Combined Actions and Interactions of Chemicals in Mixtures

The Toxicological Effects of Exposure to Mixtures of Industrial and Environmental Chemicals

Miljøministeriet
Miljøstyrelsen

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Fødevaredirektoratet

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The Danish Veterinary and Food Administration is part of the Danish Ministry of Agriculture, Food and Fisheries. The Danish Veterinary and Food Administration is responsible for the administration, research and control within food and veterinary areas "from farm to fork", as well as practical matters relating to animal protection (otherwise under the Ministry of Justice).

Making of regulations, co-ordination, research and development, take place in the Administrations center in Moerkhoej. The 11 Regional Authorities handle the practical inspection of food and veterinary matters, including import/export etc.

The central administration of The Danish Veterinary and Food Administration employ a staff of approx. 550 full-time employees, whilst the 11 regional authorities employ a further approx.1.600 full-time employees.

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Preface

This co-operation was established between the Danish Environmental Protection Agency, the Ministry of the Environment and the Institute of Food Safety and Nutrition at the Danish Veterinary and Food Administration, the Ministry of Food, Agriculture and Fisheries to summarise and evaluate the currently available scientific literature in the field of risk assessment of toxicological effects of exposures to chemical mixtures.

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Sammenfatning og konklusioner

Indledning
Efter gældende praksis baseres risikovurderinger af kemiske stoffer og efterfølgende reguleringsmæssige tiltag, som f.eks. klassifikation og mærkning, fastsættelse af grænseværdier såsom MRL værdier osv., generelt på data fra undersøgelser af de enkelte stoffer. Mennesker udsættes imidlertid for en lang række kemiske stoffer samtidigt, og disse stoffer kan potentielt have både sammenlignelige og forskellige effekter. Som følge heraf er myndighederne nødt til at tage stilling til sådanne kemiske "cocktails" for at sikre, at de ikke har uforudsete helbredseffekter.

Kombinationseffekter herunder interaktioner mellem kemiske stoffer, f.eks. lægemidler, som gives til mennesker i store doser, har været kendt i mange år inden for farmakologien. Erfaringerne herfra er dog ikke direkte anvendelige til forudsigelse af toksiske effekter af blandinger af kemikalier i miljøet og fødevarer, hvor eksponeringsniveauerne for den generelle befolkning er relativt lave, og interaktioner, som forekommer ved høje doser, er ikke nødvendigvis repræsentative for eksponering i lav-dosis niveauet. Den umiddelbare forventning er, at eksponering til forholdsvis lave doser vil resultere i mindre grad af interaktioner, når disse primært skyldes overskridelse af forskellige tærskelværdier og mætningsfænomener, for eksempel med hensyn til metabolismen (enzymatisk aktivering/deaktivering) af stofferne i organismen.

Kombinationseffekter inden for forskellige effektområder
Rapporten opsummerer de basale principper for kombinationseffekter herunder interaktioner mellem kemiske stoffer i blanding og diskuterer forskellige metoder, som er blevet foreslået anvendt ved testning og risikovurdering af sådanne blandinger. Adskillige målrettede toksikologiske undersøgelser af simple, veldefinerede blandinger diskuteres og der er foretaget en gennemgang af den nuværende viden om kombinationseffekter og interaktioner af kemiske stoffer inden for forskellige effektområder.

Der foreligger mange studier af *hud-, øjen- og luftvejsirritation* frembragt af komplekse blandinger, men kun få af disse studier muliggør en kvantitativ vurdering af de enkelte kemiske stoffers modulerende effekt i blandingen. Flere undersøgelser af modulerende effekter på hud-, øjen- og luftvejsirritation af kombinationer af kemikalier er nødvendige for at kunne vurdere mulighederne for synergistiske eller antagonistiske effekter. Forskellige *in vitro* systemer har vist sig lovende til at vurdere effekter af kemiske irritanter og disse metoder kan minimere omkostninger og varighed af undersøgelser af kombinationseffekter.

Genotoksicitet in vitro er sandsynligvis den mest hyppigt studerede effekt af komplekse blandinger, og der foreligger mange undersøgelser af komplekse miljøblandingers genotoksiske potentiale. Derimod foreligger der kun forholdsvis få undersøgelser af den genotoksiske aktivitet af simple blandinger med velkendt kemisk sammensætning. De fleste af de målrettede studier har involveret interaktioner mellem et mutagent stof og et ikke-mutagent stof, som enten har forstærket (co-mutagen effekt) eller hæmmet (anti-mutagen effekt) det mutagene stofs aktivitet. *In vitro* studier af blandinger af kendte genotoksiske stoffer har overvejende vist additive effekter og i visse tilfælde antagonistiske effekter. På grund af de mulige forskelle mellem de biologiske processer *in vitro* og *in vivo,* bør

kombinationseffekter, som er påvist *in vitro* eftervises *in vivo*. COMET-assay *in vivo* og genotoksicitetstest i transgene dyr kan vise sig lovende i den fremtidige vurdering af komplekse blandinger.

Karcinogenesen, udvikling af kræft, beskrives som regel som en fler-trins proces. Det forhold, at forskellige kemiske, fysiske og biologiske faktorer kan påvirke hvert enkelt trin i processen, gør det indlysende, at udvikling af kræft i hovedparten af tilfælde er resultatet af kombinationseffekter. Det har været kendt i mere end et halvt århundrede, at interaktion af kræftfremkaldende stoffer på forskellige trin i karcinogenesen kan føre til en synergistisk effekt, der bevirker stærkt øget tumor respons. Initierende stoffer, promotorer, konvertorer og co-karcinogener virker sammen og potentierer den endelige forekomst af tumorer. Antikarcinogener derimod kan forhindre eller hæmme hvert trin i processen. Antikarcinogener vides også undertiden at være i stand til at potentiere hinanden. Stoffer, som påvirker det samme trin i processen, men med forskellige virkningsmekanismer, kan også potentiere hinanden. Mulighederne for kombinationseffekter og interaktioner i karcinogeneseprocessen er derfor talrige.

Hvad angår effekter på *reproduktionsevnen*, så har studier med samtidig eksponering for to eller flere kemikalier vist, at interaktioner nogen gange forekommer andre gange ikke, og at det er umuligt at forudsige resultatet alene ud fra de eksperimentelle betingelser. De foreliggende data viser dog, at det hyppigst forekommende resultat som følge af *in vivo* eksponering for blandinger enten er en antagonistisk effekt eller ingen interaktiv effekt, som i nogle tilfælde er en additiv effekt. Ofte observeres én type interaktion ved et dosisniveau mens en anden type interaktion ses på et andet dosisniveau. En samlet vurdering af resultaterne antyder at lave doser af kemikalierne i blandingerne ofte foranlediger enten ingen effekt eller en additiv effekt, mens højere doser giver antagonistiske eller synergistiske effekter.

Undersøgelser med *hormonforstyrrende stoffer* har overvejende været *in vitro* eksperimenter, og kun få *in vivo* studier med blandinger af kemiske stoffer er blevet foretaget indtil nu. Hovedparten af, de især ældre studier, har konkluderet at der var tale om additive effekter, dvs. at der ikke var interaktioner, skønt detaljerede mekanistiske analyser i de fleste tilfælde ikke var foretaget. Et nyt, veltilrettelagt *in vitro* studie har klart vist, at den samlede effekt af østrogene stoffer ikke afveg fra den forventede additive effekt. Endvidere er der fundet additiv effekt af to anti-androgene stoffer indgivet *in vivo*. Med den nuværende viden er der derfor ingen evidens, der nødvendiggør, at synergisme inddrages i vurderingen af blandinger af svagt østrogene stoffer.

Med hensyn til *neurotoksicitet* er der mange muligheder for interaktioner mellem kemiske stoffer på grund af nervesystemets komplekse, hierarkiske struktur. Der er beskrevet en række eksempler herpå. De bedst kendte er den additive, narkotiske effekt af organiske opløsningsmidler og den additive effekt på acetylkolinesterase hæmning af organofosfor insekticider. Den kraftigste interaktion, der blev fundet i litteraturen, var en 5 gange forøgelse at hexans neurotoksiske effekt når methyl isobutyl keton blev indgivet samtidig. Der er imidlertid kun foretaget få kvantitative undersøgelser og interaktioner er ikke studeret systematisk. Derfor tillader den nuværende viden ikke generelle konklusioner.

De mange forskellige celletyper, som er involveret i immunsystemets funktion, giver mange teoretiske muligheder for kombinerede *immunotoksiske* effekter. Der er imidlertid kun få eksperimentelle data på området. Det ser ud til, at stoffer, som har samme virkningsmekanisme, har en additiv effekt, mens konkurrence om metaboliserende enzymer kan føre til en antagonistisk immunotoksisk effekt. Der

er ingen viden om, hvorledes den kombinerede immunotoksiske effekt vil være for stoffer, som er toksiske overfor forskellige dele af immunsystemet.

Undersøgelser har vist, at dersom en person, der lider af *allergi* over for to uafhængige allergener, provokeres med disse to allergener i blanding, så vil det efterfølgende udløste respons være enten lig med eller større end summen af de to allergeners forventede respons alene. Provokation med to allergener i blanding, med hvert allergen i doser lavere end allergenets tærskelværdi, kan udløse et respons, som ingen af stoffer alene ville udløse. Konsekvensen er, at en person kan vise sig negativ i en diagnostisk prik-test med enkeltstoffer, skønt en blanding af stofferne ville udløse en reaktion. I praksis kan det betyde, at et allergen kan tolereres i en situation, men ikke i en anden, hvor det optræder i en blanding med et andet allergen.

Kemiske stoffer, som faciliterer et kontaktallergens indtrængning i huden, eller som har effekt på hudens immunsystem, kan øge risikoen for udvikling af overfølsomhed.

Risikovurdering af kemiske stoffer i blandinger
Forudsigelse af de toksikologiske egenskaber af en blanding af kemiske stoffer kræver ideelt detaljeret information om blandingens sammensætning og om virkningsmekanismerne for hvert enkelt stof i blandingen. Og for at en ordentlig risikovurdering kan gennemføres kræves tillige viden om menneskers eksponering for blandingen. Som regel er sådanne detaljerede oplysninger ikke til stede. Komplekse kemiske blandinger kan indeholde hundredvis, måske endda tusindvis af stoffer, og hverken den kvalitative eller kvantitative sammensætning kendes fuldt ud, og kan ændre sig over tid. Adækvat testning af sådanne blandinger er ofte ikke mulig, fordi de enten ikke kan fremskaffes eller kun kan fremskaffes i så begrænsede mængder, at der ikke kan testes et tilstrækkeligt antal dosis niveauer. Hertil kommer, at høje dosis niveauer af en blanding af kemiske stoffer kan have andre typer effekter end lave doser, hvorved ekstrapolation fra høje til lave doser bliver vanskelig. Inden for luftforureningsområdet har det dog været muligt at teste blandinger, enten i form af udstødningsgasser eller andre emissioner eller ved hjælp af opkoncentrering af luftforureningskomponenterne.

Et af de vigtigste punkter, som indledningsvis skal afklares, er hvorvidt der enten *ikke vil forekomme interaktioner* eller der kan forekomme *interaktioner* i form af synergistiske eller antagonistiske effekter. Disse tre basale principper (ingen interaktion, synergi, antagonisme) for kombinationseffekter af kemiske stoffer er helt teoretiske og ofte er man nødt til at tage hensyn til to eller alle tre koncepter samtidig når blandinger består af mere end to stoffer og toksicitets målene er komplekse.

En Hollandsk forskergruppe startede et forskningsprogram for at afprøve en hypotese om, at eksponering for blandinger af kemikalier i (lave) ikke-toksiske doser af de individuelle stoffer, som en regel ikke ville være sundhedsmæssigt betænkeligt. En af grundene var, at de fleste retningslinier fra nationale og internationale myndigheder ofte foreslår at anvende modeller baseret på simpel ”dosis addition” eller ”respons addition” til vurdering af stofblandinger og herved totalt ignorerer ethvert kendskab til stoffernes virkningsmekanismer.

Ud fra resultaterne af eksperimentelle kort-tids studier konkluderede den Hollandske gruppe, at eksponering for blandinger af arbitrært udvalgte kemiske stoffer klart viste fravær af fuld additiv effekt, og gav en vis evidens for delvis additivitet når alle stofferne i blandingen blev indgivet i doser svarende til deres egne nul-effekt-niveauer (NOAELs). Ved en smule lavere dosis niveauer, blev der

ikke fundet tegn på toksiske effekter. Denne konklusion gjaldt kombinationer af stoffer som enten havde forskellige målorganer og/eller forskellige mål i det samme organ (dvs. de havde forskellige virkningsmekanismer). Derfor udgør eksponering for sådanne blandinger ikke en større risiko end eksponering for det enkelte stof, under forudsætning af, at eksponeringsniveauerne er enten omkring eller lavere end de individuelle NOAELs. Ved eksponeringer, som er højere end NOAEL niveauerne, kan der ses både synergistiske og antagonistiske effekter, afhængigt af stofferne. Gruppen konkluderer samlet, at når eksponeringsniveauerne for de enkelte stoffer i en blanding er på ADI/TDI niveau, så kan der ikke forventes nogen øget risiko.

Den Hollandske forskergruppe mener, at anvendelse af "dosis addition" metoden i risikovurderingen af kemiske stoffer i blandinger kun er videnskabeligt forsvarlig når kemikalierne i blandingen virker på samme måde, med den samme virkningsmekanisme, og kun er forskellige med hensyn til deres potenser.

En ILSI arbejdsgruppe har behandlet spørgsmålet om samme virkningsmekanisme. Arbejdsgruppen konkluderede, at samme virkningsmekanisme kunne være til stede hvis to stoffer:

- Forårsager samme kritiske effekt
- Virker på samme molekylære mål i det samme målorgan, og
- Virker gennem den samme farmakologiske mekanisme og eventuelt danner den samme toksiske metabolit

Man må imidlertid gøre sig klart, at et præcist kendskab til mekanismerne bag mange stoffers toksiske effekter er mangelfuld, med undtagelse af nogle få stofgrupper, såsom organofosfor og carbamat pesticider og visse polychlorerede dibenzo-*p*-dioxiner, - dibenzofuraner og - biphenyler.

Et andet kritisk emne er spørgsmålet om samtidig eksponering. Hermed menes samtidig eksponering inden for den givne tidsperiode under vurdering for mere end et kemisk stof, som kan interagere med et specifikt mål i et specifikt målorgan eller væv. Det er vigtigt at skelne mellem samtidig "ekstern" eksponering, som refererer til tidspunktet for oral indtagelse, dermal eksponering eller eksponering ved inhalation, og samtidig "intern" eksponering, som relaterer sig til den dosis, der aktuelt opnås ved et givent biologiske mål på det givne tidspunkt. I forbindelse med risikovurderingen er det den "interne" dosis, der er af toksikologisk betydning, men den kendes sjældent. Sammenfaldende tidspunkt og varighed af ekstern eksponering, persistens (biologisk halveringstid) af stofferne i kroppen og varigheden af effekten er de faktorer, som bestemmer om en kumulativ effekt er sandsynlig som følge af eksponering for flere stoffer med samme virkemåde.

Effekten af et kemisk stof på sit biologiske målorgan afhænger af stoffets evne til at opnå en koncentration på målstedet, der overskrider den tærskel, som kræves for at en effekt kan udløses. Intensiteten og varigheden af responset afhænger af stoffets toksikokinetiske egenskaber (absorption, fordeling, metabolisme og udskillelse) og arten af interaktion med målstedet (reversibel/irreversibel). Hvis der sker fuld genoprettelse mellem successive eksponeringer, så forventes der ikke at optræde kumulativ toksicitet. Dog kan en kortvarig eksponering godt tænkes at kunne bidrage signifikant til belastningen med et persistent kemikalie og være relevant for størrelsen af en kronisk effekt.

Risikovurderingen af eksponering for blandinger af veldefinerede kemiske stoffer bør udnytte de toksikologiske databaser optimalt. Ideelt bør udgangspunktet (point of departure (POD)) for vurderingen være en dosis, der er associeret med en

bestemt biologisk respons (ED10, ED20), da dette tager hensyn til alle tilgængelige dosis-respons data. Et udgangspunkt baseret på doser, som giver et bestemt respons bør altid foretrækkes frem for at anvende NOAEL, fordi NOAEL er en enkelt punktværdi og ikke et mål for et biologisk respons, og dets størrelse afhænger i høj grad af det eksperimentelle design. Udgangspunktet for de individuelle stoffer bør ideelt også baseres på studier med samme dyreart og med samme administrationsvej. De data, der er tilgængelige for de fleste kemiske stoffer tillader imidlertid ikke en estimering af for eksempel ED10 og relative potenser må derfor baseres på NOAEL som udgangspunkt.

I en række tilfælde er det dog foreslået at anvende ADI eller TDI, når sådanne findes, som udgangspunkt for vurderingen af kendte stoffer i blandinger, f.eks. pesticider (ATSDR 2002, Reffstrup 2002). Det største problem i forbindelse med disse metoder til kumulativ risikovurdering er, at man ikke tager hensyn til de forskelle i usikkerhedsfaktorer (UF), som måtte være anvendt til at fastsætte de regulatoriske værdier, såsom ADIer og TDIer. Hvis der er anvendt samme usikkerhedsfaktor for alle stofferne i blandingen, så vil alle metoderne give sammenlignelige resultater. Men dette er som regel ikke tilfældet, og variationer i størrelsen af usikkerhedsfaktorer anvendt på de forskellige stoffer, kommer til at påvirke resultatet af risikovurderingen.

Rigid anvendelse af en enkelt, særligt udvalgt metode til risikovurdering af alle blandinger af kemiske stoffer kan ikke anbefales. Fremgangsmåden foreslået af Reffstrup (2002) (se afsnit 4.4.3.1) kan bruges til at vurdere de relativt simple blandinger af pesticidrester i fødevarer, mens metoderne foreslået af Groten *et al.* (2001) (se afsnit 4.5) og ATSDR (2002) (se afsnit 4.6) bør foretrækkes, når der er tale om mere komplekse blandinger. Af de to sidstnævnte forekommer metoden foreslået af ATSDR (2002) at være den, der er lettest at anvende, men folk der vælger at anvende denne fremgangsmåde vil have stor nytte af at konsultere og forstå metoden beskrevet af Groten *et al.* (2001).

Summary and conclusions

Introduction
Following the current practices, health risk assessments of exposures to chemicals and the subsequent regulatory measures, e.g. classification and labelling, establishment of limit values such as MRLs, etc. are generally based upon data from studies on the individual substances. However, humans are simultaneously exposed to a large number of chemicals that potentially possess a number of similar or different toxic effects. Consequently, the authorities are challenged to consider that this "chemical cocktail" or "total chemical load" does not produce unforeseen health effects.

Combined action and interactions between chemicals, e.g. medicines administered to humans at high doses have been known for many years in the field of pharmacology. However, these experiences are not directly useful for predicting toxic effects of mixtures of environmental chemicals because the exposure levels of the general human population are relatively low and interactions occurring at high doses may not be representative for low-dose exposures. The expectation is that relatively low exposures will result in less interactions when they are primarily caused by various thresholds and saturation phenomenons, for instance with respect to the metabolism of chemical substances (enzymatic detoxification/activation) in the organism.

This report summarises the basic concepts in combined action and interaction of chemicals in mixtures and discuss various approaches that have been suggested for use in testing and risk assessment of such mixtures. A number of targeted toxicological studies on simple, well-defined mixtures are discussed and the current knowledge of combined actions of chemicals in different effect areas are reviewed.

Combined action and interaction in various effect areas
Many studies have been performed on *skin, eye and respiratory tract irritation* mediated by complex mixtures, but only few studies allows a quantitative evaluation of the modulating effects of the combination of single chemicals. Further studies on the modulating effects of the combination of chemicals concerning skin, eye and respiratory tract irritation will be required in order to evaluate the possibilities for synergistic or antagonistic effects being mediated by mixtures of chemicals. Various types of *in vitro* systems have shown promise for use to evaluate effects of irritant chemicals, and this approach may minimise the cost and duration of studies to investigate combined effects.

Genotoxicity in vitro is probably the most frequently studied effect of complex mixtures and many studies have been performed on the genotoxic potential of complex environmental mixtures, but only relatively few studies have been performed on the genotoxic activity of simple mixtures of known chemical composition. Most of these targeted studies have involved interactions between a mutagen and a non-mutagen that either enhances (co-mutagens) or inhibit (anti-mutagens) the potency of the mutagen. However, *in vitro* studies of mixtures of known genotoxic compounds tend to show additive effects and in some cases antagonistic effects. Due to the potential differences in the biological processes in *vitro* and *in vivo,* combined effects demonstrated *in vitro* should be confirmed *in*

vivo. The COMET-assay *in vivo* and genotoxicity tests in transgenic animals might be promising *in vivo* tests for future evaluation of complex mixtures.

Carcinogenesis is often described as a multi-step process. The fact that different chemical, physical or biological agents may affect either step makes it obvious that cancer is most often the result of combined actions. The interaction of carcinogens affecting different steps in carcinogenesis has been known for half a decade to cause synergistic effects, strongly increasing the tumour response. Thus, initiators, promoters, converters, and co-carcinogens all act in concert to potentiate the final tumour outcome. Anticarcinogens may prevent or inhibit cancer at either step, and are also known to potentiate each other in some cases. Compounds affecting the same step by different mechanisms can also cause potentiation. The possibilities for combined effects in carcinogenesis are therefore many

In the area of *reproductive effects*, studies using combined exposure of two or more chemicals have shown that interactions may or may not occur, and that it is impossible to predict the outcome based on experimental conditions alone. However, the published data suggest that the prevailing outcome of exposure to mixtures deduced from *in vivo* experiments is either an antagonistic effect or no interactive effect, including an additive effect. Frequently, one type of interaction was noted at some doses, while another type of interaction was noted at other doses. An evaluation of the results suggests that low doses of the chemicals in combination often produced either no effect or additive effects, whereas higher doses produced antagonistic or synergistic effects.

Most of the work made within the field of *endocrine disruption* is based on *in vitro* experiments and only very few *in vivo* experiments on mixtures have been performed so far. Such experiments will be one of the future challenges within the field of endocrine disruption. The majority of the -especially older- studies concluded that additive effects, i.e. no interaction between the compounds were found, although detailed mechanistic analysis were not applied in most cases. However, recent well-designed *in vitro* studies clearly show that the combined effects of estrogenic compounds do not deviate from the expected additivity. In addition, additive effects of two antiandrogenic compounds given *in vivo* were found. Therefore, at present there is no evidence pointing to the necessity of incorporating synergism in the hazard assessment of weakly estrogenic chemical mixtures.

In *neurotoxicity*, there are many possibilities for interaction between chemicals because of the complex hierarchical structure of the nervous system. A number of examples have been described of which the most well known are the additive narcotic effect of organic solvents, and the additive effect on acetyl cholinesterase inhibition by organophosphorus insecticides. The strongest interaction found in the literature was a 5-fold increase in the neurotoxicity of hexane when methyl isobutyl ketone was co-administered. However, very few quantitative studies have been performed and interaction has not been studied systematically. Therefore, the present state of knowledge does not allow general conclusions.

The many different cell types involved in the function of the immune system gives many theoretical possibilities of combined *immunotoxic* effects. However, there are as yet only few experimental data. Chemicals sharing a common toxic mechanism seem to have an additive effect. Competition for metabolising enzymes may antagonise the immunotoxic effect. How mixtures of chemicals that are toxic to different branches of the immune system will exert their combined immunotoxic effect remains to be established.

Studies have shown that if a person *allergic* to two unrelated allergens is challenged with these two allergens in combination the subsequent elicitation response will be the sum or greater than the expected sum of response to the two allergens alone. Challenge with two allergens combined at sub threshold doses of each can elicit a response that none of the allergens alone would elicit. The consequence of this is that a person may be negative in a diagnostic patch test with single chemicals although a mixture of chemicals would produce a reaction. In practical life an allergen may be tolerated in one situation but not in another where it is in combination with another allergen. The threshold dose for elicitation may be very low for allergens in mixture e.g. fragrances in perfume.

A chemical or a physical condition (occlusion, disease) that facilitates penetration of the skin by a contact allergen or have an effect on the skin immune system may enhance the possibility of sensitisation.

Risk assessment of chemicals in mixture

The prediction of the toxicological properties of a chemical mixture ideally requires detailed information on the composition of the mixture and the mechanism of action of each of the individual compound. In order to perform a risk assessment, proper exposure data are also needed. Most often such detailed information is not available. Adequate testing of mixtures is often not possible because they are either virtually unavailable for testing or only available in such limited amounts that a sufficient number of dose levels cannot be applied. In addition, high dose levels of a chemical mixture may have different types of effects than low dose levels and high to low dose extrapolation may be difficult. However, in the area of air pollution it has been possible to test some mixtures, either in the form of exhaust-gases or other emissions or by extraction and up-concentration of the air pollution components.

