drug Administration required toxicity testing of combinations of acetylcholinesterase-inhibiting pesticides for any new anticholinesterase insecticides. This requirement resulted from the observation of marked potentiation of malathion toxicity by another organophosphate insecticide and the elucidation of the underlying mechanism (viz. inhibition of the carboxylesterases which detoxify malathion) (page 364 in Calabrese, 1991). In 1967, however, this requirement was dropped on the grounds that 'it failed to serve any useful purpose' and 'no single, rigid, practical experimental design has been developed by which it is possible to detect all cases of potentiation' (cited in Boyd *et al*, 1990).

Also in the 1960s, the American Conference of Governmental Industrial Hygienists (ACGIH) first recommended its additivity approach to determine whether exposure to multiple chemicals is likely to pose an occupational hazard (ATSDR, 2001; chapter 23 in Calabrese, 1991). According to this approach the mixture's toxicity should be assessed on the basis of dose additivity [Hazard Index (HI) approach] unless there is good reason to expect that the components act independently. The HI-approach for components with similar toxic effects involves summation of the ratios (hazard quotients) of the exposure concentration to the threshold limit value (TLV) for each component. If the sum exceeds unity then the TLV for the mixture is considered as being exceeded. For independently acting components, the TLV for the mixture is exceeded only if at least one component has a hazard quotient that exceeds unity (this resembles dose addition with completely positive correlation of tolerances). ACGIH further recommends to evaluate synergistic or antagonistic interaction on a case by case basis and states that synergism is characteristically exhibited at high concentrations, and probably less at low concentrations.

The concept of dose addition has also been applied to the standard setting of some food additives. Examples are the polyols and poorly absorbed bulk sweeteners (based on their laxative effect at high intake levels) and esters of allyl alcohol (based on the

formation of a common metabolite which is responsible for the similar toxic effects of these flavor additives) (Groten *et al*, 2000; World Health Organization, 1987).

The first comprehensive approach for assessing health risks from multiple chemical exposures has been published by the U.S. Environmental Protection Agency (EPA) in its 'Guidelines for the health risk assessment of chemical mixtures' (EPA, 1986) and the pertaining 'Technical support document on risk assessment of chemical mixtures (EPA, 1990) which presents the scientific and technical background of the general principles and procedures described in the guidelines. More recently, EPA published the 'Supplementary guidance for conducting health risks assessment of chemical mixtures (EPA, 2000). This document does not replace the 1986 guidelines but provides more specific details on the nature of the desired information and the procedures to use in analysing the data. Current mixture risk assessment approaches adopted by other agencies, for example the Agency for Toxic Substances and Disease Registry in the United States (ATSDR, 2001) and the Health Council in the Netherlands (Health Council of The Netherlands, 2002) closely resemble those recommended by EPA.

The EPA guidelines emphasize that risk assessments should be conducted on a case-by-case basis, giving full consideration to all relevant scientific information. Guidance is given for the use of three approaches, depending on the nature and quality of the available data. Because of the uncertainties at several decision points in the flow chart summarizing the approaches, EPA recommends to perform all possible assessment paths and to evaluate the range of resulting health risk estimates.

The preferred approach is to use data on the mixture of concern (i.e. treat the mixture as a single chemical). EPA has used this method for commercial mixtures of polychlorinated biphenyls and coke oven emissions (EPA, 2000). If data are not available on the mixture of concern but health effects data are available on a similar mixture or group of similar mixtures, the risk assessment may be based on data on a sufficiently similar mixture. The determinaton of 'sufficient similarity' must be made on a case-by-case basis, taking into account chemical composition and toxicological activity. The comparative potency method, which has been applied to estimate cancer risk from emissions released upon combustion of organics, is an example of a similar-mixtures approach (EPA, 2000). The comparative potency method involves extrapolation across mixtures and across assays which monitor the same type of health effect. It is based on the assumption that the dose-response curves in the different assays have the same shape and that the relationship (relative potency) between any two mixtures is the same, whichever assay is used. In practice these two whole mixture approaches are seldom used because of a lack of data.

In the absence of data on an identical or reasonably similar mixture, most risk assessments are based on the toxic properties of the components in the mixture. When quantitative information on toxicological interactions exists, even if only on pairs of chemicals, it should be incorporated into the component-based approach. When there is no adequate interactions information, dose- or response-additive models are recommended. For carcinogens, summation of risks (response addition with completely negative correlation of tolerances) should be used. For non-carcinogenic effects, the criterion for choosing between dose and response addition is the toxicological similarity among the chemicals in the mixture.

If dose addition is most plausible, as supported by similarity of toxic modes of action or, less strongly, by target organ similarity, the risk assessment usually proceeds by the HI-method. For mixtures including chemicals that have different effects, EPA recommends the calculation of separate HIs for each endpoint of concern. Another method based on dose additivity is the Toxicity Equivalency Factor (TEF) approach which was developed as an interim procedure to assess the toxicity of complex mixtures of polychlorinated dibenzo-*p*-dioxines and dibenzofurans. The TEF-method requires more knowledge about the mode of toxic action (it assumes a common mode of action) and is, therefore, used much less frequently than the HI-method. In the TEF-approach, the dose of each mixture component is normalized against the dose of one of the components, usually the most potent and best-studied component (called the index chemical), to derive a relative potency for each component. The relative potencies are then summed to estimate the toxicity of the mixture. More details and limitations of this method can be found in the guidance documents of EPA (2000) or ATSDR (2001).

For incorporation of interaction data in the component-based approach for non-carcinogenic effects, EPA recommends a weight-of-evidence method to modify the HI (EPA, 2000). Key assumptions are that interactions can be adequately represented as departures from dose addition and that, at least for low doses, the influence of all toxicological interactions in a mixture can be adequately approximated by some function of the pairwise interactions. Higher-order interactions are assumed to be minor compared to binary interactions. In the original method developed by Mumtaz and Durkin (1992), the HI is modified by a single composite interaction factor. In EPA's currently recommended method, each component's hazard quotient is modified by the influences of all the other potentially interacting components. The interactions-modified hazard quotients are then summed to estimate the interactions-based HI. Another difference with the original method is the more flexible weight-of-evidence classification system (EPA, 2000).

Although not explicitly recommended in the EPA guidelines, physiologically based toxicokinetic (PBTK) modeling approaches are potentially useful tools for predicting quantitatively the consequences of interactions. Like the weight-of-evidence approaches, the mechanistic modeling approaches incorporate binary interaction data. In a mixture PBTK-model, the individual PBTK models for each mixture component are interconnected at the level of the tissue compartment where an interaction is hypothesized or known to occur. Such a model can be used to conduct various extrapolations (e.g. high to low dose, route to route, binary to multichemical mixtures) of the occurrence and magnitude of interactions from laboratory studies to human environmental or occupational exposure scenarios (Haddad and Krishnan, 1998; Haddad *et al*, 2000; Krishnan *et al*, 2002).

6.3.2 Evidence to support additivity assumptions

Central to the above risk assessment methods are assumptions about additivity. The experimental evidence for either dose addition or response addition as a good approximation for a mixture risk assessment is not strong, especially with respect to prolonged multiple chemical exposure at subtoxic levels. Though the validity of additivity assumptions for mixtures of more than two chemicals at fractions (down to 1/400) of the effective concentration of the individual chemicals has been studied in aquatic organisms (Könemann, 1981; Deneer *et al.*, 1988a,b), similar studies in mammals are lacking. An analysis of the U.S. EPA data base ('MixTox') on binary interaction studies in normal, healthy animals showed that nearly all studies involved two-chemical exposures which were mostly of short duration, at toxic levels, and often sequential (EPA, 1990; Simmons, 1994). Moreover, in about half of these studies statistics were not performed or not described. The papers of Pozzani (Pozzani *et al.*, 1959) and Smyth (Smyth *et al.*, 1969, 1970) report the results of a large study intended to explore the validity of the hypothesis that the acute toxicity of mixtures of randomly selected chemicals can be predicted satisfactorily by dose additivity. In single dose oral (LD50) or inhalation (LC50) studies in rats, these authors examined many binary mixtures (350 pairs were tested orally) and a few ternary or quaternary, commercial mixtures of industrial organic chemicals (selected on the basis of their large volume in commerce). The results supported the assumption of dose additivity for both equivolume and equitoxic mixtures and showed that the magnitude of the few departures from additivity was small (within a factor of 5). The Federal German Research Society reported on over 400 combinations of pesticides (organophosphates, carbamates and chlorinated hydrocarbons), most of which had only additive or less than

additive effects in acute studies (cited from Groupement International des Associations Nationales de Fabricants de Produits Agrochimiques, 1988). The few combinations that exhibited more than additive effects in acute tests showed only additive effects in follow-up studies in which rats or dogs were fed low doses of these pesticides for several months. More recently, binary combinations of clastogens were examined in a mouse micronucleus assay (Suzuki *et al.*, 1995). The results supported the use of dose addition for clastogens with similar targets (DNA or protein) and modes of action, and of response addition (independent action) for clastogens with dissimilar targets and modes of action. For combinations of clastogens with the same target but different modes of action inconsistent results were obtained. Birgelen and co-workers evaluated the validity of the TEF approach for the combination of PCB156 (a polychlorinated biphenyl found at relatively high concentrations in human milk and fat tissue) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in a subchronic feeding study in rats, using effective dose levels of the individual compounds (Birgelen *et al.*, 1994). They found non-additive effects on several endpoints. However, since the magnitude of the observed interactions was negligible compared with the uncertainty in the TEF value (which was determined in the same study), the interactive effects were suggested to have no implications for the TEF concept. Finally, conclusions from two literature reviews evaluating possible interactive effects upon exposure to more than one chemical are cited here. Ikeda (1988) reviewed environmental/occupational toxicology journals over the period 1981-1987 and concluded 'No case is detected in this search to indicate that assumption of additive effects (dose additivity, ed.) is not safe enough.'. Nelson (1994) searched the literature on developmental toxicology (period not specified) and from his subjective evaluation of about 160 *in vivo* studies he concluded that 'low doses (not defined, ed.) of combinations often produced additive effects, but higher doses produced either antagonistic or synergistic effects'. Nelson defined additivity as effect summation (i.e. response addition with completely negative correlation of tolerances).

The results of our studies with eight or nine chemicals with different target organs showed no obvious toxic effects for the mixtures at subtoxic exposure levels of the individual chemicals. Application of the HI-method (using dose addition) on these mixtures yielded HIs of about 2, indicating that the assumption of dose additivity overestimated the mixture risk. This supports the commonly adopted assumption of response addition for chemicals with different target organs (i.e. the risk of the mixture is assumed to be equal to the risk of the component with the highest risk quotient). Our study with chemicals with the same target organ but dissimilar modes of action also demonstrated absence of toxicity for the mixture at subtoxic levels of the components.

Reanalysis of the data by Mumtaz *et al.* (1998) showed that both dose addition and response addition closely predicted the mixture responses at subtoxic dose levels. At the minimally toxic level, however, neither assumption estimated the mixture response correctly, indicating that mixture data obtained at effective (toxic) levels did not accurately predict what happened at nontoxic levels of the individual chemicals. Such poor predictive value of mixture results from effect levels for no-effect levels was also apparent from our other studies, regardless of the type of mixture. The data from our study with similarly acting nephrotoxicants supported the assumption of dose additivity for chemicals with a similar target organ and similar mode of action at non-toxic levels of the mixture components.