One of the main points to consider is whether there will be no interaction or interaction in the form of either synergism or antagonism. These three basic principles of combined actions of chemical mixtures are purely theoretical and one often has to deal with two or all three concepts at the same time when mixtures consist of more than two compounds and when the toxicity targets are more complex.

A Dutch research group initiated their research programme in order to test the hypothesis that exposure to chemicals at (low) non-toxic doses of the individual chemicals, as a rule would be of no health concern. One reason being that most guidelines from national and international organisations often suggest the use of simple “dose addition” or “response addition” models for the assessment of chemical mixtures totally ignoring any knowledge on the mode of action of the chemicals.

From the results of experimental, short-term toxicity studies the Dutch group concluded that combined exposure to arbitrarily chosen chemicals clearly demonstrated the absence of full additivity, and provided some evidence of partial additivity when all chemicals in the mixture were administrated at their own individual No adverse effect levels (NOAELs). At slightly lower dose levels no clear evidence of toxicity was found. This conclusion was found valid for combinations of chemicals that have either different target organs and or different target sites within the same organ (i.e. differ in the mode of action). Therefore exposure to such mixtures is not associated with a greater hazard than exposure to the individual chemicals, provided that the exposure levels are at or below the individual NOAELs. At exposure levels higher than the NOAELs both synergistic

and antagonistic effects may be seen, dependent on the compounds. When the exposure levels are at the ADI/TDI levels no greater hazard is to be expected.

The Dutch group is of the opinion that the use of the "dose addition" approach to the risk assessment of chemical mixtures is only scientifically justifiable when all the chemicals in the mixture act in the same way, by the same mechanism, and thus differ only in their potencies. Application of the "dose addition" model to mixtures of chemicals that act by mechanisms for which the additivity assumptions are invalid would greatly overestimate the risk.

An ILSI (International Life Science Institute) Working Group has recently addressed the "common mechanism" issue. The ILSI Working Group concluded that a common mechanism might exist if two compounds:

- Cause the same critical effect
- Act on the same molecular target at the same target tissue, and
- Act by the same pharmacological mechanism of action and may share a common toxic intermediate

It should be realised that with the exception of a few groups of chemicals, such as some organophosphorous and carbamate pesticides and some polychlorinated dibenzo-*p*-dioxins, - dibenzofurans and - biphenyls, precise mechanistic information on their toxic effects are scarce.

Another critical issue is the question of concurrent exposure. This refers to co-exposure to more than one chemical able to interact with a defined target in a specific target tissue during a particular time frame of interest. It is important to distinguish between concurrent or simultaneously "external" exposure, referring to the timing of oral, dermal or inhalation exposure, from concurrent "internal" exposure that relates to the dose actually attained at a given biological target in a given time frame. For the risk assessment it is the "internal" exposure that is of toxicological significance, however, it is seldom known. The factors that determine whether a cumulative effect is likely from exposure to several different common mechanism compounds are the timing and duration of external exposure, the persistence (biological half-life) of the chemicals in the body, and the duration of the effect.

The effect of any chemical at a biological target depends of its ability to attain a target site concentration that exceeds the threshold required to elicit the response. The intensity and duration of the response depends on the toxicokinetic properties of the compound (absorption, distribution, metabolism and excretion) and the nature of the target site interaction (reversible, irreversible). If recovery is complete between successive exposures no cumulative toxicity is to be expected. However, a short-term acute exposure could potentially add to the long-term burden of a persistent chemical and be relevant for the magnitude of the chronic effect.

The risk assessment of exposure to mixtures of defined chemicals should make optimal use of the toxicological databases. Ideally, the point of departure (POD) for the assessment should be a dose associated with a particular biological response (ED10, ED20) since this takes into account all of the dose-response data available. A POD based on doses causing a particular response should always take preference over the NOAEL. This is because the NOAEL is a single point value and not a measure of a biological response and is largely a consequence of the experimental design. The POD should also ideally be based on studies with the same animal species using the same route of administration. However, the data available for

most chemicals will not permit an estimation of for instance ED10 and relative potencies may have to be based on NOAELs as PODs.

In a number of cases, ADIs, TDIs or RfDs, if available, have been suggested as the POD for the assessments (ATSDR 2002, Reffstrup 2002). The biggest problem associated with these methods of cumulative risk assessment is how to accommodate the different uncertainty factors (UF) that may have been applied to derive regulatory values such as ADIs or RfDs. If the uncertainty factors applied are the same for all the chemicals, all the methods will give similar results. However, this is most often not the case and the variations in the size of uncertainty factors applied to the various chemicals will influence the result of the risk assessment.

It is not advisable to recommend rigid use of any single approach for the risk assessment of all chemicals mixtures. The approach suggested by Reffstrup (2002) (see section 4.4.3.1) may be used for the assessment of the relatively simple mixtures of identified pesticides in food whereas the approaches suggested by Groten *et al.* (2001) (see section 4.5) and ATSDR (2002) (see section 4.6) should be preferred for the assessment of more complex mixtures. Of the latter two, the method proposed by ATSDR (2002) appears to be the most straightforward. However, people choosing to use that approach may benefit from consulting and understanding the Groten et al. (2001) approach.

1 Introduction

Prepared by John Chr. Larsen

1.1 Background

All living organisms are constantly and unavoidably exposed to foreign chemicals (xenobiotics) through food, air, water, and dermal contact. The list of possible exogenous compounds and their combinations is endless. Chemicals produced and used by intention include industrial chemicals, food additives, pesticides and drugs. As a result of industrial and other human activities (i.e. combustion, traffic) a large number of pollutants are unintentionally released to the environment and may subsequently contaminate air, food and water. Well-known examples are polychlorinated dibenzo-p-dioxins and dibenzofurans (dioxins), polychlorinated biphenyls (PCBs), metals, pesticides and polycyclic aromatic hydrocarbons (PAH). Other contaminants are formed during processing, storage or cooking of food. In addition, a wealth of naturally occurring compounds such as alkaloids, other secondary plant metabolites and toxins produced by moulds, plants and animals, are constantly being ingested by humans.

Following the current practices, health assessments of exposure to chemicals and the subsequent regulatory measures, e.g. classification and labelling, establishment of limit values such as MRLs, etc. are generally based upon data from studies on the individual substances. However, humans are simultaneously exposed to a large number of chemicals that potentially possess a number of similar or different toxic effects. Consequently, not only opponents against the use of chemicals but also the consumers at large are increasingly challenging the authorities to consider that this "chemical cocktail" or "total chemical load" does not produce unforeseen health effects. This question was even more highlighted in 1996 when the US Congress passed the US Food Quality Protection act (FQPA). This act requires that the US EPA consider the effects of exposure to all pesticides and other chemicals that act by a common mechanism of toxicity when tolerances for pesticide use in crops are derived. Therefore the aspect of combined actions of chemicals needs to be addressed to a greater extent in the risk assessment process. A major obstacle in doing so is the lack of data from studies on chemical mixtures employing generally accepted toxicological methods, such as short-term and long-term animal studies. Thus, about 95 % of all resources in toxicology are used to study single chemicals or the effects of pre-treatment with one chemical on the effects of another (Yang 1994). In addition, data on human exposures to chemical mixtures are in general very inadequate. Thus, the regulatory agencies are faced with the situation that they cannot always reliably predict whether the simultaneous exposure to foreign chemicals in the environment and food constitutes a real health problem. As the possible combinations of chemicals are innumerable and experimental testing of all such mixtures is not feasible from obvious reasons, there is a need for science based advise on how exposure to mixtures of chemicals can be dealt with in the risk assessment.

1.2 Objectives and Scope of the Report

The objectives of the report are to summarise and evaluate the current knowledge about the combined toxicological effects that may occur from exposures to different chemicals in mixtures. When justified from a scientific point of view, the

implications for hazard identification, hazard characterisation and risk assessment of chemicals are highlighted. In this context, special attention is paid to the low levels of exposures normally encountered from the unintended, indirect exposure to chemical mixtures through food and environment.

A better understanding of the combined effects between different chemicals is anticipated to improve the safety evaluations of such mixtures performed by the Danish Environmental Protection Agency and the Danish Veterinary and Food Administration and provide a more rational basis for making risk assessments on combined exposure to chemicals.

It has to be recognised that it is not possible for this report to cover all possible combined exposures to chemicals. In order to focus on the mutual needs of the Danish Environmental Protection Agency and the Danish Veterinary and Food Administration, *the main emphasis is paid on the identification of the basic principles for combined actions and interactions of chemicals and on the current knowledge on effects of exposures to mixtures of industrial chemicals, including pesticides and environmental contaminants*. Thus, interactions between natural components in food, such as inherent plant constituents, as well as their interactions with industrial chemicals and contaminants are not the topic of this report. In addition, influences of dietary habits are not dealt with. However, when studies on interactions between natural components of food provide significant understanding of the modes of action of chemical mixtures such information is included. These basic principles on combined actions and interactions of chemicals in mixtures are summarised and discussed in chapters 2-6. Chapter 7 contains a comprehensive review of different relevant effect areas, including examples of combined effects and interactions.

1.3 Key historical developments

Interactions between chemicals administered to humans at high doses have been known for many years in the field of pharmacology. However, these experiences are not directly useful for predicting toxic effects of mixtures of environmental chemicals because the exposure levels of the general human population are relatively low and interactions occurring at high doses may not be representative for low-dose exposures (Könemann & Peters 1996).

Toxicity studies with mixtures have been performed for several decades. Initially, most studies were done with binary mixtures. Later, studies with defined mixtures of more than two compounds have been reported. Studies have also been performed with complex mixtures of environmental chemicals, such as exhaust condensates, in order to gain insight into the toxic effects of such a particular mixture. However, the interpretation of the toxicity seen in these latter studies is complicated because the exact composition of such mixtures is normally not known, and the “real life” mixtures may vary considerably in composition. Therefore extrapolation to other situations may be difficult. This fact is often ignored for the sake of simplicity.

A major issue in the assessment of the combined toxicological effect of chemicals in a mixture is the type of combined action to be expected. What kind of toxicity may be expected, given the toxicity profiles of the individual components? Bliss (1939) was the first to provide a conceptional framework for the combined action of chemicals and later contributions were made by Finney (1942), Hewlett and Placket (1959 and 1964), Placket and Hewlett (1952, 1963 and 1967), Ashford and Cobby (1974) and Ashford (1981). Placket and Hewlett (1952) provided a scheme of possibilities of combined (joint) actions (Table 1.3.1).

Table 1.3.1: Classification of combined (joint) toxic actions of two compounds in mixture

	Combined action	
Interaction	**Similar action**	**Dissimilar action**
Absent (No interaction)	Simple similar action (Dose addition)	Independent action, Response addition
Present (Interaction)	Complex similar action (Antagonism or synergism)	Dependent action or complex dissimilar action (Antagonism or synergism

Modified after Placket and Hewlett (1952)

A major clue that can be taken from this scheme is that in the initial assessment it is important to evaluate whether interactions are actually occurring (present) or not (absent).

Interaction was defined as the influence of one chemical on the biological action of another (either qualitatively or quantitatively). The scheme represents the extremes of combined actions. In many cases there are not adequate information about the underlying mechanisms of combined actions. This led Berenbaum (1985 and 1989) to propose three classes of combined action: zero interaction, synergism and antagonism.

In more recent years several reference books, review articles and reports have been published on the issue of toxicological effects from combined exposure to chemicals. The more important are outlined in chapter 8.

2 Basic concepts and terminology used to describe the combined action of chemicals in mixtures

Prepared by John Chr. Larsen

2.1 Introduction

The major objective in the risk assessment of exposure to mixtures of chemicals is to establish or predict how the resulting toxicological effect might turn out. Will the toxic effect be determined by simple additivity of dose or effect, or will it deviate from additivity, either by an effect stronger or less than expected on the basis of additivity?

The prediction of the toxicological properties of a chemical mixture requires detailed information on the composition of the mixture and the mechanism of action of each of the individual compound. In order to perform a risk assessment, proper exposure data are also needed. Most often such detailed information is not available. Complex chemical mixtures may contain hundreds, or even thousands of compounds, and their composition is qualitatively and quantitatively not fully known and may change over time. Adequate testing of such mixtures is most often impossible because they are either virtually unavailable for testing or only available in such limited amount that a sufficient number of dose levels cannot be applied. In addition, high dose levels of a chemical mixture may have different types of effects than low dose levels and high to low dose extrapolation may be meaningless.

In the following, several terms used to describe interactions between chemicals are mentioned as well as basic concepts used in the toxicological evaluation and risk assessment of chemical mixtures. The descriptions of the basic concepts of the toxicology of chemical mixtures, first outlined by Bliss (1939) and Placket & Hewlett (1952) are taken from Könemann & Pieters (1996), Cassee *et al.* (1998) and Groten *et al.* (2001). The definitions of additivity, synergism, antagonism and potentiation are taken from Klaasen (1995) and Seed *et al.* (1995).

As has already been outlined in the introduction one of the main points to consider is whether there will be no interaction or interaction in the form of either synergism or antagonism. These three basic principles of combined actions of chemical mixtures are purely theoretical and one often has to deal with two or all three concepts at the same time especially when mixtures consist of more than two compounds and when the toxicity targets are more complex.

Interactions between chemicals may be of a physicochemical and/or biological nature. Examples of physicochemical interactions are the reaction of nitrite with alkylamines to produce carcinogenic nitrosamines and the binding of toxic chemicals to active charcoal, resulting in a decreased absorption from the gastrointestinal tract. It is held that physicochemical interactions will normally only occur at high doses and therefore are of lesser importance for low dose scenarios.

Physicochemical interactions will therefore not be considered in any detail in this report.

The processes leading to a biological and/or toxicological response from exposure of animals and humans to a given chemical can be divided into two distinct parts, toxicokinetics and toxicodynamics (Renwick 1993, IPCS 1994). Toxicokinetics relates to those processes that determine the extent and duration of exposure at the target organ or site of toxicity to the active chemical species (parent compound or metabolite). Toxicokinetics involves processes such as absorption, distribution, biotransformation, and excretion of the compound and metabolites. Toxicodynamics refers to the processes involved in the translation of such exposure of the target organ or site of action into the generation of a toxic effect. Toxicodynamics may involve a large number of different processes that determine the mechanisms of action of a given chemical. These processes may involve inhibition of cellular enzymes, damage through binding to proteins or DNA, or interactions at endogenous receptor sites, just to mention a few events that may pertubate the normal homeostasis of the organism or tissue.

2.2 No interaction

According to Plackett and Hewlet (Table 1.3.1) there are two types of combined action without interaction: simple similar action (dose addition, Loewe additivity) and simple dissimilar action. This latter type contains two concepts: effect or response additivity and Bliss independence. The independence criterion seems not to be widely used in toxicology (Groten *et al.*, 2001). The response to a mixture of compounds depends not only on the dose, but also on the correlation of tolerances between the effects of the chemicals in the mixture, which can vary between –1 and +1 (Bliss 1937). There is a complete negative correlation (r =-1) between the effects of two chemicals if the individuals that are most susceptible to one toxicant are least susceptible to the other, while a complete positive correlation (r = +1) exists if the individuals most susceptible to one toxicant are also most susceptible to the other.

2.2.1 Simple similar action (dose addition, Loewe additivity)

Simple similar action (simple joint action or concentration/dose addition) is a non-interactive process in which the chemicals in the mixture do not affect the toxicity of one another. *All the chemicals of concern in the mixture act on the same biological site, by the same mechanism of action, and differ only in their potencies.* The correlation of tolerances is completely positive (r = +1) and each chemical contributes to the toxicity of the mixture in proportion to its dose, expressed as the percentage of the dose of that chemical alone that would be required to obtain the given effect of the mixture. Thus the individual components of the mixture act as if they were dilutions of the same toxic compound and their relative potencies are assumed to be constant throughout all dose levels. An important implication is that in principle no threshold exists for dose additivity.

Simple similar action serves as the basis for the use of "toxic equivalency factors" often used to describe the combined toxicity of isomers or structural analogues. Additive effects are described mathematically using summation of doses of the individual compounds in a mixture adjusted for differences in potencies. This method is assumed to be only valid for compounds that produce linear dose response curves. Probably, the best validated example of a group of compounds that obey the principles of simple similar actions are the dioxins (polychlorinated dibenzo-*p*-dioxins and dibenzofurans) that produce most (if not all) of their toxicities through interaction with the Ah-receptor.

2.2.2 Simple dissimilar action (response or effect additivity, Bliss independence)

Simple dissimilar action (simple independent action, independent joint action, Bliss independence and effect addition or response addition) is also a non-interactive process where the toxic effect of each chemical in the mixture is not affected by the other chemicals present. *However, the modes of action of the constituents in the mixture will always differ and possibly, but not necessarily, the nature and site of action also differs among the constituents.* Response addition is referred to when each individual of a population (e.g. a group of experimental animals or humans) has a certain tolerance to each of the chemicals in a mixture and will only exhibit a response to a toxicant if the concentration exceeds the tolerance dose. In such a case, the number of responders within the group will be recorded rather than the average effect of a mixture on a group of individuals. By definition, response addition is determined by summing the responses of the animals to each toxic chemical in the mixture.

Three different concepts have been developed for effect/response additivity depending on the correlation of susceptibility of individuals to the toxic agents:

2.2.2.1 Complete negative correlation
There is a complete negative correlation between the effects of two chemicals if the individuals that are most susceptible to one toxicant are least susceptible to the other. This is the simplest form of response additivity. The proportion (P) of individuals responding to the mixture is equal to the sum of the responses to each of the components:

$P_{\text{mixture A,B}} = P_A + P_B$ less than or equal to 1

2.2.2.2 Complete positive correlation
There is a complete positive correlation between the effects of two chemicals if the individuals most susceptible to one toxicant are also most susceptible to the other. The proportion (P) of individuals responding to the mixture is equal to the response to the most toxic compound in the mixture:

$P_{\text{mixture A,B}} = P_A$ if toxicity $A \geq B$

2.2.2.3 No correlation, Bliss independence
This situation is equal to Bliss independence. There is no correlation if the proportion of individuals responding to the mixture is equal to the sum of proportions of individuals responding to each of the toxicants taking into account that those individuals that respond to constituent A cannot react to B as well:

$$P_{\text{mixture A,B}} = P_A + P_B \cdot (1 - P_A)$$

Although this type of correlation seems to be similar to complete negative correlation, the difference is that in this case an individual can respond to both compound A and B but not to both at the same time.

The approach of response addition can be easily applied to simple problems, such as acute toxicity of pesticides. However, more complex effects are not always easy to summate. Experimental animals are usually obtained from inbred strains, while human populations are more heterogenic. In addition, various effects on different organ systems may occur within different time frames in experimental animals.

US EPA (1986) applied the concept of response addition to the determination of cancer risks, assuming a complete negative correlation of tolerance. This assumption is considered to contribute to a conservative estimation of risk, since the correlation of tolerances may not be strictly negative in inbred homogenous experimental animals. There is a major difference between the concepts of response addition and dose addition when the human situation of low exposure levels is assessed. Response addition implies that when doses of chemicals are below the no-effect-levels of the individual compounds (i.e. the response of each chemical equals zero) the combined action of all compounds together will also be zero. In contrast, dose addition can also occur below the no-effect-level and the combined toxicity of a mixture of compounds at individual levels below the no-effect level may lead to a response.

For compounds with presumed linear dose-response curves, such as genotoxic and carcinogenic compounds for which it is assumed that a no-effect-level does not exist and for which the mechanism of action may be regarded as similar, response addition and dose addition will provide identical results (Könemann & Pieters 1996).

2.3 Interactions

Chemicals may interact with one another and modify the magnitude and sometimes also the nature of the toxic effect. According to Table 1.3.1 the combined action of chemicals that interacts can be divided into two categories: Complex similar action and dependent action (complex dissimilar action). Interactions may take place in the toxicokinetic phase and/or in the toxicodynamic phase. The interactions may result in either a weaker (antagonistic) or stronger (potentiated, synergistic) combined effect than would be expected from knowledge about the toxicity and mode of action of each individual compound.

2.3.1 Antagonism

An antagonistic effect occurs when the combined effect of two chemicals is less than the sum of each chemical given alone. Synonyms sometimes used for antagonism are: Interaction, depotentiation, desensitisation, infra-addition, negative synergy, less than additive, subaddition, inhibition, antergism, competitive antagonism, noncompetitive antagonism, uncompetitive antagonism or acompetitive antagonism.

2.3.2 Synergism

A synergistic effect occurs when the combined effect of two chemicals is greater than the sum of the effects of each chemical given alone. Synonyms sometimes used to describe synergism are: Coalitivity, interaction, unisynergism, augmentation, sensitisation, supra-addition, independent synergism, dependent synergism, degradative synergism, greater than additive, cosynergism, superaddition, conditional independence or potentiation.

2.3.3 Potentiation

Potentiation, being a form of synergism, occurs when the toxicity of a chemical on a certain tissue or organ system is enhanced when given together with another chemical that does not have toxic effects on the same tissue or organ system. This form of interaction is especially well described in mutagenesis and carcinogenesis where a number of compounds have been identified as co-mutagens or co-carcinogens.

The ultimate toxicological response following exposure to a chemical substance is most commonly the result of the action of this substance on a definite site or receptor. For a given concentration of the agent at the target site the intensity of the response will depend on the quality of the action (the intrinsic activity) and the affinity of the compound for the receptor.

When two compounds exert the same action by acting at different sites their interaction will often result in a synergistic effect but a simple additive effect is also a possibility (the synergism between smoking and asbestos exposure is the classical example).

2.3.4 Complex similar action

In the case of complex similar action two compounds acting on the same target receptor do not produce an additive effect as would be expected from simplicity, but either an antagonistic or synergistic effect. This phenomenon is well known for substances competing for the same hormonal or enzymatic receptor sites. In such cases, lower than additive effects are often observed. An example could be two chemicals that exert the same action - e.g. accumulation of acetylcholine - by acting in the same manner - e.g. by inhibition of acetylcholine esterase. An additive effect may occur if the intrinsic activities and affinities of the two substances are identical but most often an antagonistic effect is observed as both compounds compete for the same receptor. A maximal antagonism is found when the substance with the lowest intrinsic activity possesses the higher affinity for the receptor or has been the first to get into contact with the target.

In order to predict the effect of a mixture of chemicals with the same target receptor but with different non-linear dose-effect relationships either physiological or mathematical modelling can be applied. For interactions between chemicals and a target receptor or enzyme the Michaelis-Menten kinetics (first order kinetics but with saturation) are often applicable. This kind of action can then be considered a special case of similar combined action (dose addition).

It is highly likely that for compounds thought to have complex similar actions the observed deviations from the expected additivity in some cases are due to the fact that the compounds are actually not acting at the very same site at the target receptor. This means that the compounds actually have complex dissimilar actions and the combined action is misclassified as a complex similar action due to insufficient knowledge about the exact mechanisms of action.

2.3.5 Complex dissimilar actions

Complex dissimilar actions are probably the most frequently occurring interactions operating in experimental studies on mixtures applying high doses. The most obvious cases in the toxicokinetic phase involve enzyme induction or inhibition. Enzyme induction could result in a synergistic effect if more reactive (and toxic) intermediates are formed or in an antagonistic effect if the toxic agent is removed by detoxification. Compounds, which influence the amount of biotransformation enzymes, can have paramount effect on the toxicity of other chemicals. Uptake and excretion are often active processes, which may also be affected by other chemicals. Interaction between substrates for the same membrane receptors or pumps as well as for biotransformation enzymes could result in synergism and antagonism, too.

2.4 Test strategies to assess combined actions and interactions of chemicals in mixtures

Ideally, all chemicals in a mixture should be identified and the toxicity profile of each of the constituents as well as their potential combined actions and/or interactions should be determined over a wide range of exposure levels. For complex environmental mixtures this approach is not realistic, therefore a number of approaches and test scenarios have been presented to obtain toxicological information on mixtures with a limited number of test groups (Cassee *et al.* 1998).

2.4.1 Testing of whole mixtures

Although testing of the whole mixture as such seems to be the proper way to approach the risk assessment of exposure to that mixture it will not provide data on combined actions and/or interactions between the individual components of the mixture. Even if the effect of the mixture is compared with the effects of each individual component at comparable concentrations this will not allow a description of potential synergism, potentiation or antagonism, and it is even doubtful that deviations from additivity can be concluded. This can only be achieved if dose-response curves are obtained for each of the single compounds. Testing of the whole mixture as such has been recommended for mixtures that are not well characterised (Mumtaz *et al.* 1993) and has successfully been applied for assessing the combined toxicity of simple, defined chemical mixtures where the toxicological properties of the individual components were also investigated (see Chapter 5).

2.4.2 Physiologically based toxicokinetic (PBTK) modelling

For many chemicals their metabolism is the major determinant of the risk and for a number of hazardous compounds there is a considerable knowledge from experimental studies on the relationship between metabolism and toxicity. In particular, *in vitro* studies using cell cultures, subcellular fractions or pure enzymes have provided information on the nature of reactive intermediates as well as on detoxification pathways. Moreover, the significance of these processes has been demonstrated in several species of experimental animals and humans.

Most PBTK models describes the rat or man as a set of tissue compartments, i.e. liver, adipose tissues, poorly perfused tissues, and richly perfused tissues along with a description of metabolism in the liver. In case of volatile organic compounds a description of gas exchange at the level of the lung is included.

Metabolism is often described as a saturable process (equation 1) characterised by a maximal velocity (V_{max}) and a Mechaelis-Menten affinity constant (K_m), or as a first order process (equation 2):

$$RAM = V_{max}C_{vl} / (K_m + C_{vl}) \quad (1)$$

$$RAM = K_f C_{vl} V_l \quad (2)$$

where RAM, C_{vl}, K_f, and V_l refer to the rate of the amount of chemical metabolised, chemical concentration in venous blood leaving the liver, first order metabolism rate constant, and volume of liver, respectively.

Metabolism can also be described using the hepatic extraction ratio (E) as follows:

$$RAM = Q_l E C_a \quad (3)$$

Where Q_l and C_a refer to liver blood flow rate and arterial blood concentration, respectively. The use of this equation (3) permits the simulation of the theoretical limits of the impact of metabolic interactions, since the value of E can only range between 0 and 1. Enzyme induction can increase the value of E but only to a maximum value of 1 due to blood flow limitations, whereas inhibition of metabolism decreases the value of E but the value cannot be lower than 0 (Haddad *et al.* 2000 a, 2000b).

In principle, the *in vivo* human metabolism can be predicted by using *in vitro* enzyme kinetic data and can thus be compared with the *in vitro* and *in vivo* data from experimental animals. For example, experiments using microsomes or hepatocytes may predict the *in vivo* velocity of metabolism for a single metabolic pathway. Such data may be incorporated in physiologically based toxicokinetic (PBTK) modelling (Andersen *et al.* 1995, Leung and Paustenbach 1995, Yang *et al.* 1995). As a rule, the description of the rate constants such as V_{max} and K_m for the individual (iso)enzymes follows Michaelis-Menten kinetics. Therefore interindividual differences in expression levels of enzymes, and genetic polymorphism can also be modelled. Ploemen *et al.* (1997) have presented a strategy to combine PBPK modelling with human *in vitro* metabolic data to explore the relative and overall contribution of critical metabolic pathways in man.

In order to use PBTK modelling in the assessment of mixtures, Cassee *et al.* (1998) suggest that one of the components is first modelled and regarded as the prime toxicant being modified by the other components. Based on *in vitro* data on the other components, effects of for example inhibition or induction of specific biotransformation isoenzymes can be incorporated in the model. Effects of competition between chemicals in a mixture for the same biotransformation enzymes may also be incorporated by translating the effects into effects on the Michael-Menten parameters that are then incorporated into the model.

PBTK models can be extended to include the toxicodynamic phase (PBTK/TD model) if a direct relationship exists between the concentration of the active metabolite (or parent compound) and the toxic effect (Yang *et al.* 1995). As an example, both glutathione depletion and covalent binding to cellular macromolecules was easily predicted in the human situation for 1,2-dichlorobenzene based on comparison of *in vitro* data from rats and humans (Hissink *et al.* 1996).

2.4.3 Isobole methods

An isobole is a contour line representing equi-effective quantities of two agents or their mixtures (Loewe and Muischnek 1926). The theoretical line of additivity is the straight line that connects the individual doses of each of the single agents that produce a predetermined, fixed effect alone, for instance an ED50 of a given toxicity or biochemical effect. The isobole method is widely used to evaluate the effects of binary mixtures. However, a large number of different mixtures of the two compounds have to be tested in order to identify combinations that produce the fixed effect. If the graphical representation of the combinations that produce the fixed effect shows a straight line the two compounds behave in a dose-additive manner and subsequently can be regarded as chemicals that have a similar mode of action. In case of an antagonistic interaction all the equi-effect concentrations in the mixtures represent a downward concave line, whereas a synergistic interaction would produce an upward curve. Examples of isoboles produced from theoretical mixtures of two agents assumed to act either additively, antagonistic or synergistic in combinations are gives in Figures 1, 2 and 3, respectively.

Figure 2.4.3.1. Isobologram of two agents A and B that act additively

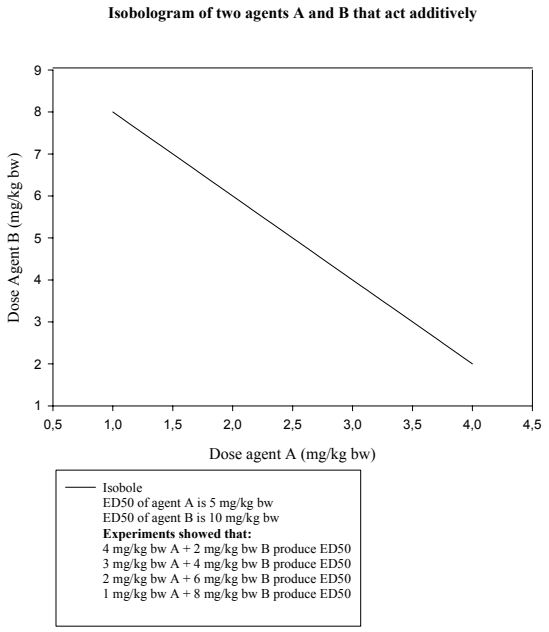


Figure 2.4.3.2. Isobologram of two agents that act synergistically

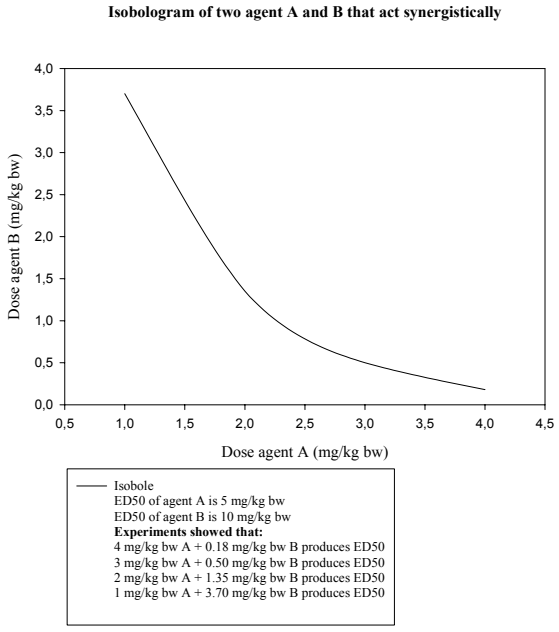
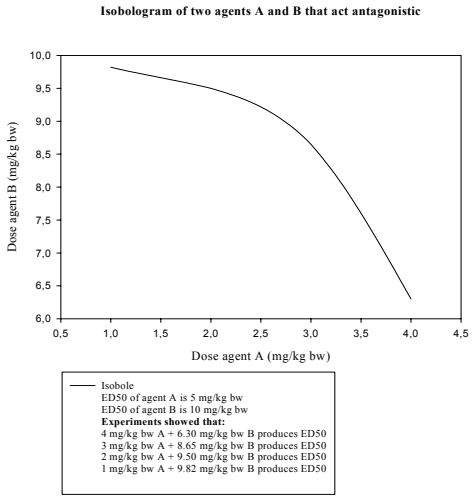


Figure 2.4.3. 3. Isobologram of two agents that act antagonistic



In practice, the interpretation of test results strongly depends on the accuracy of the estimated intercepts of the theoretical isobole with the axis, which represents the doses of the single compounds that induce the desired effect. In fact, large standard deviations of these intercepts prevent a reliable conclusion as to the deviation from additivity.

Berenbaum (1981) introduced an equation to calculate an interaction index (CI). This enables the effects of noninteractive combinations to be calculated directly from dose effect relationships of the individual compounds, regardless of the particular types of dose effect relations involved.

$CI = d_1/D_1 + d_2/D_2 + \ldots. d_n/D_n$

In this equation d_1, d_2, … d_n are the doses of the agents in the mixture and D_1, D_2, … D_n are the doses of the individual agents producing the same effect as the mixture. For binary mixtures a straight line (isobole) is produced joining D_1 and D_2 and passing through (d_1,d_2). CI is 1, <1, or >1 when the combinations show zero interaction, synergy, or antagonism using dose addition, respectively.

In cases of departure from additivity the magnitude of CI depends on the ratio of the concentrations of the constituents of the mixture. Thus the CI is not a general figure but depends on the specific concentrations of the chemicals in the mixture. One difficulty in using this approach is to determine when a specific CI actually deviates from 1 (additivity), as the method of isoboles as developed does not include measures to decide whether deviations from the line of additivity are systematic or simply due to chance or experimental error (Cassee *et al.* 1998). One way of dealing with this problem is to calculate confidence intervals for the iso-effective doses of the single compounds and to add a confidence belt to the line of additivity (Kortenkamp and Altenburger 1998). This envelope of additivity is an area in which those combinations of two compounds are lying that has a specific effect and may reasonably be considered as showing no interaction (for details see Cassee *et al.* 1998).

The isobol method can also be applied to mixtures where only one of the two agents produces the effect under consideration. In case agent A produces an effect, whereas agent B does not, the equation is reduced to:

$CI = d_1 / D_1 = 1$

In this case the iso-effective dose D_2 of the agent lacking the effect of interest can be regarded as infinitely large, so that the resulting additivity isobole runs parallel to the respective dose axis.

Combination of three agents can be analysed by constructing three-dimensional isobolar surfaces, and combinations of more than three compounds can be assessed more easily by using a generalisation of the above-mentioned equation. However, new procedures using a polynomial model have been proposed to evaluate more complex mixtures (Cassee *et al.* 1998).

One of the strengths of the isobol method is that it can be used to analyse combined effects of compounds irrespective of the shape of their dose-response curves. It is possible to assess mixtures of agents with dissimilar dose-response, even when the maximal effects are not identical (Kortenkamp and Altenburger, 1998).

Although isoboles are very illustrative a complete construction requires a large amount of data sets both on the single compounds and mixtures and large standard deviations may limit the interpretation (Cassee *et al.*1998). However, even if it is desirable to test combinations of agents at several mixture ratios so that the isoboles can be constructed reliably, Kortenkamp and Altenburger (1998) are of the opinion that this is not always a necessary prerequisite. Valid conclusions about the combination effect of mixtures can often be drawn on the basis of surprisingly few data.

2.4.4 Comparison of individual dose response curves

Comparison of dose response curves of one chemical (A) in the absence and presence of a second chemical (B) has been proposed as a tool to predict whether the combined action of the two chemicals is either additive or independent (Cassee et al 1998).

In the case of dose additivity, the dose response curve of A is determined on a linear- or log-dose scale, and an equi-effective dose of A ($d_{A,equi}$) and B (d_B) resulting in the same effect is estimated. Using the fixed dose d_B of chemical B and adding various doses ($d_A - d_{A,equi}$) of A the dose response curve should shifts to the left and reach the same maximum as the maximum for the dose response curve of A alone when the effect of B is smaller than A_{max}. However, in case of competitive agonism, the effect of B does not affect the effect of A+B at higher dose of A.

In the case of an independent effect the addition of a fixed dose of B will produce an upward shifted dose response curve. An independent effect can be calculated from the equation:

$E_{AB} = E_A + E_B - (E_A \times E_B)$ (Bliss independence)

This method is based on the idea of response addition and was developed to accommodate the observation that compounds may act on different subsystems within an organism, which may well involve different sites and modes of action. Individual mixture components are not assumed to contribute to the overall mixture effect if they are present at subthreshold levels.

2.4.5 Response surface analysis (RSA)

Response or effect surface analysis (ESA) uses multiple linear regressions to produce a statistically based mathematical relationship between the doses of each of the chemicals in a mixture and the effect parameter. The equation for a mixture containing three compounds would be:

$$E = \alpha + \beta_1 \cdot d_1 + \beta_2 \cdot d_2 + \beta_3 \cdot d_3 + \gamma_1 \cdot d_1 \cdot d_2 + \gamma_2 \cdot d_1 \cdot d_3 + \gamma_3 \cdot d_2 \cdot d_3 + \delta_1 \cdot d_1 \cdot d_2 \cdot d_3$$

Where d_n represents the dose of a chemical in the mixture. Coefficient α represents the control situation, the constants β are associated with the main effects of each of the compounds, whereas the coefficients γ and δ indicate two- or three-factor interactions, respectively. In the case of negative values for β, positive values for γ and δ indicate a less than additive interaction between two or three compounds. Zero values of γ or δ indicate absence of a particular interaction. The *p* values of these coefficients are estimated using *t* test.

Cassee *et al*. (1998) stress that it should be avoided to use high-effect levels in this method because saturation and competition will play a major role for the combined effect and might lead to erroneous conclusions about combined action at lower (and more realistic) dose levels. They advocate using a concentration range not exceeding a 50% effect level, or even less.

2.4.6 Statistical designs

When a mixture contains more than two compounds all kinds of two- or more (three)-factor interactions are possible. In order to determine these in experiments the number of possible test combinations increases exponentially with increasing numbers of compounds. In addition, the number of experimental groups will increase with the number of doses of each compound.

Factorial designs, in which n chemicals are tested at x dose levels (x^n treatment groups) have been suggested by the US Environmental Protection Agency (US EPA 1986) as a statistical approach for risk assessment of chemical mixtures. A 2^5 factorial design has been used to describe interactions between the carcinogenic activity of 5 polycyclic aromatic hydrocarbons at two dose levels (Nesnow 1994) and a 5^3 design to identify nonadditive effects of three chemicals on developmental toxicity at 5 dose levels (Narotsky *et al.* 1995).

However, full factorial designs using conventional toxicity testing are very costly, even if only two dose levels are used. This would require 2^n-1 test groups to identify interactions between all chemicals of interest. Therefore, the use of fractionated factorial designs have been suggested (Plackett and Burman 1946, Svengaard and Hertzberg 1994). Fractionated factorial designs have been used to identify interactive effects between seven trace elements and cadmium accumulation in the body (Groten *et al.*, 1991), and to determine structure-activity relationships for 10 halogenated aliphatic hydrocarbons (Eriksson *et al.* 1991). Groten et al (1997) also used a fractionated factorial design to study the interactions of nine chemicals in mixture in subacute rat studies (see chapter 5.2). They used two dose levels of each compound, and a full factorial design would have required 511 different test groups. The study applied a 1/32 fraction of the complete design involving 16 experimental groups. The design was based on a general balance of groups with and without one of the compounds and required prior knowledge on the chemicals in the mixture. For a discussion of advantages and limitations in using this approach, see Groten *et al.* (1997) and Cassee *et al.* (1998).

2.5 Toxicological test methods

When epidemiological studies form the basis for the risk assessment of a single chemical or even complex mixtures, such as various combustion emissions, it may be stated that in those cases the effects of combined action of chemicals have been incorporated. Examples can for instance be found in the updated WHO Air Quality guidelines (WHO 2001). Thus, the guideline value for e.g. ozone was derived from epidemiological studies of persons exposed to ozone as part of the total mixture of chemicals in polluted ambient air. In addition, the risk estimate for exposure to polycyclic aromatic hydrocarbons was derived from studies on coke-oven workers heavily exposed to benzo[*a*]pyrene as a component of a mixture of PAH and possibly many other chemicals at the work place. Therefore, in some instances the derivation of a tolerable intake for a single compound can be based on studies where the compound was part of a complex chemical mixture.

However, for most compounds the risk assessment has to be based on results form *in vitro* and *in vivo* studies.

The *in vitro* and *in vivo* test methods available to study combined actions and toxicological and biochemical interactions of chemicals in mixtures are essentially the same as those used for the study of single chemicals in order to examine their potential general toxicity and special effects such as mutagenicity, carcinogenicity, reproductive toxicity etc. It is not the intention to describe these methods in any details. The most often-used test methods are briefly mentioned in the later chapters dealing with various distinct toxicological effects areas. Some of them follow international guidelines while other may be specifically designed to explore special effects or mechanisms of action.

In vivo methods most often using experimental animals are the preferred choice for testing of chemicals in relation to the human health risk assessment as they integrate the toxicokinetic and toxicodynamic properties of the chemical. The limitations of *in vivo* studies are mainly due to the high cost of performing them and the limited resources available worldwide.

In vitro studies are very useful for the detection of the potential of chemicals to produce general cytotoxicity and a number of specific toxicological and biochemical effects, such as genotoxicity, cell transformation, embryotoxicity, endocrine toxicity, interaction with enzymes and other specific cell components etc. They are also the preferred choice for the initial study of mechanism of action and play an important role in determining the inherent relative toxicodynamic potencies among similar acting chemicals. *In vitro* studies are also very useful in initial studies on biotransformation pathways of xenobiotics. They also have the advantage that a large number of combinations of chemicals can be assayed within a short time frame and at relatively low costs. Testing of large series of combinations of chemicals is required for the full understanding of combined actions and interactions of chemicals in mixtures. However, care should be taken in using only results from *in vitro* studies in the prediction of effects in humans, as they are not able to incorporate the toxicokinetic behaviour of the compound in the intact mammal.

The integration of chemical and biological information is critical to any assessment of the toxicity of complex mixtures. In practice it is not possible to carry out complete chemical characterisation of a complex mixture. Accordingly, such mixtures are often partitioned in separate fractions for toxicity testing.

One especially successful method of testing complex mixtures is bioassay-directed fractionation followed by chemical identification of active compounds. Until now this method has mainly been used for the testing and identification of genotoxic compounds in environmental mixtures such as air particulates, exhaust condensates and cooked foods. In this approach, each fraction is bioassayed until the major class of specific chemical(s) responsible for the activity can be isolated and chemically characterised, which make a risk assessment of the mixture possible.

The advantage of fractionation includes the separation of active constituents from inactive or otherwise toxic components. Disadvantages include the limited amount of sample available for testing following processing, the likelihood of "spillover" of chemical classes between fractions, and the possible loss or modification of components with fractionation.

Alternatively (or initially) the mixture is treated as a whole and tested in its crude state. The advantage of this strategy includes the relevancy of the tested sample to its environmental counterpart, decreased potential for artefact formation, and inclusion of combined effects of chemicals in the mixture. Moreover if the mixture is representative of others in its class (e.g. diesel emissions from different sources would share certain characteristics), it may be possible to extrapolate results across samples. This method also circumvents the labour-intensive process of individually testing of multiple chemicals. But sometimes a complex mixture is to cytotoxic to be tested directly in a bioassay. Furthermore, it may be incompatible with the test system because of the physical matrix. Other disadvantages include the inability to specify the constituent of the mixture responsible for the toxicity, as well as potential masking effect (e.g. the masking of mutagenicity by cytotoxicity).

3 General concepts in the risk assessment of single chemicals

Prepared by John Chr. Larsen

3.1 Introduction

It is often stated that the current practice of establishing acceptable or tolerable exposure levels of single chemicals do not provide adequate health protection of the population because humans are always exposed to cocktails of chemicals. In order to examine and bring into perspective the potential health risks from exposure to complex mixtures of chemicals in the food, air, water, environment and workplace it is essential to relate the outcome of potential combined actions of chemicals to the current protection levels based on single chemical assessments. Therefore the principles of risk evaluations are briefly outlined below.

There is worldwide agreement that the measures taken to protect human health from exposure to chemicals in food, air, water, and the environment at large should be based on sound scientific assessments and various international organisations have proposed that the risk analysis be divided into three distinct and separate steps:

- Risk assessment,
- Risk evaluation (including risk management)
- Risk communication (to consumers, industry, stakeholders, etc.).

Principles for the risk assessment of new and existing chemicals have been laid down by the European Union in the Commission Directive 93/67/EEC (EC 1993) and the Commission Regulation (EC) 1488/94 (EC 1994) supported by Technical Guidance Documents (EC 1996). The international Programme on Chemical Safety (IPCS) also provided guidance on the assessment of human health risks of chemicals (IPCS 1994). There is also international agreement within the World Trade Organization (WTO) on the principles used in the safety evaluation of food additives, pesticides, veterinary drugs and other chemicals in foods such as food contaminants. These principles were agreed upon at the Uruguay Round and are laid down in "Agreement on the Application of Sanitary and Phytosanitary Measures" (the SPS Agreement). This agreement requires health and safety measures to be based on sound scientific risk assessment and WTO recognises FAO/WHO Codex Alimentarius Commission (CAC or "Codex") standards as a reference point for the safety of foodstuffs traded internationally. These standards are established in the Codex Committee on Food Additives and Contaminants (CCFAC), which uses the Joint FAO/WHO Expert Committee on Food Additives (JECFA) as an advisory committee with regard to the safety evaluation of food additives and contaminants.

3.2 Steps in the risk assessment

In the human risk assessment of chemicals an attempt is made to identify the hazards of the substances and relate them to exposure. According to WHO the risk assessment can be divided into four steps:

- *Hazard identification*. In this step, the toxic effects (hazards) of the chemical are identified, based on reviews of the scientific literature and other information.
- *Hazard characterisation*. For compounds for which a threshold for toxicity is assumed to exist, a no-observed-adverse-effect level (NOAEL) is derived from the most sensitive effect in the most sensitive species (if relevant for humans) or if this is not possible a lowest-observed-adverse-effect level (LOAEL). The NOAEL is the highest concentration or amount of the substance found by experiment or observation that causes no detectable adverse alterations of morphology, functional capacity, growth, development, or life span of the target organism under defined conditions of exposure (IPCS 1987). In most risk assessments the NOAEL is divided by an assessment factor (safety factor, uncertainty factor, adjustment factor, conversion factor, etc.) in order to derive an Acceptable Daily Intake (ADI), Tolerable Daily intake (TDI), Human Limit Value (HLV), Reference Dose (RfD), Acute Reference Dose (ARfD) or others.
- New approaches are emerging which by use of statistical and probabilistic techniques try to determine the "true" no-adverse-effect level ($NAEL_{true}$) instead of directly using the NOAEL, which is an imprecise estimate. One such alternative is the Benchmark Dose approach, which has the advantage of using all available (and relevant) dose response data. The Benchmark Dose concept can be extended to obtain an uncertainty distribution of the Critical Effect Dose (CED) using a probabilistic approach. In addition, it has also been suggested to use a probabilistic approach to combine the CED distribution with the estimated uncertainty distributions for assessment factors in order to derive HLV, ADI, TDI, etc. as a distribution in stead of a point estimate. For a discussion of these new concepts see Vermeire *et al.* (1999). One major problem in using these new approaches is that they require more data on dose response relationships, than are normally produced in standard toxicological investigations.
- For compounds where a threshold for the effect cannot be assumed, such as genotoxic carcinogens, most risk assessments would use probabilistic estimates of the risk. The use of probabilistic models, such as the linearized multistage model, assumes cancer as a non-threshold effect. The US EPA uses a descriptor that addresses upper bound risk, the Risk Specific Dose (RsD). In establishing a RsD for cancer it is attempted to describe the lowest possible dose, which could be interpreted to result in a specific risk e.g. one in one million. Or, as it is more commonly stated, the upper bound risk that the RsD could be projected to cause. The actual dose needed to generate a one in one million risk, for instance, could be much higher; conversely, the actual risk from the RsD could be much lower and, in fact, could even be zero. The use of the one in one million probability of cancer is an arbitrary convention which, when used consistently across chemicals, allows for the comparison of relative cancer potency.
- *Exposure assessment*. This assessment should take into account all foreseeable exposures by relevant routes (oral, dermal, inhalation). It should also relate to exposure pattern and different populations groups (e.g. workeers, consumers, man and the environment) potentially exposed that may differ in their sensitivity to the compound/effects in question.
- *Risk characterisation.* In this step the exposure is compared to the ADI, TDI, RfD, ARfD etc. If the exposure is less than ex. the ADI, it is by definition assumed that there is no appreciable risk. However, according to the EU Technical Guidance Documents the human risk to industrial chemicals is characterized by comparing the estimated or measured human exposure (air, water, skin, diet, inhalation, dermal, oral) to the results of the effect assessment, i.e. a NOAEL. If If the exposure estimate is higher or equal to

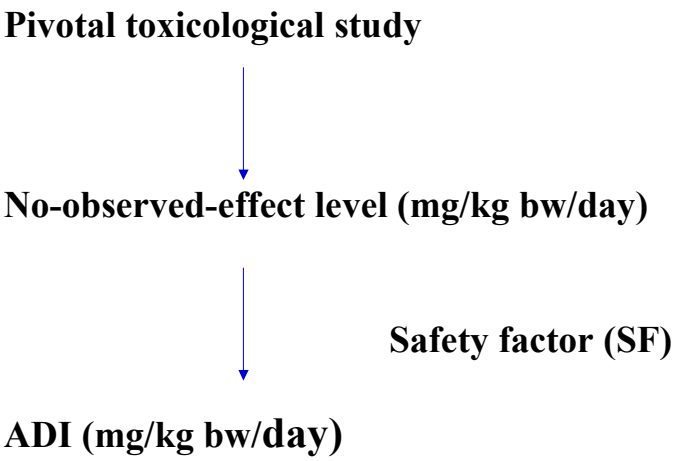
NOAEL, this indicates that the substance is of “concern” with regard to the exposed population. Where the exposure estimate is less than the NOAEL, the risk assessor should determine whether the magnitude by which the NOAEL exceeds the estimated exposure (i.e. the “margin of safety”) is of concern. This evaluation requires expert judgement to weigh the parameters of relevance for the margin of safety (e.g. intra-/interspecies variation, nature and severity of the effect, dose/response relationship, differences in exposure (route, duration, frequency and pattern)). In practise, lower “margin of safety” are normally seen for workers compared to those for consumers or humans indirectly exposed via the environment.