A search in recent literature yielded no other studies designed to evaluate the assumption of dose additivity for non-carcinogenic effects under conditions of repeated exposure to more than two chemicals at no-effect levels. The search did yield one 14-day rat study which supported dose additivity at a minimal effect level (Gennings *et al.*, 1997; Teuschler *et al.*, 2000). The mixture in this study consisted of four trihalomethanes (by-products of water disinfection) with a similar target organ (viz. the liver; plasma sorbitol dehydrogenase activity was used as endpoint for hepatotoxicity). The authors did not indicate whether these chemicals also had a similar mode of action. Another study evaluated deviations from response additivity (effect summation) for carcinogenic effects at minimally effective dose levels (Nesnow *et al.*, 1998). Lung tumorigenic interactions of a five-component mixture of environmental polycyclic aromatic hydrocarbons were examined in a medium-term bioassay (A/J strain mouse), using a 2^5 factorial design and response surface analysis. Though the results showed statistically significant, dose-related interactions (synergism at lower doses and antagonism at higher doses), the departures from additivity were relatively small (between -55% and +97% of the predicted response).

6.3.3 Other studies on mixture effects at low or subtoxic dose levels

Whereas our studies focused on mixture toxicity at exposure levels round the toxicity thresholds of the individual chemicals, the studies of the U.S. National Toxicology Program (NTP), initiated late 1980s, aimed to investigate the long-term health effects in laboratory animals given drinking water contaminants at environmentally realistic levels (Yang and Rauckman, 1987; Schwetz and Yang, 1990). About a dozen of studies at NTP were conducted with a defined mixture of 25 contaminants (19 organic chemicals and 6 metals) frequently detected in U.S. groundwater sources. In most of these studies, the highest concentration tested was

comparable to that in the heavily polluted groundwater near hazardous waste-disposal sites but probably several orders of magnitude lower than that in drinking water consumed by humans (Yang *et al.*, 1989a,b). Seed and colleagues compared the concentrations of the contaminants as present in the mixture with their individual toxicity thresholds, using data from the critical studies used by U.S. EPA to derive reference doses (Seed *et al.*, 1995). This comparison, which could be made for only 15 of the 25 mixture components, showed that the highest concentrations tested in the NTP studies were well below the NOAELs from the critical studies. The findings for the 25-chemical mixture ranged from no treatment-related changes (reproductive toxicity and many conventional endpoints for subchronic toxicity) to subtle immunosuppression, myelotoxicity, cytogenetic changes in bone marrow, microcytic anemia, nephrotoxicity, increased liver weight and inflammatory lesions in the liver, spleen, mesenteric lymph nodes and adrenals (Chapin *et al.*, 1989; Germolec *et al.*, 1989; Heindel *et al.*, 1995; Hong *et al.*, 1991, 1992; National Toxicology Program, 1993a; Shelby *et al.*, 1990; Simmons *et al.*, 1994). Though none of the studies included groups given the contaminants separately, it was stated by the authors that the immunosuppression and inflammatory lesions in several organs could not be predicted on the basis of the known toxic effects of the individual contaminants (Germolec *et al.*, 1989; National Toxicology Program, 1993a). Similarly, the unique pattern of hepatocyte proliferation transiently observed in rats given submixtures (of the 25-chemical mixture) containing 3, 4 or 7 organic and/or inorganic contaminants was not expected to have been induced by the individual components (Constan *et al.*, 1995). In a follow-up study, using a medium-term bioassay for detection of carcinogens and modifiers of hepatocarcinogenesis, these submixtures induced no changes indicative of carcinogenic promotional effects (Benjamin *et al*, 1 999).

A second, smaller series of studies conducted by NTP examined the health effects of two drinking water mixtures representing a worst-case scenario of pesticide and fertilizer contamination of groundwater in farming-intensive states , viz. California and Iowa (Yang, 1992). These mixtures consisted of 5 or 6 pesticides and one fertilizer (ammonium nitrate). At the lowest level tested, the pesticides were present at the medium survey values in groundwater under normal agricultural use and nitrate was present at the expected human exposure level. The highest dose level was 100 times higher than the lowest level but still well below the individual chemicals' toxicity thresholds as concluded from a comparison with literature data (Heindel *et al.*, 1994; Seed *et al.*, 1995). These studies revealed no signs of toxicity in conventional subchronic or reproductive toxicity studies (National Toxicology Program, 1993b; Heindel *et al.*, 1994). However, in an (unconventional) *in vivo* genotoxicity study

unexpected cytogenetic changes (viz. dose-related increases in sister-chromatid exchanges in splenocytes) were observed in rats and mice given the California mixture for 10-13 weeks (Kligerman *et al*, 1993).

Chaturvedi (1993) examined the toxicity of a mixture of 10 widely used organic pesticides of different chemical classes and/or uses in a 13-week drinking-water study in mice. The mixture was given at four levels, ranging from 10 ppb of each pesticide at the lowest level to 10 ppm at the highest level. For comparison, several of the pesticides have been found in groundwater in agricultural areas at concentrations of approximately 1 ppb. The results showed a dose-related increase in the activity of several hepatic xenobiotic-metabolizing enzymes from the lowest level, which was accompanied by an increase in liver weight and decrease in pentobarbital-induced sleep time at the higher levels. According to the author, induction of these enzymes possibly would not have been observed with the individual pesticides at similar dose levels (the study did not include single compound groups). The few other endpoints examined (viz. spleen and kidney weight, routine microscopy of the liver, and serum activities of transaminases, alkaline phosphatase and cholinesterase) were not affected by the mixture.

In contrast to the above mixtures of groundwater contaminants which induced no severe health effects at non-toxic levels of the individual components, combined exposure to non-effective doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the synthetic glucocorticoid hydrocortisone (HC) induced cleft palate, an irreversible structural change, in all exposed mouse embryos (Abbott, 1995). This marked synergistic interaction was explained by mutual upregulation of the receptors which mediate the biological activity of TCDD and HC (the aryl hydrocarbon receptor and glucocorticoid receptor, respectively).