In order to describe to use of uncertainty factors (safety factors) the principles behind the derivation and use of the ADI are outlined as an example.

3.3 The Acceptable Daily Intake (ADI)

Food additives, veterinary residues and pesticide residues in foods are regulated on the basis of the Acceptable Daily Intake (ADI). The regulation should ensure that the amounts of a given chemical permitted in various foods would not result in that the consumer has a higher daily intake than the ADI. The ADI concept was originally developed in JECFA and defined as "an estimate of the amount of a food additive, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk" (IPCS 1987). The ADI is derived from studies in humans, experimental animals and *in vitro*. Studies of acute, sub-acute (28-90 days) and chronic toxicity, carcinogenicity, reproductive toxicity/teratogenicity are mandatory as well as studies on metabolism and kinetics and short-term *in vitro* studies of mutagenicity/clastogenicity. Special studies on mechanism of actions are normally also being required. The “no observed (adverse) effect level” (NO(A)EL) is determined from the most sensitive study in the most sensitive species tested. The ADI is established from the NOEL by dividing it with a safety (uncertainty) factor (Figure 3.1.1). When the database is considered adequate a factor of 100 is used by default, but may be modified when adequate human data are available. When the database is not optimal, but there are no indications of any health problems from anticipated use, a temporary ADI may be established using a larger safety factor (200 by default, but factors >1000 have been used).

Figure 3.1.1. Derivation of the ADI



The safety factor is used to extrapolate from a group of test animals to an average human and from average humans to potentially sensitive human sub-populations. The default 100-fold factor was historically arbitrary chosen in order to cover several areas of uncertainty (Lehman and Fitzhugh 1954):

- Intra (human) species variability
- Inter (animal to human) species variability
- Allowance for sensitive human populations due to illness when compared with healthy experimental animals
- Possible synergistic action of the many intentional and unintentional food additives or contaminants

Later the safety factor has been rationalised by many to comprise a factor of 10 to allow for differences between test animals and humans (inter-species differences) and a factor of 10 to allow for human variability (inter-individual differences) (IPCS 1987).

For contaminants in food and the environment the closely similar Tolerable Daily intake (TDI) is used in most countries. The US EPA in 1988, with several modifications, also adopted the ADI approach in regulatory measures against environmental pollution (Vermeire *et al.* 1999). Instead of the term ADI and safety factor the US EPA uses the terms Reference Dose (RfD) and uncertainty factor (UF). The RfD is derived from the NOAEL by generally using one order-of-magnitude UFs:

- A 10-fold factor to account for human variation in sensitivity
- A 10-fold factor to account for uncertainty in interspecies extrapolation
- A 10-fold factor to adjust for the use of a NOAEL obtained from a subchronic study rather than a chronic study
- A 10-fold factor to adjust for the use of a LOAEL in the absence of a NOAEL
- A 10-fold factor that considers the adequacy of the database

In the early nineties Renwick (1991, 1993) proposed a scheme to further subdivide the two components of the safety factor related to inter- and intra-species extrapolation. Each factor of 10 was subdivided in order to allow for the two areas

of uncertainty i.e. differences in *toxicokinetics* (aspects such as absorption, distribution, biotransformation, and elimination which determine delivery of the chemical to its site of action/toxicity) and *toxicodynamics* (aspects such as target organ sensitivity, cytoprotective mechanisms and homeostatic control which determines the extent of any effect or response due to the presence of the chemical). The default values of the four individual factors were modified by the International Programme on Chemical Safety (IPCS) in 1994 (IPCS 1994) (Table 3.1.1). These default values may be further modified when appropriate data exist on the toxicokinetic and/or toxicodynamic properties of the compound in experimental animals and humans. Although there are still only few examples where the data on a compound are of such quality as to allow for replacement of the default values it is anticipated that this approach will gain widespread use in the future.

In connection with the elaboration of health based quality criteria for chemical pollutants in air, soil and drinking water the Danish EPA establish TDI –values using three safety factors. The safety factors account for:

- SF_1: Interspecies extrapolation (from animal to human). This factor is historically set at 10 as a default value.
- SF_2: Variation in susceptibility among humans, i.e. to account for possible increased susceptibility in the pregnant, children, elderly or sick people. This factor is often set at 10.
- SF_3: The adequacy, quality and relevance of the data set. To account for lack of a NOAEL value, lack of long-term testing, lack of data on specific end point, etc. This factor is set at a value from 1 to 100 depending on a concrete evaluation.

When using this approach in connection with chemical pollutants the overall safety factor (SF_1 x SF_2 x SF x SF_3) most often has been set in the range of 100-1000.

Table 3.1.1. Subdivision of the uncertainty factor. Default values (IPCS 1994).

Uncertainty	**Default UF**	**Default UF**
Interspecies variation	10	
Toxicokinetics		4.0
Toxicodynamics		2.5
Interindividual (human) variation	10	
Toxicokinetic		3.2
Toxicodynamic		3.2
Total	**100**	**100**

4 Approaches used in the assessment and regulation of chemical mixtures

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4.1 Introduction

Various approaches have been suggested in the scientific literature for use in the evaluation of the health risks from exposure to mixtures of chemicals. These are briefly discussed in this chapter. Most attention and effort has been devoted in the literature to procedures to assess cumulative effects of exposure to chemicals that act by a similar mechanism of action. However, the Dutch group around Victor Feron (Groten *et al.* 2001) as well as ATSDR (2002) have suggested more general approaches that cover also chemicals that differ in their modes of action (see sections 4.4 and 4.5).

Following this discussion an overview is given on approaches so far used in Denmark in the regulation of certain types of chemical mixtures (section 4.6).

4.2 Procedures used to assess cumulative effects of chemicals that act by a common mechanism of action

A number of approaches have been suggested to combine the exposure to chemicals that act by a similar mechanism of action but have different potencies and exposure characteristics (US EPA 1999, 2000). Wilkinson *et al.* (2000) have critically evaluated these approaches due to the renewed interest triggered by the US Food Quality Protection Act (FQPA) that requires the US EPA to consider the cumulative effects of pesticides and other substances that have a "common mechanism of toxicity". The approaches discussed are the hazard index (HI), toxicity equivalency factor (TEF), and combined margin of exposure (MOE) procedures and the point of departure index (PODI) and cumulative risk index (CRI) methods.

An ILSI Working Group (Mileson *et al.* 1998) has also recently addressed the "common mechanism" issue. The ILSI Working Group concluded that a common mechanism might exist if two compounds:

- Cause the same critical effect
- Act on the same molecular target at the same target tissue, and
- Act by the same pharmacological mechanism of action and may share a common toxic intermediate

It should be realised that with the exception of a few groups of chemicals, such as some organophosphorous and carbamate pesticides and some polychlorinated dibenzo(p)dioxins, – dibenzofurans and - biphenyls, precise mechanistic information on their toxic effects are scarce. In realising that the exact molecular mechanism is not known for most chemicals the term "mode of action" is used to

describe toxicities that appears to be similar albeit the mechanism is not known in details. For several groups of endocrine disrupters this terminology seems appropriate.

Another critical issue is the question of concurrent exposure. This refers to co-exposure to more than one chemical able to interact with a defined target in a specific target tissue during a particular time frame of interest (Wilkinson *et al.* 2000). It is important to distinguish between concurrent or simultaneously "external" exposure, referring to the timing of oral, dermal or inhalation exposure, from concurrent "internal" exposure that relates to the dose actually attained at a given biological target in a given time frame. For the risk assessment it is the "internal" exposure that is of toxicological significance, however, it is seldom known. The factors that determine whether a cumulative effect is likely from exposure to several different common mechanism compounds are the timing and duration of external exposure, the persistence (biological half-life) of the chemicals in the body, and the duration of the effect.

The effect of any chemical at a biological target depends of its ability to attain a target site concentration that exceeds the threshold required to elicit the response. The intensity and duration of the response depends on the toxicokinetic properties of the compound (absorption, distribution, metabolism and excretion) and the nature of the target site interaction (reversible, irreversible). If recovery is complete between successive exposures no cumulative toxicity is to be expected. However, a short-term acute exposure could potentially add to the long-term burden of a persistent chemical and be relevant for the magnitude of the chronic effect.

For acute and short-term exposures difference in the toxicokinetic properties, which will result in different times to maximum effect for the individual compounds, are critical in determining concurrency at the target site. Therefore, exposure intervals and the sequence of exposures to different chemicals may have significant impact on the potential cumulative effect.

The risk assessment of exposure to mixtures of defined chemicals should make optimal use of the toxicological databases. Ideally, in the opinion of Wilkinson *et al.* (2000) the point of departure (POD) for the assessment should be a dose associated with a particular biological response (ED10, ED20) since this takes into account all of the dose-response data available. A POD based on doses causing a particular response should always take preference over the NOAEL. This is because the NOAEL is a single point value and not a measure of a biological response and is largely an artefact of experimental design. The POD should also ideally be based on studies with the same animal species using the same route of administration. However, the data available for most chemicals will not permit an estimation of for instance ED10 and relative potencies may have to be based on NOAELs as PODs.

In describing the various procedures proposed to evaluate the risk associated with combined exposure to a group of chemicals with a common mechanism of action Wilkinson *et al.* (2000) emphasise that the biggest problem associated with all methods of cumulative risk assessment is how to accommodate the different uncertainty factors (UF) that are applied to derive regulatory values such as ADIs or RfDs. If the uncertainty factors applied are the same for all the chemicals, all the methods will give the same result. However, this is most often not the case and the different uncertainty factors applied to the various chemicals will dominate the result of the risk assessment. In order to illustrate this, exposure to a hypothetical group of four common mechanism chemicals, differing in potency by 100-fold and having exposures ranging from 0.01 to 0.5 mg/kg bw/day, was assessed assuming

they had either the same UF of 100 (Table 4.2.1a) or UF ranging from 10 to 1000 (Table 4.2.1b).

Table 4.2.1. Hypothetical example for cumulative risk assessment (adapted from Wilkinson et al (2000))

Compound	**ED_{10} (mg/kg/d)**	**Uncertainty factor (UF)**	**RfD (mg/kg/d)**	**Exposure (mg/kg/d)**
a. Chemicals with the same UF				
I	100	100	1	0.5
II	500	100	5	0.5
III	25	100	0.25	0.01
IV	5	100	0.05	0.01
b. Chemicals with different UF				
I	100	10	10	0.5
II	500	100	5	0.5
III	25	1000	0.025	0.01
IV	5	100	0.05	0.01

4.2.1 Hazard index (HI)

The hazard index is the sum of the hazard quotients (HQ) of the individual chemicals, i.e. the sum of exposure to each chemical expressed as a fraction of its RfD/ADI/TDI. The HI should not exceed 1 since this indicates that the FQPA (US Food Quality Protection Act) "risk cup", a kind of combined RfD for the common mechanism group, is full.

$HI = HQ_I + HQ_{II} + HQ_{III} + HQ_{IV}$
or
$HI = Exp_I/RfD_1 + Exp_{II}/RfD_{II} + Exp_{III}/RfD_{III} + Exp_{IV}/RfD_{IV}$

Although the HI method is transparent, easily understandable and directly relates to the RfD, the major disadvantage is that the RfD is not an appropriate metric to use as a POD for cumulative risk assessment, since the RfD is normally derived by using NOAELs and uncertainty factors, which are not data based, but may incorporate significant policy-driven assumptions.

Use of the information in Table 4.2.1a where the UF values for each compound is the same gives the following result:

HI = 0.5 + 0.1 +0.04 + 0.2 = 0.84 Risk units

Whereas use of the information in Table 4.2.1b where the UF values differ gives:

HI = 0.05 + 0.1 + 0.4 + 0.2 = 0.75 Risk units

Although the overall HI is quite similar, this example illustrates that the contribution of each chemical is highly dependent on the UF. Moreover, the method do not reflects that the components of the mixture do not all have the same critical effect.

The hazard index method has been refined by the introduction of the target-organ toxicity dose (TTD) method. This method suggests that separate hazard indexes

should be estimated for all endpoints of concern. This implies that a TTD should be established for all relevant endpoints for each chemical using the same principles as used in the "normal" derivation of the RfD/ADI/TDI and that "hazard quotients" be calculated for the relevant effects of each chemical (for details see ATSDR 2002).

4.2.1.1 Weight-of-Evidence (WOE) modification to the hazard index

The hazard index method does not incorporate information on interactions among components of the mixture (ATSDR 2002). Mumtaz and Durkin (1992) proposed a weight-of-evidence (WOE) method to systematically address this need. The method was designed to modify the hazard index to account for interactions, using the weight of evidence for interactions among pairs of mixture components. Thus, the basic assumption is that pair wise interactions will dominate in the mixture and adequately represent all the interactions. For example, if chemicals A and B interact in a certain way, the presence of chemical C will not cause the interaction to be substantially different.

It should be noted that experience with the method has revealed that it is mainly useful for a qualitative prediction as to whether the hazard may be greater or less than indicated by the hazard index (ATSDR 2002).

The method evaluates the data relevant to joint actions for each possible pair of chemicals in the mixture in order to make qualitative binary weight-of-evidence (BINWOE) determinations for the effect of each chemical on the toxicity of every other chemical. Two BINWOEs are needed for each pair: one for the effect of chemical A on the toxicity of chemical B, and another for the effect of chemical B on the toxicity of chemical A. The BINWOE determination indicates the expected direction of the interaction, such as greater than additive, less than additive, additive, or intermediate. It scores the data qualitatively by using an alphanumeric scheme that takes into account mechanistic understanding, toxicological significance, and relevance of the exposure duration, sequence, bioassay, and route of exposure. The alphanumeric terms are finally converted into a single numeric score. The BINWOE evaluations should be target organ specific. A more detailed description and discussion has been provided by ATSDR (2002).

4.2.2 Point of Departure Index (PODI)

A scientifically more appropriate method of addition is summing the exposures of each compound expressed as a fraction of their respective PODs instead of the ADI or RfDs. These POD fractions (PODF) are reciprocals of the individual margin of exposures (MOE) of each compound. This approach sums the exposures to the compounds in terms of their relative potencies. In this example the ED_{10} (Table 4.2.1) are used as PODs:

PODI = 0.005 + 0.001 + 0.0004 + 0.002 = 0.0084 Risk units

The PODI can be converted into a "risk cup" unit by multiplying by an appropriate group UF. For example, a group UF of 100 would result in a combined risk of 0.84 risk units.

4.2.3 Toxicity equivalency factors (TEF)

The TEF approach normalises exposures to common mechanism chemicals with different potencies to yield a total equivalent exposure (TEQ) to one of the chemicals, the "index compound". TEFs are derived as the ratio of the POD of the index compound to that of each member in the group. The exposure to each

chemical is then multiplied by the respective TEF value to express exposure in terms of the index compound. Summation of these values result in the total combined exposure (TEQ) expressed in terms of the index compound.

This approach was initially developed to estimate the potential toxicity of mixtures of polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and dioxin-like biphenyls (PCBs). Over the years a number of different TEF systems for PCDDs, PCDFs and PCBs have been used. Recently, a system was internationally agreed upon at a WHO Consultation in 1997 (WHO-TEF) as published by Van den Berg *et al.* (1998) (Table 4.2.3.1).

WHO only assigned TEFs for compounds that:

- Show a structural relationship to the PCDDs and PCDFs
- Bind to the aryl hydrocarbon (Ah) receptor
- Elicit Ah receptor-mediated biochemical and toxic responses
- Are persistent and accumulate in the food chain.

A TEF for a compound is determined as the toxicity of the compound relative to the toxicity of the index compound 2,3,7,8-TCDD based on available *in vitro* and *in vivo* data (Van den Berg *et al.* 1998). In contrast to previous evaluations, WHO besides establishing TEFs for humans/mammals also provided TEFs for fish and birds. For humans/mammals the differences between the new WHO system and previous systems were that 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD) was considered as toxic as 2,3,7,8-TCDD and assigned a TEF of 1, that octachlorodibenzo-*p*-dioxins (OCDD) and octachlorodibenfuran (OCDF) were assigned a TEF value 10 times smaller than previously, and that dioxin-like PCBs were included in the scheme (Van den Berg *et al.* 1998; SCOOP 2000).

The majority of studies assessing the combined effects of PCDD, PCDF and dioxin-like PCB congeners in complex mixtures have supported the hypothesis that the toxic effects of combinations of congeners follow dose additivity. Therefore, the concentrations and TEFs of individual congeners in a mixture may be converted into a toxic equivalent (TEQ) concentration by multiplying the analytically determined amounts of each congener by the corresponding TEF and summing the contribution from each congener using the following equation:

$$TEQ = \sum (PCDD_i \times TEF_i) + \sum (PCDF_i \times TEF_i) + \sum (PCB_i \times TEF_i)$$

(Van den Berg *et al.* 1998, WHO 2000)

TABLE 4.2.3.1. Toxicity equivalency factors (TEFs) for dioxins and dioxin-like PCBs (van den berg *et al.* 1998).

PCDDs and PCDFs	**Toxic Equivalency Factor (TEF)** WHO-TEF (van den Berg *et al.*, 1998)
2,3,7,8-TCDD	1
1,2,3,7,8-PnCDD	1
1,2,3,4,7,8-HxCDD	0.1
1,2,3,6,7,8-HxCDD	0.1
1,2,3,7,8,9-HxCDD	0.1
1,2,3,4,6,7,8-HpCDD	0.01
OCDD	0.0001
2,3,7,8-TCDF	0.1
1,2,3,7,8-PnCDF	0.05
2,3,4,7,8-PnCDF	0.5
1,2,3,4,7,8-HxCDF	0.1

1,2,3,6,7,8-HxCDF	0.1
1,2,3,7,8,9-HxCDF	0.1
2,3,4,6,7,8-HxCDF	0.1
1,2,3,4,6,7,8-HpCDF	0.01
1,2,3,4,7,8,9-HpCDF	0.01
OCDF	0.0001

PCBs (IUPAC number)	**Toxic Equivalency Factor (TEF)**
	WHO-TEF (van den Berg *et al.*, 1998)
Non-*ortho PCBs*	
3,3',4,4'-TCB (77)	0.0001
3,4,4',5-TCB (81)	0.0001
3,3',4,4',5-PnCB (126)	0.1
3,3',4,4',5,5'-HxCB (169)	0.01
Mono-*ortho PCBs*	
2,3,3',4,4'-PnCB (105)	0.0001
2,3,4,4',5-PnCB (114)	0.0005
2,3',4,4',5-PnCB (118)	0.0001
2,3,4,4'5-PnCB (123)	0.0001
2,3,3',4,4',5-HxCB (156)	0.0005
2,3,3',4,4',5'-HxCB (157)	0.0005
2,3',4,4',5,5'-HxCB (167)	0.00001
2,3,3',4,4',5,5'-HpCB (189)	0.0001

Abbreviations: PnCDD, pentachlorodibenzo-*p*-dioxin; HxCDD, hexachlorodibenzo-*p*-dioxin; HpCDD, heptachlorodibenzo-*p*-dioxin; OCDD, octachlorodibenzo-*p*-dioxin; PnCDF, pentachlorodibenzofuran; HxCDF, hexachlorodibenzofuran; HpCDF, heptachlorodibenzofuran; OCDF, octachlorodibenzofuran; TCB, tetrachlorobiphenyl; PnCB, pentachlorobiphenyl; HxCB, hexachlorobiphenyl; HpCB, heptachlorobiphenyl.

TEFs were also used by the NRC Committee on Pesticides in the Diet of Infants and Children to estimate the aggregate risk to children from dietary exposure to a mixture of pesticides (NRC 1993). The Committee examined five organophosphate pesticides, acephate, chlorpyrifos, dimethoate, disulfoton, and ethion, which all are cholinesterase inhibitors and may be present as residues on fruits and vegetables. Chlorpyrifos was used as the index compound. The TEF was defined as the ratio of the NOAEL or LOAEL for each pesticide to the NOAEL or LOAEL for chlorpyrifos. TEFs based on LOAELs were used as no NOAEL could be established for two of the compounds. Based on US FDA residue data on the five pesticides total chlorpyrifos equivalents concentrations were estimated for each food item included.

The UK Pesticide Safety Directorate (PSD) has decided to use the TEF approach for assessment of combined risk from exposure to mixtures of acetyl cholinesterase inhibitors (organo-phosphorous (OP) compounds and carbamates) (PSD 1999). Despite clear differences in the action of carbamates and OP compounds, the index compounds selected for all acetyl cholinesterase inhibitors were either aldicarb (carbamate) or chlorpyrifos (OP). The POD for determining relative potency was predetermined as the dose level that produced 20% inhibition of red blood cell cholinesterase in a 90-day dietary study in rats.

The use of the method for risk assessment of chemical mixtures has recently been discussed by EPA (EPA 1999, 2000).

Wilkinson *et al.* (2000) used the information in Table 4.2.1, choosed compound IV as the index compound (TEF = 1), assigned TEF values to compounds I (0.05), II (0.01), and III (0.2) and calculated the total compound IV equivalent exposure

(TEQ) to 0.042 mg/kg bw/day. When this TEQ was compared to the RfD of compound IV (0.05 mg/kg bw/day) a value of 0.84 was obtained, representing a kind of combined hazard quotient that indicates that 84% of the risk cup was filled. This risk estimate will be the same regardless which compound is selected as the index compound, provided that the UF for each member in the group is the same (Table 4.2.1a).

There are no specific guidance criteria available for the selection of the index compound. EPA (1986) has suggested that the index compound should be the member of the group that is the best studied and has the largest body of scientific data of acceptable quality. This will be associated with a low UF and lead to the lowest combined risk. However, this has been criticised for using data on well-studied compound to improve the acceptability of compounds that have poor toxicological databases. By using the information in Table 4.2.1b Wilkinson *et al.* (2000) illustrated that if the UF for each compound is different, the selection of the index compound is critical. If compound 3 (UF of 1000) was selected as index compound the TEQ exposure would be 0.21 and the combined risk estimate 8.4-fold higher than considered acceptable.

4.2.4 Margin of exposure (MOE)

The MOE is the ratio of the POD (e.g. NOAEL, ED_{10}) to the level of exposure.

$$MOE = \frac{ED_{10}}{Exposure}$$

The MOE approach is often used to determine the acceptability of acute risks for single chemicals and MOEs of >100 or >10 are usually considered acceptable when derived from toxicological data from animal and human studies, respectively. The US EPA favours this concept for performing aggregate and cumulative risk assessments (Whalan and Pettigrew, 1997). The combined MOE (MOE_T) is the reciprocal of the MOEs of each compound in the mixture.

$$MOE_T = \frac{1}{(1/MOE_1) + (1/MOE_2) + MOE_3) + MOE_4}$$

Using the hypothetical data in Table 4.2.1:

$$MOE_T = \frac{1}{0.005 + 0.001 + 0.0004 + 0.002} = 119$$

There are no established criteria to define the magnitude of an acceptable MOE_T for exposure to mixture of chemicals. If the compounds act through a common mechanism of toxicity then a MOE_T of 100 may by intuition be considered acceptable as this value is considered acceptable for single compounds. However, as the number of compounds in the mixture increases the MOE_Ts decreases and combinations of two, three and four compounds, each having acceptable MOEs of 100, will yield MOE_Ts of 75, 33, and 25, respectively. In such cases, to obtain a MOE_T of > 100 for mixtures containing two, three, or four compounds, the individual MOEs have to be greater than 200, 300, and 400, respectively. Alternatively, the exposure level to each compound should be reduced by 2, 3, or 4 times, respectively. In the example, the MOE_T of 100 results from summation of compounds that have MOEs of 200 (I), 1000 (II), 2500 (III), and 500 (IV). This

shows the pronounced influence of the compound (I) that has the lowest MOE. In particular, the $MOE_T > 100$ approach seems inappropriate when the individual MOEs originate from data (NOAELs, ED_{10}s) that would relate to application of different UF (, e.g. data from animals and humans). Therefore, a stepwise reduction in the magnitude of the acceptable MOE_T has to be considered as the size of the group increases.