The effects of binary combinations of solvents at occupationally relevant concentrations were examined in two human volunteer studies (Tardif *et al.*, 1991; Engelen *et al.*, 1997). In both studies, acute (4 hours for toluene plus *m*-xylene, 15 minutes for methyl ethyl ketone plus *n*-hexane) concurrent exposure to air concentrations around the TLV of the individual solvents resulted in a significant toxicokinetic interaction (metabolic inhibition), as indicated by the changes in the blood concentrations of parent compounds (toluene and *m*-xylene increased) or metabolite (2,5-hexanedione decreased). The interaction between toluene and *m*-xylene, which potentially augments their neurotoxicity, was not demonstrable at exposure concentrations which were about two times lower than the individual TLVs. Indirectly, this suggests that a dose-additivity based occupational exposure limit for

simultaneous exposure to toluene and *m*-xylene adequately protects against the acute neurobehavioral effects of these two solvents.

The following studies addressed carcinogenic effects of defined chemical mixtures. Though this thesis focused on non-carcinogenic effects, these studies are mentioned here because of their relevance in relation to the question whether multi-chemical exposure at low, non-toxic levels of the individual chemicals is of health concern.

In Japan, Ito and co-workers have extensively studied the effects of pesticide mixtures using their medium-term rat bioassays for the detection of genotoxic and non-genotoxic carcinogens (Ito *et al*, 1995a,b). Dietary administration of a mixture of 20 pesticides (mainly organophosphate pesticides) enhanced diethylnitrosamine-initiated liver preneoplastic lesion development when each pesticide was present at 100 times its own acceptable daily intake (ADI) level, but had no effect when the pesticides were present at their ADI. In a multi-organ assay, dietary administration of mixtures of 20 (suspected carcinogens) or 40 (high production volume) pesticides at their ADI did not enhance carcinogenesis in any organ initiated by a combination of 5 potent carcinogens. The studies did not include single compound groups. The authors concluded that these results support the safety factor (usually 100) approach presently used for risk evaluation of pesticides.

Ito and co-workers further used their medium-term bioassays to examine the effects of low doses of mixtures of (carcinogenic) heterocyclic amines which might be simultaneously generated in foods during cooking (Ito *et al.*, 1991; Hasegawa *et al.*, 1994a,b). The amines were given in the diet, either alone or in combinations of five or ten, at fractions of the doses found to be carcinogenic in lifespan rat studies. Two different five-component mixtures containing the amines at levels which were non-effective upon single administration (viz. 1/25 of the carcinogenic dose) induced effects which were similar to those induced by the individual amines at five times higher dose levels. This indicated that the mixture responses could be explained by dose additivity when the individual amines were present at no-effect levels. The responses to the five-component mixtures at the effective levels (1/5 fractions), however, were inconsistent (viz. similar to or larger than the sums of the individual responses). The ten-component mixture was non-effective when each amine was present at 1/100 of the carcinogenic dose but induced a larger than expected response when each amine was present at 1/10 of the carcinogenic dose (the 1/10 fraction was a no-effect level for eight of the ten amines when given alone).

An unexpected tumor response (viz. induction of follicular cell tumors in the thyroid) was also observed in a lifespan carcinogenicity study in rats fed a diet

containing 40 environmental carcinogens, with many different target organs, at low doses (viz. 1/50 of the TD50 doses which induced tumors in 50% of the animals in previous studies with the individual carcinogens) (Takayama *et al.*, 1989). In a follow-up study with three known thyroid carcinogens, given simultaneously or alone at 1/3 of their TD50, the mixture response was much higher than the sum of the individual responses (viz. 100% incidence instead of 15%) (Hasegawa *et al*, 1994c).

6.4 Conclusions

The studies presented in this thesis demonstrated that simultaneous exposure to eight chemicals with different primary target organs or to four chemicals with a similar target organ (kidney) but dissimilar modes of action, at dose levels equal to or slightly below the No-Observed-Adverse-Effect Level of the individual chemicals, was not associated with obvious adverse effects. These results support the hypothesis that as a rule exposure to mixtures of chemicals at (low) non-toxic doses of the individual chemicals is of no health concern. A review of other studies on the effects of defined chemical mixtures at non-toxic levels of the individual chemicals yielded some additional evidence in support of this hypothesis. However, the review also showed a few unexpected exceptions to the rule, even at levels far below the individual toxicity thresholds.

The study with four chemicals with the same target organ (kidney) and same mode of action provided support for the assumption of dose additivity for mixtures of similarly acting systemic toxicants under conditions of concurrent, repeated exposure at dose levels below the toxicity thresholds of the individual chemicals.

Additionally, our studies showed that mixture toxicity results obtained at dose levels above the individual chemicals' toxicity thresholds were not predictive of the mixture's effect at levels below the individual thresholds, regardless of the type of mixture.

The data from our work and a few other studies do not indicate that (uncertainty about the toxicological consequences of) combined action should be incorporated routinely (by means of an extra safety or uncertainty factor) in the health-based standards for individual non-carcinogenic substances. It should be noted, however, that these data were not obtained under people's actual (long-term, low-level, multi-chemical) exposure conditions, and that the few mixtures examined are not representative of the many different mixture exposure scenarios of people. Further, the current knowledge and understanding of mixture toxicity also suggests that it is prudent to consider the possibility of increased toxicity due to simple similar (dose additive) joint action or synergistic interactions when setting standards, taking into account all available information on (similarities in) mechanisms of toxic action, toxicokinetic processes and human exposure levels and scenarios.