4.2.5 Cumulative risk index (CRI)

The CRI (also referred to as aggregate risk index (ARI)) has been suggested by US EPA (Whalan and Pettigrew 1997) to combine MOEs for chemicals with different UF. The risk index (RI) of a chemical is the MOE divided by the UF or simply the reference dose divided by exposure and is the reciprocal of the hazard quotient (HQ).

$$RI = \frac{POD}{Exposure \times UF} = \frac{RfD}{Exposure} = \frac{1}{HQ}$$

The CRI is thus defined as:

$$CRI = \frac{1}{1/RI_I + 1/RI_{II} + 1/RI_{III} + 1/RI_{IV}}$$

$$\text{or} \quad = \frac{1}{Exp_I/RfD_{II} + Exp_{II}/RfD_{II} + Exp_{III}/RfD_{III} + Exp_{IV}/RfD_{IV}}$$

The CRI has the same disadvantages as described for the Hazard index (HI) and in addition, since it is derived from the MOE approach, the CRI is not as transparent and understandable as the HI. It also involves more complex calculations.

4.3 Use of response addition (simple dissimilar action) in the risk assessment of mixtures of polycyclic aromatic hydrocarbons (PAH).

The application of the concept of response addition has been suggested by the US EPA (1986) to determine the cancer risk from mixtures containing carcinogenic compounds. The assumption was that such compounds show simple dissimilar action with a complete negative correlation of tolerance. However, as pointed out by Könemann and Pieters (1996) for compounds with presumed linear dose-response curves, such as genotoxic and carcinogenic compounds for which it is assumed that a no-effect-level does not exist and for which the mechanism of action may be regarded as similar, response addition and dose addition will provide identical results. Therefore, various authors have used different terminology in the assessment of PAH, relative response factors, relative potency factors or toxic equivalency factors (TEF).

A number of PAH as well as coal-tar and some occupational exposures to combustion emissions containing these compounds have shown carcinogenicity in experimental animals and genotoxicity and mutagenicity *in vitro* and *in vivo* (IPCS, 1998). Several attempts have been made to derive relative potency factors, often expressed as toxic equivalency factors (TEF) for individual PAH (relative to benzo[*a*]pyrene, the best studied PAH) with the purpose of summarising the

contributions from individual PAH in a mixture into a total benzo[*a*]pyrene equivalent dose, assuming additivity in their carcinogenic effects (Nisbeth and Lagoy 1992; Rugen *et al.* 1989; Thorslund and Farrar 1990; Krewski 1989; Larsen and Larsen, 1998). Because there is a total lack of adequate data from oral carcinogenicity studies on PAH others than benzo[*a*]pyrene, TEF values for PAH in food have been suggested based on studies using skin application, pulmonary instillation and subcutaneous or intraperitoneal injections.

There are several problems in using the TEF approach in the risk assessment of PAH in food. The use of the TEF approach requires that the compounds in question exert the toxicological effect by the same mechanism of action, such as is the case for the polychlorinated dibenzo-p-dioxins and – dibenzofurans, which act through binding to the Ah-receptor. Although a number of PAH bind to the Ah receptor, this effect is not the only effect that determines the carcinogenic potency of PAH. DNA binding and induction of mutations are other significant effects in the carcinogenesis of PAH, and there is no indication that different PAH are activated via the same metabolic route, binds DNA in the same positions, and induce the same types of mutations in the same organs or tissues. In fact, the study by Culp *et al.* (1998) showed that a coal-tar mixture of PAH also produced tumours in other tissues and organs than those affected by benzo[*a*]pyrene alone, and that the additional PAH in the mixture did not significantly contribute to the incidence of stomach tumours observed after benz[*a*]pyrene alone.

Furthermore, studies on mixtures of individual PAH have shown that they may interact metabolically in a number of ways resulting in not only additive but also synergistic and/or antagonistic effects (Montizaan *et al.*, 1989).

The limitations in using the TEF approach for the assessment of PAH carcinogenicity following oral administration was illustrated when it was used on the carcinogenicity data and the analytical data on the PAH composition in the coal tars used in the study by Culp *et al.* (1998). When the TEF values derived by Larsen and Larsen (1998) (Table 4.3.1) were used the carcinogenic potency of both coal tar mixtures was predicted to be only approximately 1.5 times that of the benzo[*a*]pyrene content. However, the observed potencies of the coal tar mixtures were up to 5 times that accounted for by the benzo[*a*]pyrene content. In this case, the use of the TEF approach for PAH carcinogenicity would underestimate it.

Schneider *et al.* (2002) also examined the use of the TEF approach on the data from the Culp *et al.* (1998) study and from several other studies using dermal or lung application of PAH mixtures of known composition. They used the TEF derived by Brown and Mittelsman (1993) (Table 4.3.1) and concluded that the benzo[*a*]pyrene equivalency factors do not adequately describe the potency of PAH mixtures and lead to underestimation of the carcinogenic potencies in most cases.

Table 4.3.1 Estimates of carcinogenic potencies of various PAH, relative to benzo[*a*]pyrene (BaP)

Compound	Studies using rat lung installation		Studies using mouse skin painting			Combined estimates from different types of studies				
	Calc. A)	Publ. B)	Calc. C)	Calc. D)	Publ. E)	Publ. F)	Publ. G)	Publ. H)	Publ. I)	Publ. J)
Anthracene				<0.0046		0.32		0.01	0.0005	0.01
Fluorene								0.001	0.0005	0
Phenanthrene	0.0004							0.001	0.0005	0
Benz[*a*]anthracene					0.0039-0.0055	0.145			0.005	0.1
Chrysene	0.030			0.013		0.0044	0.0044	0.01	0.03	0.01
Cyclopenteno[*cd*]pyrene			0.0084				0.023		0.02	
Fluoranthene				<0.105				0.001	0.05	0.01
Pyrene				<0.0046		0.081	0.081	0.001	0.001	0
Benzo[*b*]fluoranthene	0.089	0.123	0.18	0.037	0.023	0.140	0.140	0.1	0.1	1
Benzo[*j*]fluoranthene	0.053	0.052	0.022	0.040	0.075		0.061		0.05	0.1
Benzo[*k*]fluoranthene	0.052	0.053	$4x10^{-8}$	0.0004		0.066	0.066	0.1	0.05	0.1
Benzo[*ghi*]fluoranthene	0.012	0.021				0.022			0.01	0.01
Benzo[*a*]pyrene	1	1	1	1	1	1	1	1	1	1
Benzo[*e*]pyrene	0.0019	0.007		0.0039			0.004		0.002	
Dibenz[*a,h*]anthracene	1.23			0.65	0.59	1.1	1.1		1.1	1
Anthanthrene	0.340	0.316					0.320		0.3	
Benzo[*ghi*]perylene							0.022	0.01	0.02	0.01
Dibenzo[*a,e*]pyrene				0.221					0.2	0.1
Dibenzo[*a,h*]pyrene				0.843					1	1
Dibenzo[*a,i*]pyrene				0.082					0.1	1
Dibenzo[*a,l*]pyrene				1.27					1	1
Indeno[1,2,3-*cd*]pyrene	0.102	0.278	$4x10^{-8}$	0.035	0.0059	0.232	0.234	0.1	0.1	0.1
Coronene			0.007						0.01	

A) Calculated by Nielsen *et al.*, 1995; B) Thorslund and Farrar, 1990; C) and D) Calculated by Nielsen *et al.*, 1995; E) Rugen *et al.*, 1989; F) Clement, 1988; G) Krewski *et al.*, 1989; H) Nisbet and Lagoy, 1992; I) Larsen and Larsen, 1998; J) Brown and Mittelsman 1993 (US EPA OPPTS).

4.4 Approach to assess simple and complex mictures suggested by the Dutch group

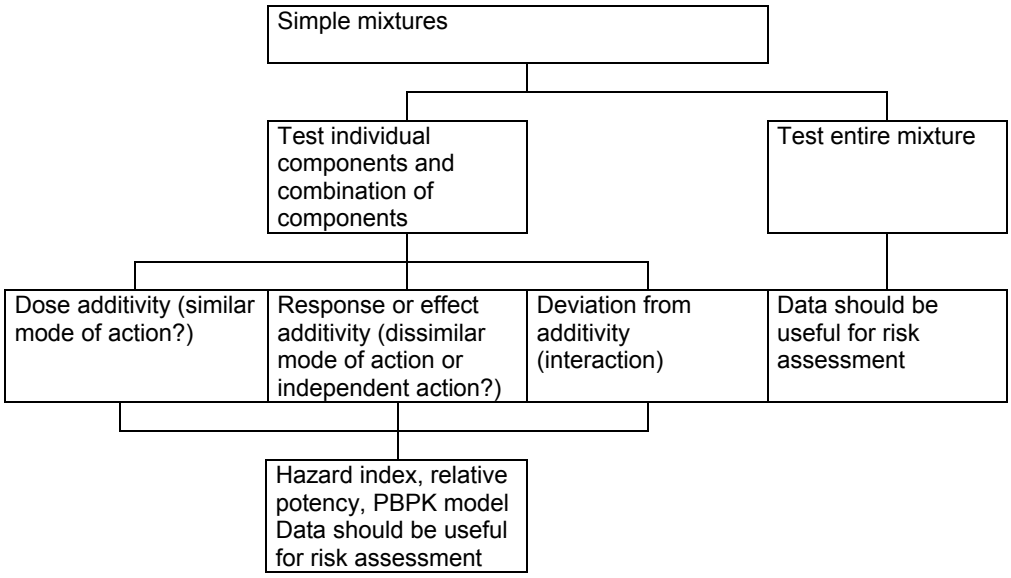
The Dutch group around Feron group initiated their research programme in order to test the hypothesis that exposure to chemicals at (low) non-toxic doses of the individual chemicals, as a rule would be of no health concern. One reason being that most guidelines from national and international organisations often suggest the use of simple “dose addition” or “response addition” models for the assessment of chemical mixtures totally ignoring any knowledge on the mode of action of the chemicals. Clearly, such an approach would greatly overestimate the risk in case of chemicals that act by mechanisms where the additivity assumptions are invalid. The group clearly recognises that for mixtures of compounds known to act by the same mechanism, and therefore not will show interactions, a cumulative approach is the valid choice using dose or response addition.

They consider it important to distinguish between simple and complex mixtures. According to Feron *et al.* (1998) a simple mixture consists of a relatively small number of chemicals (e.g. 10 or less) and the composition of the mixture is known, both qualitatively and quantitatively. An example would be a cocktail of pesticide residues in food. A complex mixture comprises tens, hundreds or thousands of chemicals, and the qualitative and quantitative composition is not fully known. They also emphasise to distinguish between whole-mixture analysis (top-down approach) and component-interaction analysis (bottom-up approach), the latter requiring an understanding of the basic concepts of combined action of chemicals.

4.4.1 Simple mixtures

A general scheme for the safety evaluation of simple mixtures has been proposed (Groten *et al.*, 2001). The most pragmatic and perhaps simplest approach is to test the toxicity of the mixture without identifying the type of interactions between the individual components. However, the results of the testing can only be used for hazard characterisation following exposure to that particular mixture. A more detailed approach is to assess the combined action of the individual components in the mixture. Several experimental designs can be used, primarily depending on the complexity and number of compounds in the mixture. The major concern in the analysis of the data is whether the components act via similar toxicological processes, by the same mode of action or their modes of action are functionally independent (Figure 4.4.1.1).

Figure 4.4.1.1. Scheme for safety evaluation of simple mixtures



4.4.2 Complex mixtures

As regards complex mixtures the Feron group initially recommended a two-step approach: First to identify the "n" (e.g. 10) most risky chemicals in the mixture, and then to perform hazard identification and risk assessment of the defined mixture of the (10) priority chemicals using procedures appropriate for simple, defined mixtures (Feron *et al.* 1995a, 1995b, Cassee *et al.* 1998, Feron *et al.* 1998):

Figure 4.4.2.1. Two-step procedure for the safety evaluation of complex mixtures (Feron *et al.* 1995b)

Step 1: Identification of priority chemicals

Select a limited number of chemicals (e.g. ten) with the highest risk potential, using the risk quotient (RQ) $RQ = \frac{\text{Level of exposure}}{\text{Level of toxicity}}$ In other words, identify the "top ten" chemicals

Step 2: Hazard characterisation and risk assessment

Identify the hazard and assess the health risk of the defined mixture of the ("ten") priority chemicals, using approaches appropriate for simple mixtures of chemicals
A pragmatic approach: carry out limited toxicity studies e.g. one four-week rat study and one screening assay for genotoxicity with the defined mixture of ("ten") priority chemicals, using exposure concentrations e.g. 3 – 10 times higher than those occurring in the complex mixture

Selection of the "top ten" chemicals in the first step should be based on the level of exposure and level of toxicity of the individual chemicals. The higher the value of the risk quotient (RQ) the higher the probability of adverse health effect in human (e.g. higher risk) and the higher the chemical should rank on the list of priority chemicals. The hazard identification and risk assessment of the mixture of selected chemicals (the "top-ten" chemicals) should be based on toxicity data and on the mechanism of action of the individual compounds and on the prediction of presence or absence of additive or potentiating interactive effects. In order to predict combined action (additivity) or interactions between the selected chemicals knowledge about the presumed mechanism of action is necessary. Therefore, a classification system of chemicals on the basis of their mechanism of action would be extremely helpful. A classification could be based on:

- Similar or identical biotransformation pathways, including ability to induce or inhibit biotransformation enzymes
- Similar or identical receptors for the compounds or their active metabolites
- Structural similarities pointing to either of the above.

The group recognises that a major practical problem is lack of information of biotransformation and relevant receptor or target site of many chemicals. In such cases, the chemicals should be classified using computer-based structure-toxicity relationships and expert judgement and experience.

The evaluation assumes that the hazard and possible risk of the defined ("top-ten") mixture of chemicals are representative for the hazard and risk of the entire complex mixture. For some mixtures, that are relatively easily available (e.g. combustion fumes, food products, pesticide mixtures), this assumption could be validated by comparing the toxicity of the "top ten" mixture with the toxicity of the original complex mixture in short-term test.

The group has elaborated on their approach and provided a scheme (decision tree) for hazard identification and risk assessment of complex mixtures (Feron et al 1998, Groten *et al.*, 2001) (Figure 4.4.2.2). In this scheme it is suggested that:

- For complex mixtures that are virtually unavailable for testing as a whole (such as workplace atmospheres, coke oven emissions, atmospheres at waste sites) the "top-ten" approach as mentioned above is suggested.

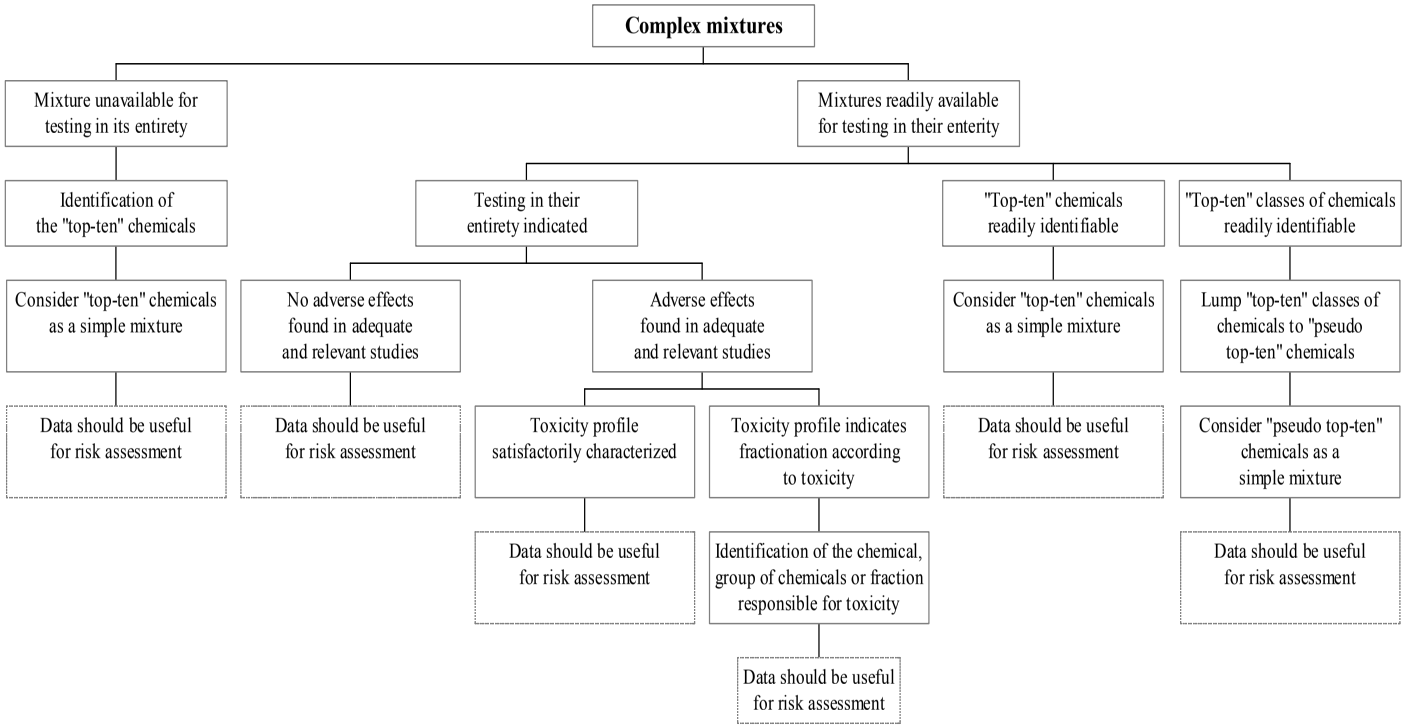
- For complex mixtures that are readily available for testing as a whole (such as drinking water, diesel exhaust, welding fumes, tobacco smoke, pesticide mixtures, food products) three possible approaches are suggested:

    - Testing as a whole. This may characterise the toxicity profile of the mixture and eventually verify the (presumed) safety or hazard from exposure to mixture. One problem may be that incorporation of the test material in the diet at a sufficiently high dose may result in an unbalanced diet and nutritional deficiencies. Another problem is that most mixtures may change in chemical composition over time.
    - Identification of the "top-ten" chemicals to be treated as a simple mixture. This should be considered as the primary option if the available data on the composition and the toxicity profile of the mixture indicate that the hazard is driven by a small number of the constituents.
    - The "pseudo top-ten" approach should be considered for mixtures which consist of a large number of widely varying chemicals with no

obvious ranking of individual constituents according to their potential health risks and the "top-ten" chemicals of the mixture are not easily identified. This approach involves identification of the "top-ten" classes of chemicals to be lumped together by class to the "top-ten" chemicals to be treated as a simple mixture. The lumping technique is based on grouping chemicals with relevant similarity such as the same target organ or similar mode of action. The selected "top-ten" chemicals are either chemicals representative of each class or pseudo components representing a fictional average of a certain class. This technique has been described by Verhaar *et al.* (1997) who proposed it to be combined with QSAR and PBPK/PD (physiologically based pharmacokinetic/pharmacodynamic) modelling in predicting the toxicity of complex mixtures of petroleum products.

Groten *et al.* (2000) used the principle that joint actions and/or interactions could possibly occur if chemicals shared a common target organ and produced similar adverse effects to analyse all approved food additives allocated a numerical ADI (a total of 65 additives). Target organs were identified based on the adverse effects reported above the NOAELs in animal or human studies. Description of pathological and other changes found were used to assess whether different additives, sharing the same target organ, would produce a common toxic effect. In many cases the adverse effects were considered to be non-specific and/or related to nutritional/palatability problems, such that a clear target toxicity could not be identified. Twelve different target organs were identified, and each group of additives, sharing the same target organ, was studied in detail for possible joint actions and/or interactions. In all but four cases, the possibility of joint actions and/or interactions could be excluded on scientific grounds. The exceptions were groups of additives with critical effects on the liver (curcumin, thiabendazole, propyl gallate and butyl hydroxyl toluene), the kidneys (diphenyl, o-phenylphenol and ferrocyanide salts), the blood (azorubine and propyl gallate), and the thyroid (erythrosine, thiabendazole and nitrate). However, an in-depth analysis of both the specific use and the intake levels of these four groups of additives led the authors to conclude that joint actions or interactions among the additives in a group was a theoretical rather than a practical concern (Groten *et al.* 2000).

Figure 4.4.2.2 Scheme for safety evaluation of complex chemical mixtures



4.5 Approach for assessment of joint toxic action of chemical mixtures suggested by ATSDR

The American Agency for Toxic Substances and Disease Registry (ATSDR) in 2002 issued a "Guidance Manual for the Assessment of Joint Toxic Action of Chemical Mixtures" (ATSDR 2002) in which strategies for exposure-based assessments are proposed for noncarcinogenic and carcinogenic effects, respectively. The strategies are based on a number of sequential questions asked and are outlined in the following:

4.5.1 ATSDR strategy for noncarcinogenic effects

Step 1 and step 2 ask questions as to whether the mixture has already been either regulated or scientifically evaluated in which case this information should be used. If this is not the case further steps are proposed:

Step 3
Are the hazard quotients (HQs, see 4.2.1) equal to or greater than 0.1 for at least 2 of the mixture components?

If no, additivity and interactions are unlikely to result in health hazard.

If yes, further evaluation of additivity and interactions is necessary for the components of concern (HQ equal to or greater than 0.1).

Step 4
Is a relevant PBPK/PD model or studies on joint toxic action available for mixture of components of concern?

If yes, use model or study to evaluate potential health hazard.

If no, go to step 5.

Step 5
Do components of concern have the same critical effects?

If yes, go to step 6a. If no go to step 6b.

Step 6a
Apply the hazard index (HI) method (see 4.2.1).

Step 6b
Apply target-organ toxicity dose (TTD, see 4.2.1) modification of HI method for overlapping targets of toxicity or access any unique critical effect with separate HQ.

If HI or separate HQ are equal to or less than 1 go to step 7a, if greater than 1 go to step 7b (HI > 1: potential health hazard due to additivity; separate HQ >1: potential health hazard due to unique critical effect.

Step 7a
Apply qualitative WOE (see 4.2.1.1, and ATSDR 2002): is combined score positive?

If no, health hazard is unlikely

If yes, there may be potential health hazard due to interactions and/or additivity.

Step 7b
Apply qualitative WOE: is combined score positive?

If no, health hazard likely to be less than indicated by HI or by separate HQ for unique critical effect.

If yes, there may be potential health hazard due to interactions and/or additivity.

4.5.2 ATSDR strategy for carcinogenic effects

Step 1 and step 2 ask questions as to whether the mixture has already been either regulated or scientifically evaluated in which case this information should be used. If this is not the case further steps are proposed:

Step 3
Are estimated risks equal to or greater than $1x10^{-6}$ for at least 2 of the individual mixture components?

If no, additivity and interactions are unlikely to result in health hazard.

If yes, further evaluation of additivity and interactions is necessary for the components of concern (risk equal to or greater than $1x10^{-6}$).

Step 4
Is a relevant PBPK/PD model or studies on joint toxic action available for mixture of components of concern?

If yes, use model or study to evaluate potential health hazard.

If no, go to step 5.

Step 5
Is the sum of the risks for the components equal to or greater than $1x10^{-4}$?

If no, go to step 6a. If yes (potential health hazard due to additivity; evaluate interactions), go to step 6b.

Step 6a
Apply qualitative WOE: is combined score positive?

If no, health hazard is unlikely

If yes, there may be potential health hazard due to interactions and/or additivity.

Step 6b
Apply qualitative WOE: is combined score positive?

If no, health hazard likely to be less than indicated by the sum of risks.

If yes, there may be potential health hazard due to interactions and/or additivity.

4.6 Approaches currently used by regulatory agencies in Denmark

4.6.1 Danish Working Environment Authority

When several substances are present at the same time at the work place, they may have an over-all intensifying (synergistic) or weakening (antagonistic) effect, or the interaction may be additive. Limited information and data are available (in the literature) and the Danish Working Environment Authority requires the following procedure in controlling combined exposure:

If no specific information on the interaction of the substances is available, at least an aggregate (additive) effect should be considered. The following formula is used when calculating the aggregate effect:

$$C_1 / GV_1 + C_2 / GV_2 + C_3 / GV_3 + \ldots + C_n / GV_n$$

Where C is the concentration in the air of the respective substances, and GV is the corresponding exposure limit values. A fraction sum of 1 corresponds to the exposure limit value for the aggregate effect/combination of substances.