6.5 References

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Summary

Summary

The work described in this thesis concerns the toxicity of chemical mixtures. Historically, possible adverse health effects of chemicals have been examined almost exclusively by studying substances one at a time. The results of studies on a single chemical are then used to establish an exposure standard for that chemical, for example a Threshold Limit Value (TLV) for exposure in occupational settings, or an Acceptable Daily Intake (ADI) for food additives. When establishing exposure standards for single chemicals, no allowance is generally made for the possibility that a chemical's toxicity is increased by another chemical (synergistic interaction), or that similarly acting chemicals are additive in their effects. In both situations, the effect from exposure to a mixture will be stronger than expected on the basis of the exposure levels of the individual chemicals, which may result in unforeseen adverse health effects upon exposure to levels which are regarded as save on the basis of the exposure standards for the individual chemicals. This phenomenon is well known, and reasonably well studied, for combined exposures to drugs or to drugs and alcohol. For other chemicals to which humans are exposed voluntarily or involuntarily, there are much less examples of unexpected toxicity upon combined exposure and systematic research on mixture toxicity is lacking. Moreover, most data on mixture toxicity were obtained in short-term studies in which mixtures of only two or three chemicals were examined at very toxic or even lethal exposure levels of the individual chemicals. In contrast, people are usually exposed, lifelong, to multiple chemicals through, among other things, their food and drinking water, the air they breath, or the personal care products they use. The actual human exposure levels are generally far below the toxic levels of the individual chemicals. It is, therefore, important to gain insight into the joint action of chemicals under conditions of prolonged exposure to levels below the toxicity threshold of the individual chemicals. Such insight is not only of importance for establishing exposure standards, but also for assessing health risks from actual mixture exposure situations and possible measures required to reduce the risk.

In 1985, the Health Council of The Netherlands, in her advisory report on the setting of health-based standards for non-carcinogenic substances, addressed the question as to whether uncertainty about the toxicological consequences of combined action should be incorporated in the standards for individual substances. The Health Council concluded that there was no immediate reason to take combination effects into account at concentrations well below the standards for the individual substances. Furthermore, the Council was not aware of useful data on the effects of prolonged exposure to combinations of substances at concentrations around the experimental No-

Observed-Adverse-Effect Levels of the individual substances and, therefore, recommended research to fill this data gap. A few years later, a research programme on mixture toxicity was started at TNO Nutrition and Food Research, funded by the Ministry of Housing, Spatial Planning and Environment. One of the major aims was to test the hypothesis that as a rule exposure to mixtures of chemicals at (low) non-toxic doses of the individual chemicals is of no health concern. For this purpose, we conducted a series of toxicity studies in rats with defined mixtures of chemicals with similar or dissimilar target organs and/or modes of action. Four of these studies are described in detail in chapters 2 through 5 of this thesis, two other studies are summarized in chapter 6.

In chapter 1 an overview is given of terminology and basic concepts in relation to mixture toxicity, followed by a short explanation on mechanisms of interaction and experimental approaches to assess the toxicity of mixtures. This chapter also contains a summary of a few older studies on mixture toxicity which addressed, according to widely differing experimental approaches, the toxicity of contaminants in (drinking) water. Chapter 1 ends with the scope and objectives of the work described in this thesis.

The objective of the first study (chapter 2) was to examine whether a mixture of eight chemicals with different primary target organs would be toxic when the individual chemicals were present at their No-Observed-Adverse-Effect Level (which was 5 to 3 times lower than the minimally toxic level of the individual chemicals in most cases). The chemicals were administered to rats via their feed or drinking water during four weeks, at four different levels around the No-Observed-Adverse-Effect Level of the individual chemicals. Possible adverse effects were measured by means of about 70 common toxicity parameters (as is routinely done in toxicity studies with individual chemicals). The mixture caused no obvious signs of toxicity at non-toxic exposure levels of the individual chemicals. When the exposure levels of the individual chemicals were minimally toxic, the mixture was also toxic. Moreover, at the toxic level several effects of the mixture were more severe than those of individual chemicals whereas other mixture effects were less severe.

As a sequel to this study with chemicals affecting different organs, a 4-week repeated-dose study (chapter 3) and an acute study (chapter 4) were conducted with mixtures of four chemicals which affected the same organ, viz. the kidney, by different modes of action. Again the objective was to examine whether exposure to a mixture at non-toxic levels of the individual chemicals would result in toxic effects. As in the first study, the mixture caused no renal damage at non-toxic exposure levels of the individual chemicals. The results of these studies also showed that simultaneous

exposure to minimally toxic levels of the individual chemicals resulted in adverse effects which ranged from less severe to clearly more severe compared with the effects of the individual chemicals at these levels.

The last study (chapter 5) was conducted with mixtures of three or four chemicals which affect the same organ, again the kidney, through a similar mode of action. For a mixture of similarly acting chemicals which do not influence each other's toxicity, it is commonly assumed that, at low exposure levels, the toxicity of the mixture can be calculated from the toxicity data of the individual chemicals using a mathematical formula for dose addition. According to this formula, each component contributes to the total toxicity of the mixture in proportion to its dose. Though this additivity assumption has been shown to be valid in acute, high-dose studies in rats, its validity under conditions more relevant to human exposure situations has been examined poorly. The major goal of the last study was, therefore, to test the additivity assumption (dose addition) under conditions of concurrent, repeated exposure to non-toxic levels of the individual chemicals. To this end, rats were treated with mixtures of three or four chemicals during 32 days, at non-toxic levels which were three or four times lower than the minimally toxic levels of the individual chemicals. As shown by the increase in the weight of the kidneys, individually non-toxic doses of different chemicals elicited toxicity when given simultaneously. The magnitude of the mixture effect at the non-toxic levels of the individual chemicals corresponded to that predicted on the basis of dose additivity. At a slightly higher, minimally toxic level, however, the additivity assumption was not valid: the effect of the mixture was less than expected under dose addition.