This formula is not normally applied to combination of benzene and tetrachloromethane, and lead and sulphuric acid (At-Guideline C.0.1 2000).

4.6.2 The Danish Environmental Protection Agency

In the harmonized EU-regulation for classification and labelling the classification of preparations (chemical mixtures) is based on the classification of the single chemical substance where the content of the substances classified for the same harmful effect (acute toxicity and irritation) are summarised i.e. the preparations are classified in accordance with the principle of additivity of similar effects. Furthermore substances and preparations (mixtures) may be classified as corrosive due to their physical-chemical properties on the basis of the pH value and the acidic or basic capacity.

With respect to classification and labelling for other specific effects such as sensitizing effects, reproductive, mutagenic and carcinogenic effect the classifications depends on specific concentration limits of the single substances and thus no addition of contents is made.

For some substances i.e. oil- and coal derived substances where each substance comprises of a great amount of constituents a specific amount of a specific indicator have been chosen (e.g. the content of benzene, 1,3-butadiene, benzo[*a*]pyrene and the DMSO extractable fraction) to decide whether the complex substance should be classified as carcinogenic or not.

With respect to harmful effects in connection with aspiration risk to the lung after ingestion, the oil-/coal- derived substance (or preparation) is classified according to physical-chemical properties with regard to its flow rate and viscosity.

In the guidelines for regulation of air emission the regulation is mainly based on the tolerable outdoor air concentrations for the single substances (Danish EPA 2002). However, some considerations have been made in connection with simultaneous emission of multiple substances and from an operational point of view the following is recommended:

In case of substances with different toxic effects and mode of action the C-values for each substance should be complied with separately.

In case of substances that affect each other's effect and mode of action the C-values for each substance should be complied with separately, as well, as no interaction is expected to occur at concentrations below the C-values, which represent human no-effects levels of the specific compounds. The assumption is that interaction only occurs at levels above the no-effect levels.

In case of substances with identical effects and mode of action *and* in case of substances from the same chemical homologous group (e.g. alcohols, ketones, ethers, etc) the contribution from each substance (in relation to their respective C-value) should be added together, i.e. simple additivity of the effect is assumed.

In relation to PAH-emissions, the emission value is based on a specific benzo[*a*]pyrene equivalent value based on the contribution and relative potency factors of 15 PAH-substances.

4.6.3 The Danish Veterinary and Food Administration

The Danish Veterinary and Food Administration follows the international evaluations of chemicals in food, which in a number of cases consider the possibility of combined actions of chemicals. One of the most well known examples is the use of toxicity equivalency factors for the evaluation of mixtures of polychlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls in food.

For a number of food additives, group ADIs have been established. Examples are groups of food preservatives like sorbic acid and sorbates, benzoic acid and benzoates and the parabenes (ethyl-, methyl- and propyl-para-hydroxybenzoates). They have all been allocated group ADIs, which means that the intake of the sum of the amounts of each compounds in the group (obtained through simple addition) should not exceed the group ADI. For benzoic acid and the benzoates, the group ADI of 0-5 mg/kg bw/day also includes the intake of the flavouring agents benzyl acetate and other benzyl esters, benzyl alcohol and benzaldehyde. The rationale behind this is that these compounds are all quickly and efficiently metabolised into benzoic acid.

4.6.3.1 Risk assessment of pesticide residues in food

Prepared by Trine Klein Reffstrup

The current practice in risk assessment of multiple pesticide residues in food is generally based upon data from studies on single compounds although humans at the same time are exposed to more than one pesticide that potentially possesses similar or different toxic effects. Thus, Chambers and Dorough (1994) discussed the fact that almost all pesticides occur in mixtures. "Pesticides are applied to crops, forests, home gardens, households and buildings as formulated products that contain solvents, emulsifiers, and "inert" ingredients. Moreover, technical grades of pesticide compounds contain isomers, analogues, breakdown products, or rearrangement products that form during synthesis and are not removed or that form after synthesis and/or formulation and during storage."

These compounds may interact causing a higher or lower toxic effect than would be expected from the single compounds. Consequently potential combined actions of pesticides need to be addressed in the risk assessment process. However, there is

no internationally accepted procedure for such a toxicological evaluation except for the few groups of pesticides sharing a group ADI.

A well-known example is the group ADI of 0-0.03 mg/kg bw/day allocated to dithiocarbamate fungicides. Thus, the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1993 in its evaluation of Mancozeb concluded that "the data on mancozeb would support an ADI of 0-0.05 mg/kg bw, based on the NOAEL of 4.8 mg/kg bw/day for the thyroid effects in rats using a 100 fold safety factor. However, the Meeting established a group ADI of 0-0.03 mg/kg bw for mancozeb, alone or in combination with maneb, metiram, and/or zineb, because of the similarity of the chemical structure of these compounds, the comparable toxicological profiles of the dithiocarbamates based on the toxic effect of ethylenethiourea (ETU), the main common metabolite, and the fact that parent dithiocarbamate residues cannot be differentiated using the presently-available analytical procedures".

The Danish Veterinary and Food Administration have up till now evaluated mixtures of pesticides found as residues in crops by using the following two practices:

- Summation of the concentrations of all residues and applying the ADI for the most toxic pesticide found – this is the most restrictive assessment.
- Using the sum of the percentage of the ADI of the individual pesticides found.

However, the report from the Bichel-commitee recommended that knowledge of combined action should be taken more into account in the risk assessment of pesticides (Bichel-udvalget, 1999). Therefore the Danish Veterinary and Food Administration carried out a project in which the current knowledge about combined toxic effects of mixtures of pesticides (the active substances of pesticide formulations) published in the scientific literature was summarised and evaluated (Reffstrup, 2002). The objective was to examine whether there is a scientific basis for using a general standard formula in the risk assessment of pesticide mixtures. This was done in order to ascertain and/or improve the toxicological risk assessment of pesticide mixtures which humans are exposed to via food. The main points and conclusions in the report were:

Two different kinds of approaches for health risk assessment of chemical mixtures have been recommended in the literature, namely whole mixture approaches and component-based methods. The assessment of whole mixtures can be done on the mixture of concern, on a sufficiently similar mixture, or on a group of similar mixtures. These assessments would be ideal for risk assessment of pesticide residues in food, however they are not applicable in these cases since the methods are very data intensive. This leaves the single compound approaches as the more realistic ones.

For mixtures in which the compounds are toxicologically similar (e.g. same mechanism of action), four methods based on simple similar action (Loewe additivity) have been suggested: the hazard index, the relative potency factor method, and the special type of the relative potency factor method named the toxicity equivalency factor, and the margin of exposure approach. These methods differ by the required data on toxicological processes but in all cases the exposure levels are added after having been multiplied by a scaling factor that accounts for differences in the toxicological potency. For compounds acting independently (different mechanism of action) the response addition approach have been

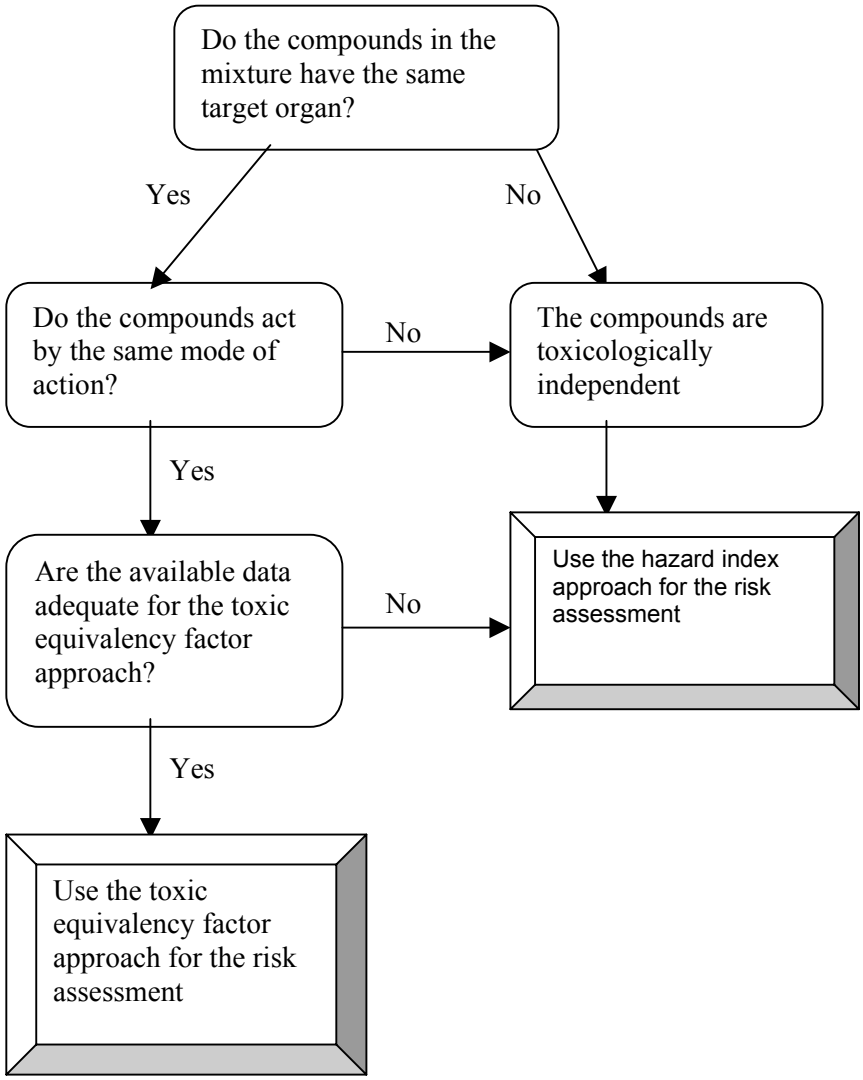
suggested, and for compounds that interact, use of interaction hazard index have been suggested.

Several reports have suggested that since the pesticides are found in food at levels well below their respective no observed adverse effect levels (NOAEL), the approaches based on toxicologically similar mechanism (same mechanism of action) and toxicological independence (different mechanism of action) should be used for risk assessment of pesticide residues. In fact, it has been suggested that methods for toxicologically similar compounds could be used in most cases, even when the compounds are not toxicologically similar.

The present knowledge about combined toxic effects of mixtures of pesticides (the active substances of pesticide formulations) that have been published in the scientific literature were summarised and evaluated in the report in order to test the hypothesis behind the risk assessment models presented. The *in vivo* studies on pesticide mixtures were performed at high doses (ten times NOAEL or higher) compared to the expected pesticide residues in food. Studies employing high doses have shown both additivity and interactions such as synergism or coalism as well as antagonism. None of the studies reported in the literature used low pesticide doses, in the range normally found for residues in food. Furthermore, the number of theoretical possible pesticide mixtures is enormous compared to the number of mixtures studied and published in the scientific literature and the overall quality of the data in the studies is not good enough to reach a clear conclusion. *Therefore it was concluded that there is no scientific background for establishing a general standard formula for risk assessment of pesticide mixtures in food*. However, studies on other chemical mixtures at low doses have been found not to demonstrate a risk different from that of the single compounds in the mixture. The authors of these studies have concluded that combined exposure to arbitrarily chosen chemicals did not demonstrate more than an additive action when all chemicals in the mixture were administrated at their own individual NOAELs (see chapter 5).

In the report it is suggested to use the flow chart shown in figure 4.6.3.1 for risk assessment of pesticide mixtures in crops. The risk assessment of pesticide mixtures in crops should be done by a case-by-case evaluation in which the available chemical and toxicological data on the pesticides are evaluated in a weight of evidence process. Then the hazard index with the ADI (where the individual safety factors are included) and not the NOAEL's (where the safety factors are not included) as the acceptable level in the denominator is recommended for use. However, in cases where the weight of evidence process points out that the compounds in the mixture share a common mechanism (e.g. for the organophosphorus pesticides), the toxicity equivalency factor (TEF) approach should be used, if possible (Reffstrup 2002).

Figure 4.6.3.1 Flow chart of the risk assessment approach for pesticide mixtures found in food.



5 Experimental studies using simple, well-defined mixtures

Prepared by John Chr. Larsen

5.1 Introduction

In recognising the difficulties in the risk assessment of chemical mixtures the group around Victor Feron at the TNO Nutrition and Food Research Institute in Zeist, The Netherlands, initiated a research programme to obtain some basic information on the toxicological interactions between toxicologically well-characterised chemicals in well-defined mixtures. The objective was to establish knowledge about some general principles for the interaction of chemicals in mixtures that would be useful in the risk assessment of complex mixtures. The group used relatively simple mixtures (not more than 10 different compounds), which were tested in short-term repeated-dose toxicity studies in order to examine the concepts of simple similar action or simple dissimilar action and its implication for the risk assessment of chemical mixtures.

5.2 Chemicals with different target organs and/or different modes of action

Two four-week studies of the toxicity (clinical chemistry, haematology, biochemistry, and pathology) in rats were performed on combinations of compounds with different target organs and/or different modes of actions. The study designs are given in Tables 5.2.1 and 5.2.2. NOAELs and LOAELs ("minimum-observed-adverse-effect level"), expressed in mg per kg of bodyweight per day, had been previously established for each single compound in the same laboratory using the same strain of rats and comparable experimental conditions. In the first study (Jonker *et al.* 1990, Feron *et al.* 1995a) the test compounds were arbitrarily chosen. Groups of 10 four-week old male and female rats were administered diets containing stannous chloride, sodium metabisulphite, metaldehyde, loperamide, Mirex, lysinoalanine, and di-*n*-octyltin dichloride and drinking water containing potassium nitrate at levels that for each compound corresponded to one tenth the NOAEL, one third the NOAEL, the NOAEL, or the LOAEL (Table 5.2.1). Proper control groups were included.

In the groups receiving one tenth or one third of the NOAEL no treatment related adverse effects were found. At the NOAEL level slightly decreased haemoglobin concentration and slightly increased kidney weight in male rats were the only treatment related adverse effects recorded.

As was expected from the studies of the toxicity of the individual compounds, a wide range of adverse effects was seen at the LOAEL. The effects included growth retardation (stannous chloride, loperamide, Mirex), reduced food and water intake (stannous chloride, metaldehyde, loperamide, Mirex), changes in haematological (stannous chloride, Mirex) and biochemical parameters (Mirex, di-*n*-octyltin dichloride), increased relative testes and thyroid weights (Mirex), increased liver weights (metaldehyde, Mirex), swollen and vacuolated hepatocytes (stannous chloride, Mirex), hyperplasia and hyperkeratosis of the forestomach (sodium metabisulphite), and reduced weight and lymphoid depletion of the thymus (di-*n*-

octyltin dichloride). A number of adverse effects seen at the LOAEL, such as decreased prothrombin time, increased liver enzyme activity (ALAT and ASAT) in plasma, increased kidney weights, 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 found at doses higher than the LOAEL of the individual compounds. Although some of these changes may be related to the severe growth retardation, they suggested some kind of interaction resulting in a slightly more severe and maybe broader range of toxic response. On the other hand the changes in the thymus were less pronounced on combined exposure than after exposure to di-*n*-octyltin dichloride alone, suggesting interaction leading to less severe toxicity.

Table 5.2.1. Toxicological studies of chemicals with different target organs and/or modes of action.

Compound	**NOAEL/10**	**NOAEL/3**	**NOAEL**	**LOAEL**	**Target organ**
	Mg/kg bw/day				
Potassium nitrate	10	33	100	300	Adrenals
Stannous chloride	100	330	1000	3000	Body weight, blood, liver
Sodium metabisul-phite	500	1670	5000	20000	Blood, stomach, liver
Metalde-hyde	20	70	200	1000	Liver
Loperamide	0.5	1.7	5	25	Body weight
Mirex	0.5	1.7	5	80	Body weight, liver blood
Lysino-alanine	3	10	30	100	Kidneys
Di-*n*-octyltin dichloride	0.6	2	6	30	Thymus, liver

The authors concluded that the study demonstrates absence of a simple additive effect and synergism, 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 appeared to exist.

The other experiment (Feron *et al.* 1995a, Groten *et al.* 1997) was a 4-week oral/inhalatory study in male Wistar rats in which the toxicity of combinations of nine compounds was examined (Table 5.2.2). In this study a combination was used of compounds highly relevant to the general population in terms of use patterns and levels and frequency of exposure. The study included 20 experimental groups, four groups of eight rats in the main study and 16 groups of five rats in a satellite study. In the main study, the rats were simultaneously exposed to mixtures of all nine chemicals. Dichloromethane and formaldehyde were administered by inhalation and aspirin, di(2- ethylhexyl)phthalate (DEHP), cadmium chloride, stannous chloride, butyl hydroxyanisol (BHA), loperamide, and spermine were given in the diet at concentrations equal to the "minimum-observed-adverse-effect level" (LOAEL), the NOAEL, or one third the NOAEL. The satellite study was designed as a fractionated two-level factorial study in which the rats were simultaneously exposed to combinations of maximally five compounds at their LOAEL. These 16

combinations of nine factors (9 chemicals) jointly comprise a 1/32 fraction of a complete study.

Table 5.2.2. Toxicological studies of chemicals with different target organs and/or modes of action.

Compound	NOAEL/3	NOAEL	LOAEL	Target organ
	Mg/kg bw/day			
Aspirin	330	1000	5000	Liver, stomach
Cadmium chloride	3	10	50	Red blood cell, liver
Stannous chloride	260	800	3000	Red blood cell
Spermine	130	400	2000	Hearth, liver
Loperamide	2	6	30	Liver
BHA	330	1000	3000	Stomach
DEHP	65	200	1000	Liver
Dichloro-methane	30	100	500	Blood
Form-aldehyde	0.3	1	3	Nose

A number of effects were observed at the LOAEL. Growth retardation and reduced food intake were signs of general toxicity. In haematology, a decrease in mean corpuscular volume, MCV (cadmium) and thrombocyte count (aspirin) and an increase in mean corpuscular haemoglobin, MCH (DEHP/aspirin) and carboxyhaemoglobin (dichloromethane) was seen. A number of biochemical/clinical chemistry parameters were also affected, notably decreased alkaline phosphatase, glucose, triglycerides, and cholesterol concentrations, and an increase in ALAT, ASAT and palmityl CoA activities, albumin, bilirubin, and total protein concentrations. The absolute weights of the liver and kidney were increased while spleen and hearth weights were decreased and the relative weights of all organs except spleen and hearth were significantly higher in all animals. Treatment related histopathological changes were observed in the liver and nasal cavity of all rats of the LOAEL group. The lesions consisted of hepatocellular hyperthropy and hyperplasia of the transitional epithelium and/or squamous metaplasia of the respiratory epithelium in the nose.

Based on the toxicity data of the individual compounds most of these effects were expected. A few effects seen in the toxicity studies with the individual compounds had disappeared in the combination, whereas some effects not seen in the range-finding studies with the individual compounds appeared in the combination.

For most of the end points studied in the satellite groups, the factorial analysis revealed main effects of the individual compounds and interactions (cases of nonadditivity) between the compounds. As an example, the factorial analysis revealed that two compounds, aspirin and DEHP were able to induce palmityl CoA activity, whereas BHA slightly reduced the activity. In addition, there was a slight and unexpected interaction between BHA and DEHP, which resulted in a decreased palmityl CoA activity. As another example, it was found that cadmium chloride, aspirin, and loperamide were able to increase the ASAT activity whereas stannous chloride was able to decrease it. In addition, two cases of interactions were identified, namely the interaction between cadmium chloride and loperamide resulting in a higher ASAT activity than would be expected on the basis of summation of the effects of the two compounds and the interaction between

stannous chloride and cadmium chloride resulting in a lower ASAT activity than expected on the basis of additivity.

As expected, the carboxyhaemoglobin formation was mainly due to dichloromethane and no interactions with other compounds were observed. Aspirin, DEHP, and surprisingly stannous chloride increased the incidence of hepatocellular hypertrophy and there were no significant cases of interaction in the liver. The factorial analysis revealed a slightly increased incidence of epithelial hyperplasia in the nasal cavity in rats exposed to dichloromethane in the absence of formaldehyde. However, in the presence of formaldehyde most of the rats showed epithelial hyperplasia independent of the presence or absence of dichloromethane.

In the main study the only treatment related effects observed in the NOAEL group were decreased alkaline phosphatase activity and triglyceride concentration and slight and infrequent hypertrophy of liver cells and epithelial cells of the nasal cavity. Liver changes had previously been observed after administration of DEHP, aspirin or spermine at higher doses, and changes in the nasal cavity is characteristic for the effect of formaldehyde. Relative kidney weights were increased in rats of the NOAEL and one-third the NOAEL groups. The satellite part of the study revealed that BHA, aspirin, or stannous chloride might increase the kidney weight. The effect on kidney weight at the LOAEL could well be explained by summation of the effects of BHA, aspirin and stannous chloride and a similar joint (additive) action of these compounds might also explain the in crease in kidney weight at the NOAEL.

It was concluded that simultaneous exposure to these nine chemicals did not constitute an evidently increased hazard compared to exposure to each of the chemicals separately, provided the exposure level of each chemical in the mixture was at most similar to or lower than its own NOAEL (Groten *et al.* 1997).

In comparison with the adverse effects produced by the individual compounds, both more severe and less severe effects were observed at the LOAEL of the combined compounds, indicating interactions of effects at this exposure level. Several of the effects (growth retardation, reduced food intake, and liver damage) were more severe than seen with the individual compounds.

5.2.1 Other studies

Ito *et al.* (1995) examined the combined dietary administration to rats of 19 organophosphorus compounds and one organochlorine pesticide, all permitted for use in Japan, each at its acceptable daily intake (ADI) level. The dietary exposure at this level did not enhance the development of rat liver preneoplastic lesions initiated by diethylnitrosamine. However, a mixture administered at 100 times the ADI levels of the pesticides significantly increased the number and area of lesions. The authors concluded that the study provided direct support for the present use of the safety factor approach in the quantitative hazard evaluation of pesticides.

Swiss albino male rats were treated orally by gavage with 100- and 1000-fold the ADI of endosulfan, dimethoate and carbaryl (Akay *et al.*, 1999). The authors had previously reported that endosulfan, dimethoate and carbaryl, either alone or in mixture at 1- and 10-fold the ADIs, did not cause significant effects on immunological and haematological parameters of rats. No effects were reported on immunoglobulin concentrations or blood parameters at the 100-fold ADI level as well as after treatment with the individual pesticides. It was concluded that the mixtures acted like the single compounds and that no synergistic or antagonistic effects were observed. However, the authors ignored elevations in the red blood

cell and monocyte counts in the rats receiving a mixture of endosulfan and dimethoate. They also ignored effect on the red blood cell counts in rats receiving a mixture of dimethoate and carbaryl. At 1000-fold the ADIs indication of immune suppression was reported to depend on the presence of endosulfan. The authors suggest that carbaryl had a slight antagonistic effect on endosulfan and dimethoate at high doses.

Wade et al. (2002) studied the subchronic effects (systemic, immune, and reproductive effects) of exposure to a complex mixture of persistent contaminants in sexually mature male rats. Each chemical was included in the mixture at the "minimum risk level" (MRL), the reference dose (RfD) or tolerable daily intake (TDI) as determined by ATSDR or US EPA. For 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) the NOAEL (1 ng/kg bw/day) used to calculate the TDI in Canada in 1993 was used. The rats were exposed to the mixture at 1, 10, 100, and 1000 times the estimated safe levels daily for 70 days (see table 5.2.3).

Table 5.2.3. Composition of contaminant mixture administered to male rats

Contaminant	**1 x MRL/RfD/TDI (xg/kg bw/d)**	**10x MRL/RfD/TDI (xg/kg bw/d)**	**100 x MRL/RfD/TDI (xg/kg bw/d)**	**1000 x MRL/RfD/TDI (xg/kg bw/d)**
Aldrin	30 ng	0.3 μg	3 μg	30 μg
p,p'-DDT	30 ng	0.3 μg	3 μg	30 μg
p,p'-DDE	570 ng	5.7 μg	57 μg	570 μg
Dieldrin	50 ng	0.5 μg	5 μg	50 μg
Endosulfan	50 ng	0.5 μg	5 μg	50 μg
Heptachlor	0.5 μg	5 μg	50 μg	500 μg
Hexachlorbenzene	0.3 μg	3 μg	30 μg	300 μg
Hexachlorocyclohexane	0.3 μg	3 μg	30 μg	300 μg
Mirex	0.8 μg	8 μg	80 μg	800 μg
Methoxychlor	2 μg	20 μg	200 μg	2000 μg
1,2,3-Trichlorobenzene	0.77 μg	7.7 μg	77 μg	770 μg
1,2,4-Trichlorobenzene	2.3 μg	23 μg	230 μg	2300 μg
1,2,3,4-Tetrachlorobenzene	0.2 μg	2 μg	20 μg	200 μg
Pentachlorobenzene	0.5 μg	5 μg	50 μg	500 μg
TCDD	1 ng	10 ng	0.1 μg	1 μg
PCB (Aroclor 1254)	1 μg	10 μg	100 μg	1000 μg
Cadmium chloride	0.7 μg	7 μg	70 μg	700 μg
Lead chloride	0.1 ng	1 ng	10 ng	0.1 μg

Evidence of hepatotoxicity was seen as a significant enlargement of the liver in the 1000x group, reduced serum LDH activity (100x), and increased serum cholesterol and protein levels (both 1000x). Hepatic EROD activities (a marker of cytochrome P450 activity) were elevated in animals exposed to 10x and above. The mixture caused decreased proliferation of spleenic T cells at the highest dose and had a biphasic effect on natural killer cell lytic activity with an initial increase in activity at 1x followed by a decrease to below control levels in response to 1000x. No treatment-related effects were seen on bone marrow micronuclei, daily sperm production, serum LH, FSH, or prolactin levels or weights of most organs of the reproductive tract. The weights of the whole epididymis and the caput epididymis were significantly decreased at 10x and higher doses. However, no effect was seen on cauda epididymal weight. The sperm content of the cauda epididymis was increased at the 1x level but not significantly different from control at higher levels. A slight, but significant, increase in the relative numbers of spermatids was

seen in the animals from the 1000x group with a trend towards reduced proportion of diploid cell at that dose.