Chapter 6 starts with a discussion of the above studies and addresses the question whether combined exposure constitutes an increased health risk compared to exposure to individual chemicals. In the first three studies, the absence of obvious toxicity upon combined exposure to non-toxic levels of the individual chemicals indicated that at such exposure levels there were no synergistic interactions with significant risk enhancing implications. Also, it was clear that the assumption of dose addition for mixtures of chemicals with different target organs would overestimate the mixture toxicity. In the last study it was shown that combined exposure to chemicals with a similar target organ and mode of action constituted an increased health risk, which was explained by dose addition.

Next, an overview is given of regulatory approaches to the risk assessment of chemical mixtures and of the limited experimental support for underlying (additivity) assumptions. Chapter 6 also summarises results of (complementary) low-dose studies conducted by other investigators. Finally, the following conclusions were drawn:

Simultaneous exposure to chemicals with different primary target organs or to chemicals with a similar target organ (kidney) but dissimilar modes of action was not associated with obvious adverse effects, provided that the dose levels were equal to or slightly below the No-Observed-Adverse-Effect Level of the individual chemicals. These results support the hypothesis that as a rule exposure to mixtures of chemicals at (low) non-toxic doses of the individual chemicals is of no health concern. Literature data on the effects of defined chemical mixtures at non-toxic levels of the individual chemicals yielded some additional evidence in support of this hypothesis, but there were also a few unexpected exceptions to the rule, even at levels far below the toxicity thresholds of the individual chemicals.

The study with chemicals having the same target organ (kidney) and same mode of action provided support for the assumption of dose additivity for mixtures of similarly acting systemic toxicants under conditions of concurrent, repeated exposure at dose levels below the toxicity thresholds of the individual chemicals.

Additionally, our studies showed that mixture toxicity results obtained at dose levels above the individual chemicals' toxicity thresholds were not predictive of the mixture's effect at levels below the individual thresholds, regardless of the type of mixture.

The data from our work and a few other studies do not indicate that (uncertainty about the toxicological consequences of) combined action should be incorporated routinely (by means of an extra safety or uncertainty factor) in the health-based standards for individual non-carcinogenic substances. It should be noted, however, that these data were not obtained under people's actual (long-term, low-level, multi-chemical) exposure conditions, and that the few mixtures examined are not representative of the many different mixture exposure scenarios of people. Further, the current knowledge and understanding of mixture toxicity also suggests that it is prudent to consider the possibility of increased toxicity due to simple similar (dose additive) joint action or synergistic interactions when setting standards, taking into account all available information on (similarities in) mechanisms of toxic action, toxicokinetic processes and human exposure levels and scenarios.

Samenvatting

Samenvatting

Het onderzoek beschreven in dit proefschrift gaat over de schadelijkheid (toxiciteit) van mengsels van stoffen. Van oudsher worden eventuele ongewenste gezondheidseffecten van stoffen vrijwel uitsluitend onderzocht door elke stof apart te onderzoeken. Vervolgens worden de resultaten van dergelijk onderzoek gebruikt bij het vaststellen van normen of grenswaarden voor afzonderlijke stoffen, zoals de maximaal aanvaarde concentraties (MAC-waarden) voor blootstelling aan stoffen op de arbeidsplek of de aanvaardbare dagelijkse inname (ADI) voor toevoegingen aan voedingsmiddelen. In de meeste gevallen wordt bij de normstelling voor afzonderlijke stoffen geen rekening gehouden met de mogelijkheid dat verschillende stoffen elkaars schadelijke werking versterken (synergistische beïnvloeding of interactie), of dat bij gelijktijdige blootstelling aan meerdere stoffen met een overeenkomstige werking de effecten van de afzonderlijke stoffen als het ware opgeteld worden. In beide gevallen zal gecombineerde blootstelling een sterker effect hebben dan verwacht op grond van de blootstellingsniveaus van de afzonderlijke stoffen. Hierdoor kan gezondheidsschade ontstaan na blootstelling aan niveaus die op grond van de normen voor de afzonderlijke stoffen als veilig worden beschouwd. Dit fenomeen is reeds lang bekend, en redelijk goed onderzocht, voor combinaties van geneesmiddelen of combinaties van geneesmiddelen met alcohol. Voor andere stoffen waarmee mensen gewild of ongewild in aanraking komen zijn de voorbeelden echter veel minder talrijk en ontbreekt systematisch onderzoek. Bovendien zijn de meeste gegevens over mengseltoxiciteit afkomstig van kortdurende studies waarin combinaties van slechts twee of drie stoffen zijn onderzocht op blootstellingsniveaus waarop de afzonderlijke stoffen schadelijk of zelfs dodelijk waren. Daarentegen komen mensen dagelijks, levenslang, in aanraking met een veelheid van stoffen, onder andere via voedsel, (drink)water, ingeademde lucht of persoonlijke verzorgingsprodukten, en liggen de werkelijke blootstellingsniveaus meestal ver beneden de schadelijke niveaus van de afzonderlijke stoffen. Het is dus belangrijk om inzicht te hebben in de gezamenlijke werking van stoffen onder omstandigheden van langdurige blootstelling aan niveaus onder de toxiciteitsdrempel van de afzonderlijke stoffen. Zulk inzicht is niet alleen van belang bij de normstelling, maar ook bij het beoordelen van gezondheidsrisico's van in de praktijk voorkomende mengselblootstellingssituaties en eventueel benodigde risicoreducerende maatregelen.