The authors concluded that the mixture induced effects on the liver and the kidney and on the general metabolism at high doses but caused only minor effects on immune function, reproductive hormone levels, or general indices of reproductive function measures. The results suggest that additive or synergistic effects of exposure to contaminants resulting in residue levels representative of contemporary human tissue levels are unlikely to result in adverse effects on immune function or reproductive physiology in male rats (Wade *et al.* 2002).

5.3 Same target organ with dissimilar or similar modes of action

5.3.1 Nephrotoxicants with dissimilar modes of action

The toxicity of mixtures of chemicals with the same target organ was examined in rats using nephrotoxicants with similar or dissimilar modes of action. In a four-week feeding study groups of 10-week old male and female rats were administered lysinoalanine, mercuric chloride, hexachloro-1,3-butadiene (HCBD) and *d*-limonene, each affecting renal proximal tubular cells but through different modes of action. The nephrotoxicity of HCBD results from initial conjugation to glutathione followed by several metabolic processes to produce a β-lyase activated and cytotoxic metabolite while *d*-Limonene , via its 1,2-dioxide, produce accumulation of the male rat-specific protein $\alpha_{2\mu}$-globulin. The processes involved in the nephrotoxicity of mercuric chloride are still poorly understood but probably involve mitochondrial dysfunction from inhibition of enzymes and proteins by binding to sulphhydryl groups. Lysoalanine may disturb protein functions either by acting as a metal chelator, by incorporation in proteins, or by inhibition of lysyl-tRNA-synthetase. The compounds were administered simultaneously at their individual lowest-observed-nephrotoxic-effect level (LONEL), no-observed-nephrotoxic-effect level (NONEL) and one-quarter of the NONEL (Jonker *et al.* 1993, Feron *et al.* 1995a).

Table 5.3.1.1. Toxicological studies of chemicals with the same target organ but different modes of action.

Compound	NONEL/4 (ppm in diet)	NONEL (ppm in diet)	LONEL (ppm in diet)	Mode of action
Lysino-alanine	7.5	30	240	Metal ion chelator
Mercuric chloride	3.75	15	120	Mitochondrial dysfunction
Hexachloro-1,3-butadiene	5	20	100	β-Lyase mediated activation
d-Limonene	125	500	4000	$\alpha_{2\mu}$-Globulin accumulation

The individual nephrotoxins caused slight growth depression in males at the LONEL, but not at the NONEL, whereas the combination depressed growth slightly at the NONEL and severely at the LONEL. In females at the LONEL, 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 LONEL, and a dose-dependent increase in kidney weight and number of epithelial cells in the urine at the NONEL and the LONEL. The males treated with a single agent showed slightly increased kidney weights, and/or slight histopathological changes in the kidneys at the LONEL, and (with *d*-limonene only) epithelial cells in the urine at the NONEL and LONEL. 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 NONEL. The authors concluded that combined exposure to the four nephrotoxins at their individual NONEL did not constitute an obviously increased hazard, indicating absence of clear additivity and synergistic interaction, whereas at the LONEL clearly enhanced renal toxicity occurred in males, although not in females (Jonker *et al.* 1993, Feron *et al.* 1995a).

5.3.2 Nephrotoxicants with similar mode of action

In a subsequent study the additivity assumption (dose addition) was tested, using the similarly acting nephrotoxicants tetrachloroethylene (Tetra), trichloroethylene (Tri), hexachloro-1,3-butadiene (HCBD) and 1,1,2-trichloro-3,3,3-trifluoropropene (TCTFP) (Jonker *et al.* 1996). The compounds were given to female rats by daily oral gavage for 32 days either alone, at the LONEL and NONEL (= LONEL/4), or in combinations of four (at the NONEL and LONEL/2) or three (at the LONEL/3), see Table 5.3.21.

Relative kidney weight was increased on exposure to the individual compounds at their LONEL and, to about the same extent, on combined exposure at the NONEL or the LONEL/3.The other end-points studied (histopathology, concentrating ability, urinary excretion of glucose, protein and marker enzymes, and plasma creatinine and urea) were not or only scarcely affected upon combined exposure at the NONEL or LONEL/3. As assessed by the effect on kidney weight, the renal toxicity of the mixtures corresponded to the effect expected on the basis of the additivity assumption. (Feron *et al.* 1995a, Jonker *et al.* 1996).

Table 5.3.2.1. Four-week oral toxicity study in female rats with mixtures of nephrotoxicants having similar mode of action

Treatment (mg/kg bw/d)		**Total doses in toxicity units**
Control: corn oil 10 ml/kg bw/d		
Individual compounds at NONEL[e]		
Tetra[a]	600 mg/kg bw/d	¼
Tri[b]	500 mg/kg bw/d	¼
TCTFP[c]	1.5 mg/kg bw/d	¼
HCBD[d]	1.0 mg/kg bw/d	¼
Individual compounds at LONEL[f]		
Tetra	2400 mg/kg bw/d	1
Tri	2000 mg/kg bw/d	1
TCTFP	6.0 mg/kg bw/d	1
HCBD	4.0 mg/kg bw/d	1
Combination of all 4 compounds		
At NONEL		1
At LONEL/2		2
Combination of 3 compounds		
Tetra + Tri + TCTFP at LONEL/3		1
Tetra + Tri + HCBD at LONEL/3		1
Tetra + TCTFP + HCBD at LONEL/3		1
Tri + TCTFP + HCBD at LONEL/3		1

[a] Tetra = tetrachloroethylene
[b] Tri = trichloroethylene

[c] TCTFP = 1,1,2-trichloro-3,3,3-trifluoropropene
[d] HCBD = hexachloro-1,3-butadiene
[e] NONEL = No observed nephrotoxic effect level (=LONEL/4)
[f] LONEL = Lowest observed nephrotoxic effect level

5.4 Mixtures of chemicals affecting the same target organ but with different target sites

A number of 3-day inhalation studies (6 h/d) were carried out in male rats with formaldehyde, acetaldehyde, and acrolein and mixtures of two or three of these toxicants (Table 5.4.1). They all produce the same type of adverse effect (nasal cytotoxicity) but with different target sites (different regions of the nasal mucosa). Formaldehyde had no effect on the nasal epithelium at 1 ppm, but produced clear changes at 3.2 ppm. Acrolein induced marked nasal changes at 0.67 ppm and less severe changes at 0.25 ppm. Both dose levels of acetaldehyde only produced minor changes. The nasal changes seen after exposure to mixture 1 and mixture 2 were very similar in site, type, degree and incidence to those induced by 0.25 ppm acrolein alone. These changes were therefore considered to be induced by acrolein and not influenced by co-exposure to 1,0 ppm formaldehyde or to 1.0 ppm formaldehyde + 750 ppm acetaldehyde. Mixture 3 induced pronounced changes that were more severe than those found after exposure to the individual compounds at comparable concentrations. The changes indicated that the combined effect of formaldehyde and acrolein on the nasal respiratory epithelium was at least additive and that formaldehyde and/or acrolein probably potentiated the effect of acetaldehyde on the olfactory epithelium.

The authors conclude that neither effect addition nor potentiating interactions occur, providing the exposure concentrations of the aldehydes are at NOAELs. They also state that the type of combined action or interaction found at clearly toxic effect levels is not very helpful in predicting what will happen at levels that are not toxic (Feron *et al.* 1995a, Cassee *et al.* 1996, 1998).

Table 5.4.1. Exposure concentrations (ppm) used in 3-day (6 h/d) inhalation toxicity studies in male rats with nasal toxicants

Groups[a]	Formaldehyde	Acetaldehyde	Acrolein
Formaldehyde/low	1.0		
Formaldehyde/high	3.2		
Acetaldehyde/low		750	
Acetaldehyde/high		1500	
Acrolein/low			0.25
Acrolein/high			0.67
Mix 1	1.0		0.25
Mix 2	1.0	750	0.25
Mix 3	3.2	1500	0.67

[a] Each separate study included a control group exposed to clean air only.

5.5 Conclusions of the Dutch studies

The overall conclusion that the Dutch group draws from their experiments is that combined exposure to arbitrarily chosen chemicals clearly demonstrated the absence of full additivity, and provided some evidence of partial additivity when all chemicals in the mixture were administrated at their own individual NOAELs. At slightly lower dose levels no clear evidence of toxicity was found. This conclusion was found valid for combinations of chemicals that have either different target organs and or different target sites within the same organ (i.e. differ in the

mode of action). Therefore exposure to such mixtures is not associated with a greater hazard than exposure to the individual chemicals, provided that the exposure levels are at or below the individual NOAELs. At exposure levels higher than the NOAELs both synergistic and antagonistic effects may be seen, dependent on the compounds. When the exposure levels are at the ADI/TDI levels no greater hazard is to be expected. The group is of the opinion that the use of the "dose addition" approach to the risk assessment of chemical mixtures is only scientifically justifiable when all the chemicals in the mixture act in the same way, by the same mechanism, and thus differ only in their potencies. Application of the "dose addition" model to mixtures of chemicals that act by mechanisms for which the additivity assumptions are invalid would greatly overestimate the risk (Feron *et al.* 1995b,c; Cassee *et al.* 1998).

6 Interactions in toxicokinetics

Prepared by John Chr. Larsen

6.1 Toxicokinetics

Toxicokinetic interactions occur when the disposition of a toxic compound, i.e. its absorption, distribution (including localisation at the target site), biotransformation or excretion is altered by exposure, either simultaneously or displaced in time, to another compound. The toxicological net-outcome of a toxicokinetic interaction depends on whether a higher or lower level of the biologically active species is achieved at the target site and/or whether the target site is exposed for a shorter or longer duration.

6.1.1 Interactions with absorption

Absorption of chemicals from the gastro-intestinal tract is usually a passive diffusion-driven process. Interactions are mainly to be expected when an active transport process or a specific transporter is involved (Feron *et al.* 1995c). For example, iron is known to decrease the gastro-intestinal absorption of cadmium presumably by competing for the proteins involved in the transport of cadmium, and thus protects against cadmium accumulation and toxicity in experimental animals (Groten *et al.* 1991). This makes iron deficient women a particular risk group for cadmium toxicity due to increased uptake from the gastrointestinal tract.

As regard absorption through the skin it is well known that surface-active compounds and skin irritants can enhance the absorption of other chemicals (see chapter 7.1.2.2)

6.1.2 Interference with distribution

Chemicals are distributed throughout the body via the bloodstream (or the lymph in special cases). Lipophilic compounds are to a large extent bound to proteins in the blood instead of just dissolved in water. A more lipophilic compound may remove a less lipophilic substance from the binding site and thus severely increase the concentration of unbound compound available for toxicological effect. This situation is well known for medical drugs administered simultaneously (Feron *et al.* 1995c).

6.1.3 Interference with biotransformation

The majority of compounds that enter the organism require metabolism in order to be excreted. If the parent compound is responsible for the toxicity and its metabolites are less toxic an increased biotransformation rate will reduce the toxicity, and conversely. However, if the chemical's toxicity is mainly due to its metabolite stimulating the biotransformation will enhance the toxicity.

There are numerous possibilities for interactions among chemicals at the level of the enzymes involved in the biotransformation processes. Such interactions may in principle be due to competition for a given enzyme or cofactor. Well known examples are the detoxification of different alkylating agents by conjugation with

glutathione, which may be reduced by compounds that compete for the glutathione-S-transferases and/or glutathione (Feron *et al.* 1995c).

Another important possibility for interactions is induction or inhibition of the drug metabolising enzymes. Inducers or inhibitors of the microsomal cytochrome P450 oxidative systems may either potentiate (via increased production of active metabolites) or reduce (via increased detoxification) the toxicity of other chemicals. Thus, ketones like acetone and methyl n-butyl ketone and methyl isobutyl ketone can potentiate the hepatotoxicity of carbon tetrachloride and 1,2-dichlorobenzene by induction of cytochrome P450. On the other hand, inhibition of cytochrome P450 by disulfiram strongly enhances the carcinogenicity of ethylene dichloride and dibromide by forcing their biotransformation through the glutathione pathway, leading to enhanced formation of the ultimate carcinogenic glutathione conjugate. The principle of enhancing the toxicity of some pesticides by adding an inhibitor of cytochrome P450 (e.g. piperonyl butoxide) in the formulation is well known (Feron *et al.* 1995c).

A review of the literature (Krishnan & Brodeur 1991) demonstrated that the majority of toxicokinetic interaction results from metabolic induction or inhibition caused by some components of the mixture. These interactions may alter tissue dosimetry and thereby the toxicity of components in the mixture. The tissue doses of chemicals in mixture can be predicted with physiologically based toxicokinetic (PBTK) models when the binary interactions between all of the components in the mixture are known (Haddad *et al.* 1999a, 1999b, 2000a, 2000b). However, the quantitative characteristics of each of these binary interactions have to be determined by experimentation. Given the complexity of the mixtures, to which humans are exposed, this would obviously require an unrealistic large number of experiments in order to characterise the qualitative and quantitative nature of the possible interactions.

Haddad *et al.* (2000b) addressed this problem by using the theoretical limits of the PBTK modulation of tissue dose that would arise from hypothetical metabolic interactions between 10 volatile organic compounds (VOCs) in the male rat. The VOCs used were dichloromethane, benzene, trichloroethylene, toluene, tetrachloroethylene, ethylbenzene, styrene, and *para*-, *ortho*-, and *meta*-xylene. All rat physiological parameters and physico-chemical (partition coefficient) and biochemical (metabolic constants) parameters used in the PBTK models were taken from the vast literature on these compounds. All model equations, except those describing metabolism, were taken from Ramsey and Andersen (1984). PBTK models predicting the blood concentrations of each mixture component were simulated using either the description of saturable metabolism presented in chapter 2.4.2 (equation 1) or the description using the hepatic extraction ratio (equation 3). In the latter case the numerical value of E was set to either 1 (maximal enzyme induction) or 0 (maximal enzyme inhibition). Data on blood concentration kinetics following exposure to binary, quaternary, quinternary, octernary and decernary mixtures of the VOCs were obtained in rats exposed for fours hours by inhalation (50 – 100 ppm each). For all chemicals the simulation lines obtained using E = 1 and E = 0 formed the boundary lines, whereas the one obtained using V_{max} and K_m values was in between. The kinetic data from mixture exposures were within the simulated boundaries of blood concentrations. However, with increasing complexity of the mixtures the impact on the blood kinetics of the single components became progressively more important, i.e. blood concentrations of unchanged parent chemicals increased with mixture complexity. This is consistent with the occurrence of metabolic inhibition among the chemicals in the mixture. In a second experiment rats were pre-exposed to the mixture of all ten chemical (50 ppm each) four hours a day for three consecutive days. On day four the rats were

once more exposed and the kinetics of the compounds followed in blood. There seemed to be a systematic decrease (although not statistically significant) in blood concentrations indicative of greater metabolism due to enzyme induction.

Chaturvedi *et al.* (1991) studied the effects of mixtures of parathion, toxaphene and/or 2,4-D on the hepatic mixed-function oxygenase in ICR male mice. They found that a seven days toxaphene pre-treatment enhanced the hepatic biotransformation of parathion and paraoxon both in the presence and in absence of NADP. However, in the absence of NADP the enhancement was minor. The authors suggest that toxaphene induced the metabolic pathways of parathion and paraoxon involving the mixed-function oxygenase and that paraoxonase is not involved in the toxaphene-induced decreases of the two compounds. Toxaphene is enhancing the NADP-dependent metabolism of parathion and paraoxon and thereby decreasing their toxicity. Carboxyl esterase is involved in decreasing the toxicity of parathion and paraoxon by acting as a pool of non-critical enzymes, which compete for the binding of paraoxon thereby preventing an inhibition of cholinesterase. The increase in the level of Carboxyl esterase and cholinesterase has the potential to enhance further the ability of toxaphene to limit the toxicity of parathion. The authors therefore anticipated the toxicity of a mixture of parathion and toxaphene to be lower than that of parathion. Thus the results of the study could indicate an antagonistic effect of toxaphene on parathion and on paraoxon.

Chaturvedi (1993) also examined the effect of mixtures of ten pesticides (alachlor, aldrin, atrazine, 2,4-D, DDT, dieldrin, endosulfan, lindane, parathion and toxaphene) administered by oral intubations or by drinking water on the xenobiotic-metabolising enzymes in male mice. He concluded, ”The pesticide mixtures have the capability to induce the xenobiotic-metabolizing enzymes which possibly would not have been observed with individual pesticides at the doses and experimental conditions used in the study.” However, it is not possible to categorise the type of combined action because Chaturvedi (1993) only examined the combined effects of the ten compounds in the mixture and did not consider the effect of the individual pesticides.

6.1.4 Interference with excretion

For excretion processes the same reasoning may be used as for absorption. Cases of interaction are only to be expected when active processes are involved. Increased excretion of a chemical following administration of an osmotic diuretic or alteration of the pH of the urine is well known examples of dispositional interaction.

7 Combined actions in different toxicological effect areas

This chapter describes the major toxicological effect areas that form the basis for most risk assessments of chemicals. When available, examples of combined actions and interactions are given within these areas.

7.1 Local irritation

Prepared by Eva Selzer Rasmussen

7.1.1 Introduction

Cytotoxic effects of chemicals may cause local tissue irritation to the skin, eyes and respiratory tract. The most severe acute effect is tissue necrosis produced by corrosive chemicals. Less severe effects include impairment of the integrity of cell membranes leading to increase in cell and tissue permeability, which may become manifest as oedema. Local irritative effects may also lead to increase in the blood flow to the tissues causing local erythema or result in capillary leakage producing oedema and blisters.

The stratum corneum of the skin and the intact epithelia of the eyes, airways and lungs constitute the main biological barriers against exposure of the underlying tissue cells to xenobiotics. Damage to these barriers may be due to the combined actions or interactions of chemicals. The tissues of the eye and airways are also protected by additional, efficient defence mechanisms such as the blinking reflexes and tear flow of the eyes or the function of the mucociliary escalator of the airways. It has been demonstrated that these defence mechanisms can be impaired following the combined action of chemicals. Finally, it is increasingly being recognised that the cells in the skin, the eyes and the respiratory tract are active in metabolising xenobiotics. Induction or depletion of the enzymatic capacity of these tissues may thus be an additional basis for combined actions of chemicals resulting in local irritative effects.

7.1.2 Skin irritation

7.1.2.1 Composition of the skin

The skin is constituted of two major tissue layers: an outer layer of thin stratified epithelium, the epidermis, and an underlying dense connective tissue, the dermis. The main function of the epidermis is to generate the stratum corneum, which functions as the major permeability barrier of the skin, primarily against hydrophilic substances.

Epidermis

The basal cell layer in the epidermis is the keratinocytes, a layer of fast dividing cylindrical cells. A layer of polygonal cells follows the keratinocytes, along with Langerhans' cells, melanocytes, and other cells, and a layer of flattened nucleated cells containing keratohyalin granules. The outermost layer, the stratum corneum, consists of several layers of thin, flat anucleated keratinized cells (corneocytes).

Dermis

The dermis consists of a supportive connective tissue composed mainly of collagen fibres together with elastic fibres in a matrix of glycosaminoglycan, salt and water. The dominant structural elements are synthesised by the dermal fibroblasts. The dermis also contains blood vessels, nerves, hair sacs, sebaceous glands and sweat glands. The three latter structures may act as an alternative "shunt" in the percutaneous absorption of chemicals. The dermis is stretching up papillae in epidermis, providing the viable epidermis with essential nutrients and draining it for waste products and penetrants. The viable epidermis and the dermis also constitute a permeability barrier, primarily against lipophilic substances.

7.1.2.2 Percutaneous absorption

Absorption of chemicals through the skin involves several individual transport processes, including adsorption to the surface of the stratum corneum, passive diffusion through this barrier, desorption into the viable epidermis, diffusion through this part of the epidermis and the papillary dermis, and ultimately transfer into the blood circulation. It has been demonstrated that the rate-limiting event for dermal absorption of xenobiotics in undamaged skin in most cases is the passive diffusion through the stratum corneum. The stratum corneum can be considered morphologically and functionally to represent a two-compartment system consisting of corneocytes largely composed of fibrous protein networks in an intercellular matrix, predominantly composed of neutral lipid.

Although not considered totally impermeable to neither water nor lipid soluble compounds, the intact stratum corneum layer is in practice impermeable for large molecules (MW>500). Among compounds of lower molecular weight, the best skin penetrants are soluble in both lipid and water. The major pathway for penetration appears to be through regions with a high lipid content (the intercellular matrix), and within series of similar substances the rate of penetration often correlates with their water/lipid partition coefficients. Hydrophilic substances may penetrate through the protein-rich, hydrated intracellular regions. Some absorption may occur through "shunts" (hair follicles and sweat ducts), but the transdermal flux through this pathway is considered to be minimal. However, for very lipophilic and large molecules together with electrolytes, the alternative shunt seems to be important for penetration. Highly lipophilic substances may penetrate the stratum corneum easily, and for such compounds the viable epidermis and dermis may be the rate-limiting barrier.

7.1.2.3 Test systems

Skin irritancy is most often studied in animal experiments or in studies with human volunteers. Skin organ cultures with human or animal skin have been used to model effects of chemical irritants (van de Sandt and Rutten, 1995). Preliminary results obtained with reconstructed human epidermal tissue cultures hold promise as future test systems (Coquette *et al.*, 1999; De Burgerolle de Fraisinette, A. *et al.* 1999).

Compounds or factors that can modulate the barrier function of the stratum corneum may dramatically affect the effects of dermal irritants. Hydration and delipidization are known to decrease the barrier function of the stratum corneum. Hydration plays an extremely important role in the rate of absorption of materials through the skin. In normal skin, a gradient in water concentration exists through the tissue corresponding to an average concentration of 0.9 g of water per gram of dry tissue. *In vitro* studies have demonstrated that this amount of water increases the rate of absorption of various materials approximately 5-10 fold compared to dry skin. The stratum corneum can ultimately absorb three to five times its own weight of water, and this further hydration may results in an additional 2-3 fold increase in the rate of absorption of water and other polar molecules (Wester & Maibach 1985). Extraction of lipids from the skin by delipidization with organic solvents appears to decrease the barrier effect

for rather short time periods, since the barrier is restored through dynamic intrinsic lipogenesis (Menczel, 1985).

7.1.2.4 Examples of combined action

Local toxic effects to the skin include irritation and corrosion (tissue necrosis). Examples of dermal irritants are strong bases and acids, oxidising or reducing substances, organic solvents and surfactants. When the skin is mildly irritated the dermal blood flow will increase and a local erythema may be produced. More severe irritants can induce capillary leakage to produce manifestations as local oedema or blisters. Very severe intoxications may result in cell and tissue necrosis, and the formation of scars.

Substances and preparations (mixtures) may be classified as corrosive due to their physical-chemical properties on the basis of the pH value and the acidic or alkaline capacity.

Changes in transepidermal water loss may be the cause of combined effects of dermal irritants. Tandem application of topical retinoic acid and sodium lauryl sulphate has been shown to cause synergistic effects concerning non-specific skin irritation. Transepidermal water loss increases dramatically shortly after application of sodium lauryl sulphate, but the increase is delayed after application of retinoic acid (Ale *et al.*, 1997).

Various chemicals have been used in dermatologic preparations in order to enhance the percutaneous absorption of drugs, and additive or synergistic effects by combined exposure to such substances and dermal irritants may be expected. The literature contains many references to skin penetration enhancers that produce minimal irritation and are of low toxicity. Among the most popular and regularly used penetration enhancers are dimethyl sulfoxide (DMSO) and propylene glycol. DMSO appear to enhance skin penetration by either solubilizing the drug in the vehicle or by preceding the drug in penetration and altering the biochemical and structural integrity of the skin, whereas propylene glycol appear to more strictly function as a solubilizer (Gummer, 1985).