In 1985 heeft de Gezondheidsraad in haar advies over normstelling voor niet-kankerverwekkende stoffen aandacht besteed aan de vraag of onzekerheid over de schadelijke werking van stoffen in combinatie verwerkt moet worden in advieswaarden en normen. De Gezondheidsraad concludeerde dat er geen directe aanleiding was om

rekening te houden met combinatiewerkingen bij blootstellingsniveaus ver beneden de normen voor afzonderlijke stoffen. Omdat destijds geen bruikbare informatie bekend was over de effecten van langdurige blootstelling aan combinaties van stoffen bij blootstellingsniveaus rond het geen-nadelig-effect niveau van afzonderlijke stoffen adviseerde de Gezondheidsraad om hiernaar onderzoek te doen. Vervolgens startte enkele jaren later bij TNO Voeding, met financiële steun van het Ministerie van Volksgezondheid, Ruimtelijke Ordening en Milieu, een onderzoeksprogramma over mengseltoxiciteit. Een belangrijk doel van dit programma was om de hypothese te testen dat blootstelling aan mengsels of combinaties van stoffen in de regel geen gevaar vormt voor de gezondheid zolang de blootstellingsniveaus van de afzonderlijke stoffen onschadelijk (laag) zijn. Hiertoe werd een serie studies uitgevoerd waarin ratten werden blootgesteld aan diverse combinaties van stoffen. Vier van deze studies zijn in detail beschreven in hoofdstuk 2 tot en met 5 van dit proefschrift, twee andere zijn kort samengevat in hoofdstuk 6.

In hoofdstuk 1 wordt een overzicht gegeven van gangbare terminologie en basisconcepten met betrekking tot mengseltoxiciteit, gevolgd door een korte uitleg over manieren waarop stoffen elkaars werking kunnen beïnvloeden en over methoden om de toxiciteit van mengsels te onderzoeken. Ook worden enkele oudere mengselstudies beschreven waarin, op sterk uiteenlopende manieren, de toxiciteit van verontreinigingen in (drink)water is onderzocht. Hoofdstuk 1 eindigt met de achtergrond en doelstellingen van het onderzoek beschreven in dit proefschrift.

De eerste studie (hoofdstuk 2) had tot doel om na te gaan of een mengsel van acht stoffen met verschillende primaire doelorganen schadelijk zou zijn wanneer de afzonderlijke stoffen aanwezig waren op het eigen geen-nadelig-effect niveau (dat was in de meeste gevallen 1/5 tot 1/3 van het minimaal-schadelijke niveau). De stoffen werden toegediend aan ratten via het voer of drinkwater, gedurende vier weken, op vier verschillende niveaus rond het geen-nadelig-effect niveau van de afzonderlijke stoffen. Eventuele schadelijke effecten werden gemeten aan de hand van een 70-tal gangbare toxiciteitsparameters (zoals gebruikelijk is bij toxiciteitsstudies met afzonderlijke stoffen). Het mengsel bleek geen noemenswaardige schade te veroorzaken bij onschadelijke blootstellingsniveaus van de afzonderlijke stoffen. Bij blootstelling aan minimaal-schadelijke niveaus van de afzonderlijke stoffen was het mengsel ook schadelijk. Bovendien bleek dat op het schadelijke blootstellingsniveau diverse effecten van het mengsel ernstiger waren dan die van afzonderlijke stoffen terwijl andere mengseleffecten juist minder ernstig waren.

De twee volgende studies hadden eveneens tot doel om te onderzoeken of blootstelling aan een mengsel op onschadelijke niveaus van de afzonderlijke stoffen

zou resulteren in schadelijke effecten. De mengsels in deze studies bestonden uit vier stoffen met een overeenkomstig doelorgaan, namelijk de nier. De werkingsmechanismen volgens welke deze stoffen nierschade veroorzaken waren echter verschillend. In de ene studie kregen ratten het mengsel gedurende vier weken via het voer (hoofdstuk 3), in de andere studie werd het mengsel eenmalig toegediend (hoofdstuk 4). Net als in de eerste studie bleek dat gelijktijdige blootstelling aan meerdere stoffen geen nierschade veroorzaakte zolang de niveaus van de afzonderlijke stoffen onschadelijk waren. Ook bleek opnieuw dat gecombineerde blootstelling aan minimaal-schadelijke niveaus van de afzonderlijke stoffen resulteerde in effecten die varieerden van minder ernstig tot duidelijk ernstiger vergeleken met de effecten van de afzonderlijke stoffen op die niveaus.

Terwijl bovenstaande twee mengsels bestonden uit stoffen die nierschade veroorzaken via verschillende werkingsmechanismen werd in de laatste studie (hoofdstuk 5) de toxiciteit onderzocht van mengsels van drie of vier stoffen die nierschade veroorzaken via een soortgelijk mechanisme (stoffen met overeenkomstige werking). Voor een mengsel van stoffen met een overeenkomstige werking die elkaars werking niet beïnvloeden (geen interactie) wordt doorgaans aangenomen dat, bij lage blootstellingsniveaus, de toxiciteit van het mengsel kan worden berekend met behulp van toxiciteitsgegevens van de afzonderlijke stoffen middels de rekenregel voor dosisadditie. Volgens deze rekenregel draagt elke mengselcomponent bij aan het totale effect van het mengsel naar rato van zijn dosis. Er zijn echter nauwelijks experimentele bewijzen voor de geldigheid van deze aanname onder omstandigheden van gelijktijdige, herhaalde blootstelling aan onschadelijke niveaus van de afzonderlijke stoffen. Het belangrijkste doel van de laatste studie was derhalve om de geldigheid van deze additiviteitsaanname onder zulke omstandigheden te toetsen. Hiertoe werden ratten gedurende ruim vier weken blootgesteld aan mengsels van drie of vier stoffen, op onschadelijke niveaus die een factor drie of vier lager waren dan de minimaal schadelijke niveaus van de afzonderlijke stoffen. Deze studie liet zien dat veilige doses van de afzonderlijke stoffen tezamen schadelijk waren, zoals bleek uit de toename van het niergewicht. Ook bleek dat de grootte van het schadelijke effect van het mengsel inderdaad overeen kwam met hetgeen verwacht was op basis van dosisadditie. Bij blootstelling aan iets hogere, minimaal-schadelijke niveaus van de afzonderlijke stoffen bleek de additiviteitsaanname onjuist: het effect van het mengsel was geringer dan verwacht op grond van deze aanname.

In hoofdstuk 6 worden de resultaten van bovengenoemde studies besproken, waarbij aandacht is besteed aan de vraag of gecombineerde blootstelling gezondheidsrisicoverhogend was. In de eerste drie studies vormde de afwezigheid van

schade na gecombineerde blootstelling op onschadelijke niveaus van de afzonderlijke stoffen een duidelijke aanwijzing dat er op dergelijke niveaus geen sprake was van synergistische interacties met sterk risicoverhogende gevolgen. Ook was duidelijk dat toepassing van de rekenregel voor dosisadditie voor stoffen met verschillende doelorganen de mengseltoxiciteit zou overschatten. Uit de laatste studie bleek dat er bij gecombineerde blootstelling aan stoffen met een zelfde doelorgaan en werkingsmechanisme wel sprake was van een verhoging van het risico en dat die verhoging verklaard kon worden met dosisadditie.

Voorts wordt in hoofdstuk 6 een kort overzicht gegeven van manieren waarop regelgevende instanties gezondheidsrisico's van blootstelling aan mengsels evalueren en van de beperkte experimentele bewijzen voor de daarbij gehanteerde additiviteitsaannames. Tevens worden resultaten gepresenteerd van een aantal mengselstudies van andere onderzoeksgroepen die zijn uitgevoerd in ongeveer dezelfde periode als de studies beschreven in dit proefschrift. Mede op grond van die resultaten werd het onderstaande geconcludeerd:

Gelijktijdige blootstelling aan stoffen met verschillende primaire doelorganen of aan stoffen met een zelfde doelorgaan (de nier) maar verschillend werkingsmechanisme veroorzaakte geen schadelijke effecten mits de blootstellingsniveaus gelijk aan of iets onder het geen-nadelig-effect niveau van de afzonderlijke stoffen waren. Deze uitkomsten ondersteunen de hypothese dat blootstelling aan mengsels van stoffen in de regel geen gevaar vormt voor de gezondheid zolang de niveaus van de afzonderlijke stoffen veilig (laag) zijn. De uitkomsten van diverse in de literatuur beschreven mengselstudies bevestigen deze hypothese eveneens, maar er waren ook uitzonderingen op de regel, zelfs bij blootstellingsniveaus ver beneden de toxiciteitsdrempel van afzonderlijke stoffen.

De studie met stoffen met hetzelfde doelorgaan (de nier) en soortgelijk werkingsmechanisme leverde bewijs voor de geldigheid van dosisadditie voor mengsels van overeenkomstig werkende stoffen onder omstandigheden van gelijktijdige, herhaalde blootstelling op niveaus onder de toxiciteitsdrempel van de afzonderlijke stoffen.

Onze studies lieten voorts zien dat, ongeacht het type mengsel, de gegevens over mengseltoxiciteit verkregen op blootstellingsniveaus boven de toxiciteitsdrempel van de afzonderlijke stoffen niet voorspellend waren voor de mengseltoxiciteit bij blootstelling aan niveaus onder de individuele toxiciteitsdrempels.

De uitkomsten van de studies uitgevoerd bij TNO Voeding en die van enkele andere studies leverden geen aanwijzingen dat onzekerheid over de schadelijke werking van stoffen in combinatie routinematig, middels een extra veiligheids- of

onzekerheidsfactor, verwerkt moet worden in advieswaarden voor afzonderlijke (niet-kankerverwekkende) stoffen. Daarbij moet echter worden aangetekend dat deze studies niet zijn gedaan onder voor de mens gebruikelijke omstandigheden (c.q. langdurige blootstelling aan lage niveaus) en dat het geringe aantal onderzochte mengsels niet representatief is voor de vele mengsels waarmee mensen in aanraking komen. Verder suggereert de huidige kennis over mengseltoxiciteit dat het verstandig is om bij normstelling aandacht te schenken aan de mogelijkheid van verhoogde toxiciteit als gevolg van overeenkomstige werking (dosisadditie) of synergistische interacties, waarbij gelet moet worden op alle beschikbare informatie over (overeenkomsten in) werkingsmechanismen, toxicokinetische processen en voor de mens mogelijke blootstellingsniveaus en -scenarios.

Curriculum vitae

and

List of publications

Curriculum vitae

Diana Jonker werd geboren op 17 juni 1961 te Ootmarsum. Na het voltooien van de middelbare schoolopleiding (VWO) aan de Gemeentelijke Scholengemeenschap 'Doetinchem' begon zij in september 1979 haar studie aan de Landbouwhogeschool Wageningen, studierichting Voeding. Het doctoraaldiploma werd behaald in juni 1986 (met lof) en omvatte de hoofdvakken voedingsleer en toxicologie, de bijvakken gezondheidsleer en pedagogiek en algemene didactiek, en een stage van 7 maanden (onderwerp levertoxiciteit) bij de afdeling biochemie van 'The British Industrial Biological Research Association' te Carshalton. Sinds september 1986 is zij werkzaam als toxicoloog (onderzoeksleider) bij de afdeling Algemene Toxicologie van TNO Voeding te Zeist. In december 1994 is zij geregistreerd als Medisch Biologisch Wetenschappelijk Onderzoeker, richting Toxicologie (SMBWO erkenning) en sinds maart 1998 is zij ingeschreven in het register van erkende toxicologen van de Nederlandse Vereniging voor Toxicologie. Het onderzoek beschreven in dit proefschrift werd uitgevoerd bij TNO Voeding.

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[1] P-woord = proefschrift; promotie.
Vragen als 'Hoe gaat het met je proefschrift?' of 'Wanneer is jouw promotie?' werden mij de laatste jaren steeds vaker met enige aarzeling gesteld.

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Hypericum perforatum (St John's wort)

St John's wort has been phenomenally successful as a herbal antidepressant (Ernst, 1999).

The U.S. Food and Drug Administration has asked health care professionals to caution patients about the risk of potentially significant interaction between St John's wort and various prescribed drugs (Henney, 2000).

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