The addition of lipids to the skin may prevent loss of skin lipids due to e.g. exposure to organic solvents or replace skin lipids extrinsically. Lipid ingredients of cream bases have been demonstrated to protect industrial workers against the effects of exposure to organic solvents (Menczel, 1985). Other skin-protective materials include different types of waxes, e.g. paraffin wax and bees wax. Application of the waxes to the skin of human volunteers before treatment with irritants or allergens has significantly suppressed the dermal irritancy of sodium lauryl sulphate and combined ammonium hydroxide/urea treatment and moreover appeared to protect against the induction of allergic contact dermatitis (Zhai *et al.*, 1998).

Several investigations have shown a pronounced dermal capacity for metabolism of xenobiotics, and induction or inhibition of dermal enzyme activities by one compound may affect the dermal effect of another compound. The viable epidermis is the most metabolically active part of the skin. For several enzyme activities, e.g. aromatic hydrocarbon hydroxylase, 7-ethoxycoumarin deethylase, aniline hydroxylase, and NADP-cytochrome c reductase, the specific activities in the epidermis and in the liver are comparable (Noonan & Wester, 1985).

7.1.3 Ocular irritancy

7.1.3.1 Composition of the eye

The conjunctiva consists of a non-keratinized epithelium, which contains many blood vessels, nerves, inflammatory cells and the conjunctival glands. These glands secrete the precorneal tear film, which is very important for the corneal function. When the eye is irritated the production of the tear film is often dramatically increased, and the irritant is diluted or removed. If not, significant changes in the tissue can be induced. The blood flow through the conjunctiva is increased and this may result in erythema and hyperaemia. In more serious cases chemosis can be the result.

The outermost part of the cornea consists of a multilayered stratified epithelium, which rests on a basal membrane. Below the epithelium, a thick layer of stromal tissue is found. The stroma consists mainly of uniform collagen fibrils organised in a lattice. When the spatial arrangement of the stromal fibrils are changed, e.g. by swelling or by dehydration, the changes in spacing alters the refractivity of the tissue and this may result in loss of corneal transparency. In normal stroma no blood or lymph vessels are present, and the tissue contains 80 per cent water. These factors are also critical for the corneal transparency. Stroma rests on a basal membrane and below this a single layer of cubic endothelial cells is present. Additionally, an endothelial pump regulates the state of hydration of the stroma.

The epithelial cell layer forms a barrier against entrance of xenobiotics and excess water into the stroma. Damage to the outer layers of epithelial cells may not lead to irreversible effects, but if the stroma is damaged serious ulcers and oedemas may be created. During the repair process ingrowths of blood vessels and of different cells may result in permanent scarring and loss of vision. As the human endothelial cells cannot be replaced, damage to these cells may also result in permanent stromal and epithelial oedema.

The iris consists of connective tissue with smooth muscles, strongly pigmented cells and a rich supply of blood vessels. The surface of the iris consists of a single layer of strongly pigmented cubic epithelial cells. Damage to the iris can result in hyperaemia of the blood vessels and oedema in the connective tissue.

Exposure of the anterior parts of the eye to toxic substances can result in reactions from mild irritation to invalidating damage. The ocular toxicity of a substance is dependent on its chemical composition, concentration, solubility and several other conditions. Strong bases and organic solvents are among the groups of substances, which most frequently are causing ocular damage. Most often the conjunctiva, the cornea or the iris are affected.

The eye contains various cytochrome P450 isozymes, although at lower levels in total cellular content than the liver. In the eye, the vascularized tissues are generally high in drug metabolising activities. The ciliary body demonstrates the highest specific activity among the ocular tissues in metabolising aromatic hydrocarbons, and the induction of various cytochrome P450 isozymes is more pronounced in the non-pigmented ciliary cells than in the pigmented cells (Shichi *et al.*, 1991). The non-vascularized tissues of the anterior eye are generally low in drug metabolising activities, but the potential for ocular metabolism of xenobiotics should not be ignored.

7.1.3.2 Test systems

Tests for ocular irritancy have most often been conducted in rabbits *in vivo* (Draize test). Several *in vitro* systems have shown promise as potential alternatives to *in vivo* tests for ocular irritancy for individual compounds and complex mixtures

(Brantom *et al.*, 1997). A few *in vitro* systems, such as the HET-CAM Test (Hens Egg Test at the Chorion Allantois Membrane), have been used to evaluate combined effects of ocular irritants.

7.1.3.3 Examples of combined action

Contamination of the eye with surfactants and detergents represents a widespread, but complex problem. Some surfactants, such as ordinary soap, cause immediate stinging and burning with little or no injury. Other surfactants produce corneal oedema and loss of corneal epithelium without any alerting discomfort. Several cationic surfactants, e.g. benzalkonium chloride and cetylpyridinium chloride, may produce severe delayed effects on the corneal epithelium and stroma. Additive or synergistic effects of combined exposure to other ocular irritants can be expected, when the barrier properties of the corneal epithelium have been compromised.

Exposure to acids and bases produce rapid, deep penetrating ocular injury as a result of the extreme pH-changes within the tissues. Immediate effects are dissolution of the epithelia and mottled clouding of the corneal stroma after alkaline substances, or coagulation of epithelia after acids. Later effects include oedema, opacification, vascularization, and degeneration of the cornea. Slight, superficial and reversible injuries involving the corneal epithelium may cause great discomfort due to irritation of the corneal nerve endings. More serious chemical burns may, however, produce little pain, because destruction of the sensory nerves of the cornea renders the cornea anaesthetic.

It is well established, that pre-treatment of the eyes of rabbits with topical anaesthetics synergize the effects of ocular irritants. Anaesthetics reduce blinking and tearing, thereby maintaining a higher concentration of the test-material concentration at the surface of the eye. The anaesthetic may also increase corneal permeability and this may bring the test agent into contact with more structures of the eye. Some anaesthetics delay healing after ocular injury. All of these various effects may result in increased irritation to the eye (Durham *et al.*, 1992; Seabaugh *et al.*, 1993).

Ocular exposure to chemically inert solvents, especially very lipophilic solvents, usually causes immediate stinging and smarting pain to the eyes, as it may cause loss of some or all of the corneal epithelium. Recently, the combined effects on ocular and nasal irritation of various solvents have been assessed. Threshold responses of nasal irritation and eye irritation were determined in human volunteers for single chemicals (1-oropanol, 1-hexanol, ethyl acetate, heptyl acetate, 2-pentanone, toluene, ethyl benzene, and propyl benzene), and their mixtures. Various degrees of additive effects were observed for each of the three sensory channels when testing mixtures. As the number of components and the lipophilicity of such components in the mixtures increased, so did the degree of agonism. Synergistic effects characterised the eye irritation response for the most complex and the most lipophilic mixtures (Cometto-Muniz and Cain, 1997).

An *in vitro* test where the uptake of [3H]-uridine by mouse fibroblasts is measured has been used to predict the ocular irritancy of 25 chemicals individually and in combination. The test compounds included alcohols, ethers, esters, ketones, amides, acids and a detergent. The concentration of the agents required to induce a 50% inhibition in uridine uptake rates after 4 hours of treatment correlated well with published data on the chemicals' capacity to induce ocular irritation in rabbits. Combinations of agents with differing functional groups produced additive effects on the inhibition of uridine uptake, suggesting the utility of this approach for the analysis of mixtures (Shopsis and Sathe, 1984).

Another *in vitro* test for ocular irritancy, the HET-CAM Test (Hens Egg Test at the Chorion Allantois Membrane) has recently been used to investigate combined action of chemicals. Compounds, which can occur as disinfecting by-products in swimming pool water, were tested. Previous studies using the rabbit eye test (Draize test) had shown that the irritating potential of typical concentrations of free and combined chlorine are insufficient to explain the degree of eye irritation that can result from exposure to swimming pool water. The compounds tested included halogenated carboxyl compounds (HCC's) which act as precursors during the formation of chloroform. Some of the compounds tested were found to have a significantly increased irritating effect when compared to a chlorine/chloramine mixture of the same concentration, several mixtures of HCC's where even more active at lower concentrations than single compounds. However, the irritating effects of individual compounds as well as of mixtures of HCC's were not sufficiently intense to allow the identification of those compounds specifically responsible for the overall observed increase in irritation. HCC's were therefore tested in the presence of aqueous chlorine solution. When combined with aqueous chlorine, a number of compounds exhibited significantly enhanced effects (Erdinger *et al.*, 1998).

7.1.4 Irritancy to the respiratory tract

7.1.4.1 Composition of the respiratory tract

The respiratory tract consists of three compartments: the nasopharyngeal, the tracheobronchial and the pulmonary region. The nasal passages are lined with vascular ciliated mucous epithelium. The nasopharynx filters out large inhaled particles, and in this region the temperature of the air is moderated and the relative humidity is increased. The airways of the tracheobronchial region are lined with ciliated epithelium and coated with a thin layer of mucus, which is secreted by goblet cells and mucus secreting cells. The surface of the airways in this region serves as a mucociliary escalator, moving particles from the lung to the oral cavity. The physical dimensions and the branching patterns of the airways are critical in determining the absorption of gases and deposition of particles in the respiratory tract.

More than 40 cell types are present in the respiratory tract, but the cells of greatest interest are the types that are unique to the region. These are the ciliated bronchial epithelium, the non-ciliated bronchial epithelium (Clara cells), and various pneumocytes and alveolar macrophages. The endothelial cells, the interstitial cells (fibroblasts and fibrocytes), and the cells lining the trachea and bronchi are of interest since they are extremely susceptible to various types of injury. The Clara cells are metabolically active, and they are the major sites of injury from xenobiotics, which are metabolized to reactive substances by the lung cytochrome P-450 enzyme systems (Boyd *et al.*, 1980).

Irritation of the air passages often results in constriction of the airways. Ammonia and chlorine are classic examples of irritant gases. They first influence the bronchial smooth muscle cells, and bronchioconstriction occurs immediately on inhalation. Ammonia and chlorine are highly water soluble, and thus they are primarily removed by the upper airways. Unless the concentration is sufficient to cause death, the acute effects do not result in chronic pulmonary damage (Weill *et al.*, 1969).

Other types of acute cell toxicity moreover cause irritation of the airways. Damage to the cells lining of the airways may result in necrosis, increased permeability and oedema. When the cells of the airways and alveoli are damaged, the permeability is increased and this leads to the release of oedema fluid into the lumen of the airways and the alveoli. The production of major oedema may take several hours to develop.

The cytotoxicity may be general, non-specific, and the effects will depend on the distribution of the toxic substance in the respiratory tract. The water solubility of the compound is one of the major determinants of the site of action. If the toxic agent is an aerosol, the prime determinant of the site of action is the particle size.

7.1.4.2 Test systems

Respiratory tract toxicity is most often modelled in animal experiments or evaluated on the basis of experiments with human volunteers. Only few studies exists on combined toxic effects using *in vitro* systems. Human alveolar macrophages have, for example, been simultaneously exposed to NO_2 and particles or fibres. The cytotoxic effects of the combined exposure were higher than particle or fibre induced cytotoxicity. Co-exposure did, however, reduce the cellular expression of proinflammatory cytokines (Drumm *et al.*, 1999).

Respiratory tract toxicity is extensively being predicted by physicochemical modelling (Ultman, 1988; Gargas *et al.*, 1993). This approach may be combined with *in vitro* studies of respiratory tract irritancy using cell cultures, isolated human or animal tissues or reconstructed tissues.

With respect to harmful effects in connection with aspiration risk to the lung after ingestion, oil-/coal- derived substances (or preparations) are classified according to physical-chemical properties with regard to its flow rate and viscosity.

7.1.4.3 Examples of combined action

Ozone and nitrogen dioxide are examples of toxic agents that may produce cellular damage and synergistic effects in the respiratory tract. The two gases are among the most critical irritants in urban air. The water solubility of both ozone and nitrogen dioxide is sufficiently low that the main site of action is the respiratory bronchioles and alveoli, and exposure to either of the gases is well known to produce a variety of morphological and biochemical changes in the lung.

Using animal models it has been demonstrated that combined exposure to ozone and nitrogen dioxide resulted in morphological changes in the airways, which were principally accounted for by ozone alone (Freeman *et al.*, 1974), biochemical effects that were additive (Yokoyama *et al.*, 1980; Mustafa *et al.*, 1984), or acute effects (pulmonary oedema and mortality) which were synergistic (Diggle & Gage, 1955). However, recent experiments with combined exposure of rats to varying levels of ozone (400-1600 mg/m^3) and nitrogen dioxide (6840-27360 mg/m^3) indicate that a threshold level for synergistic effects may exist. Clear synergistic effects were observed on the recovery of cells and proteins obtained by pulmonary lavage of combined exposure to ozone and nitrogen oxide levels of 800 mg/m^3 and 13680 mg/m^3, respectively, and at higher exposure levels. However, the effect was less than additive at the lowest level of combined exposure (Gelzleichter *et al.*, 1992).

The most likely mode of action of ozone and nitrogen dioxide is through peroxidation of cellular membranes. By combined exposure of rats to low levels of ozone (100 mg/m^3) and nitrogen dioxide (76 mg/m^3) during 5 months, synergistic effects regarding lipid peroxidation of the lung tissue were observed (Sagai & Ichinose, 1991). Based on results from an *in vitro* experiment with combined exposure of human red blood cells to ozone and nitrogen dioxide, it was concluded that the absolute and relative concentrations of the two gases as well as the time course and the sequence of administration modified the occurrence of toxic effects. Co-exposure to ozone and nitrogen oxide induced additive effects on the osmotic fragility of the cells, lipid peroxidation, acetyl cholinesterase activity and levels of reduced glutathione and methemoglobin. At low levels of the gases, a synergistic increase in

lipid peroxidation was observed, while at higher concentrations the combined effect was less than additive (Goldstein, 1976).

In several well-conducted human clinical experiments on the interaction between ozone and nitrogen dioxide there is insufficient evidence to conclude that an interactive effect on pulmonary function occurs. Both the animal studies and the *in vitro* experiment suggest, however, an interaction between ozone and nitrogen dioxide of at least an additive nature for several cellular parameters. It still has to be established if the biochemical effects of the combined exposure to the gasses are occurring in humans (Calabrese, 1991). However, based on the animal experiments, a simple additive model for the interaction between ozone and nitrogen dioxide may be established (Larsen *et al.*, 1997).

Contradictory results have been obtained concerning possible synergistic effects in humans due to co-exposure to low levels of sulphur dioxide and ozone, and no convincing evidence exists that nitrogen dioxide and sulphur dioxide interact to cause enhanced acute respiratory irritancy in humans. A study with human volunteers suggests that ozone and peroxyacetylnitrate (PAN) may interact to enhance respiratory symptoms (Calabrese, 1991).

Various aldehydes are also established as critical irritants in urban air, and the combined effects of exposure to formaldehyde and acrolein have been studied a couple of animal experiments. These aldehydes have shown similar effects concerning effects on pulmonary function in animal experiments, induction of increased epithelial cell proliferation, induction of DNA-protein cross-binding and of DNA strand-breaks. Acrolein appears to be more cytotoxic than formaldehyde, but the latter is the most DNA damaging agent (Roemer *et al.*, 1993; Leikauft, 1992; Grafström, 1990). In two studies with rats, the animals were exposed to combinations of formaldehyde, acetaldehyde and acrolein. Cassee *et al.* (1995) measured the reduction of breathing frequency as an indicator for the irritative response, and found that the combined effect of the aldehydes was 20% lower than the sum of the responses on the single substances. In another study, the irritancy was monitored by measurements of changes in the tissue of the nasal epithelium. At low exposures no potentiated effects of the combined exposure was observed, but a more than additive effect was observed at the highest exposure level (Feron *et al.*, 1995). Based on these observations, a simple additive model for the interaction between the aldehydes has been suggested (Larsen *et al.*, 1997).

7.1.5 Conclusions

Many studies have been performed on skin, eye and respiratory tract irritation mediated by complex mixtures, but only few studies allows a quantitative evaluation of the modulating effects of the combination of single chemicals.

Effects on several types of endpoints may serve as a basis for combination effects, including:

- Damage to biological barriers against exposure of the cells to xenobiotics, like the stratum corneum of the skin.

- Impairment of defence mechanisms such as the ocular blinking and tearing reflexes or the function of the mucociliary escalator of the upper airways.

- Induction or depletion of the capacity of the xenobiotic metabolising enzymes of the tissues.

Substances and preparations (mixtures) may currently be classified as corrosive due to their physical-chemical properties on the basis of the pH value and the acidic or alkaline capacity. Further studies on the modulating effects of the combination of chemicals concerning skin, eye and respiratory tract irritation will be required in order to evaluate the possibilities for synergistic or antagonistic effects mediated by the mixture of industrial chemicals. Various types of *in vitro* systems have shown promise for use to evaluate effects of irritant chemicals, and this approach may minimise the cost and duration of studies to investigate combination effects. Draft OECD guidelines have been prepared for *in vitro* skin penetration tests (Howes *et al.*, 1996) and for *in vitro* skin corrositivity testing (Botham *et al.*, 1998). Several *in vitro* systems have shown promise as potential alternatives to *in vivo* tests for ocular irritancy for individual compounds and complex mixtures (Balls *et al.*, 1995; Brantom *et al.*, 1997), and promising *in vitro* systems for skin irritation are moreover under validation (Botham *et al.*, 1998). Respiratory tract toxicity is extensively being predicted by physicochemical modelling (Ultman, 1988; Gargas *et al.*, 1993), and this approach may be combined with *in vitro* studies of respiratory tract irritancy using cell cultures or isolated and reconstructed tissues.

7.2 Genotoxicity

Prepared by Mona-Lise Binderup

7.2.1 Introduction

Genotoxic compounds interact with and cause damage to the hereditary material (DNA). When the DNA of a cell is damaged, the lesion may either be correctly repaired or lead to the death of the cell and there will be no genetic consequences. However, if the lesion is misrepaired, either spontaneously or during cell replication the DNA sequence may be changed. This may result in mutations.

Mutations are defined as DNA alterations that are propagated through subsequent generations of cells or individuals. During cell division, DNA is normally transferred to the daughter cell very precisely. However, mutations can occur either spontaneously or induced by chemical substances or radiation. Mutations induced by genotoxic compounds may cause irreversible, adverse health effect at even low exposure levels. Thus, mutations and genotoxic events in somatic cells can lead to cancer, and mutations in reproductive cells can potentially lead to reproductive diseases.

Human exposure to chemicals in the environment will almost always be to complex mixtures, which may also contain genotoxic compounds and/or compounds that may be able to modify the responses of genotoxic agents. Well-known examples of such complex mixtures are polluted air (from a number of industrial sources, motor vehicles or domestic heating), cigarette smoke condensate, contaminated waters and soils, toxic components formed during cooking of food and migrates from food packaging materials. An extensive body of literature exists on the study of genotoxic effects of individual chemicals using both *in vitro* and *in vivo* test methods. In addition, a number of complex mixtures of partly unknown chemical composition have also been tested, in particular *in vitro*. However limited information is available about how combined actions or interactions between chemicals in mixtures may affect the net genotoxic outcome, in particular *in vivo*. Interactions that affect the bioavailability (e.g. absorption or transport through the cell membrane), metabolism, DNA binding, or repair of DNA damage may have pronounced influence on the ultimate genotoxicity of a complex

mixture. These interactions may deviate from additivity (no interactions) and be expressed as synergistic or antagonistic effects due to interactions in either the toxicokinetic and/or toxicodynamic phase.

Although the ultimate target molecule for genotoxic agents is the DNA there are a number of different mechanisms by which chemicals can damage DNA. This makes the prediction of the net outcome of the genotoxicity of complex mixtures very difficult.

7.2.2 Types of damages to the hereditary material (DNA)

7.2.2.1 Primary DNA alterations

Primary DNA alterations can be caused by radiation (UV or ionising radiation) or by reactive electrophilic agents. Chemicals may be either reactive *per se* or be metabolically activated to reactive molecules. These reactive agents can react with nucleophilic centres in DNA and cause different primary DNA alterations (figure 7.2.2.1).

Chemicals may react with numerous sites in all four bases in DNA. However, not all nucleophilic sites have the same reactivity. Ring oxygen and -nitrogen are nucleophilic centres, and in general ring nitrogen atoms are more nucleophilic than ring oxygen atoms, with N^7 in guanine and N^3 in adenine being the most reactive.

Primary DNA lesions include:

DNA Adducts

A large number of chemicals and/or their metabolites are able to bind covalently to DNA forming DNA adducts. Well known examples are polycyclic aromatic hydrocarbons (PAH) like benzo[*a*]pyrene, heterocyclic compounds, aromatic amines, and aflatoxins, just to mention a few groups of compounds that produce bulky adducts. A number of small molecular-size compounds, such as nitrogen mustards and N-nitroso compounds, are able to alkylate DNA. The formation of DNA adducts may subsequently result in mutations in daughter cells or in a number of the following other primary DNA damages.

DNA strand breaks

Single and double stranded DNA breaks may be caused by radiation, reactive chemicals or failure in repair of other DNA damages such as DNA adducts.

DNA base modifications

Alkylating agents can cause DNA base modifications like deamination.

Loss of DNA bases

Alkylating agents and agents forming bulky adducts can cause loss of DNA bases (depurimidation/depyrimidation). Such agents can be either mono-functional or bi-functional (with one or two reactive groups).

DNA cross-links

Inter- or intrastrand cross-links are formed as a result of reaction with bi-functional alkylating agents (with two reactive groups like e.g. mitomycin or nitrogen mustard) with nucleophilic centres in the DNA strands. If the sites of reaction are on opposite polynucleotide strands, *interstrand* DNA cross-links result. If these sites are situated on the same polynucleotide chain, the reaction product is referred to as an *intrastrand* cross-link.

Some chemicals and radiation can *indirectly* cause damage to DNA by forming reactive oxygen species (e.g. the $OH^{\cdot}$ radical) that are able to produce modified bases like 8-hydroxyguanine.

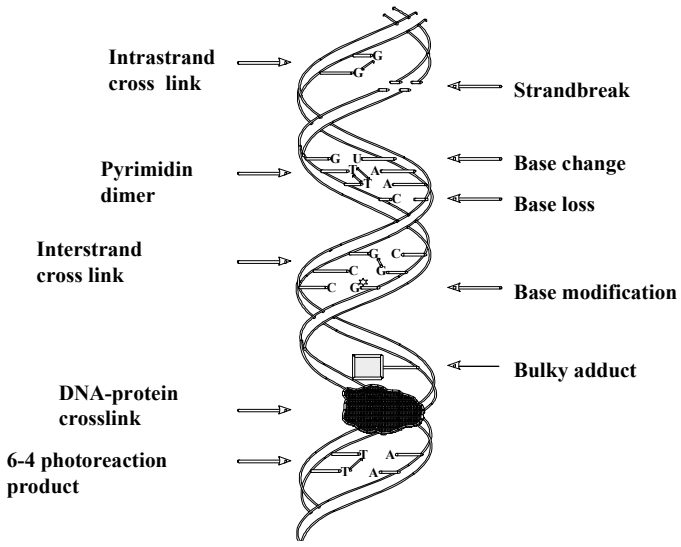


Figure 7.2.2.1. Different primary DNA damages.

7.2.3 Types of mutations

Mutations arise when the primary DNA damages are not repaired or are being misrepaired. Mutations often result in the elimination or alteration of gene functions, and if the damage is not lethal it will lead to inheritable changes. Mutations can vary in character and size and can roughly be divided in:

7.2.3.1 Point (gene) mutations
A gene is the simplest functional unit in the DNA molecule. Gene (or point) mutations are changes in one or a few base pairs in a gene. Base pair, or base substitution, mutations occur when one nucleotide base is replaced with another. Frameshift mutations occur through the addition or deletion of one or a few bases, which alters the reading frame in DNA (and RNA).

7.2.3.2 Chromosomal mutations (structural aberrations)
Chromosomal mutations (structural aberrations) are seen as morphological alterations in the structure of eukaryotic chromosomes. The most common chromosomal aberrations are shown in figure 7.2.3.1. Most of the aberrations seen are lethal to the cell because of loss of vital genetic information or, loss of vital cell functions. However, these aberrations are used as indicators for more biological relevant non-lethal aberrations like small deletions, inversions and translocations (for explanation see “test systems” below). These more subtle changes may have important consequences in both germ cells and somatic cells. The presence of structural (or numerical) aberrations in germ cells can lead to spontaneous abortion, congenital malformation, and infertility. It has been estimated that up to 40% of spontaneous abortions have chromosomal defects, and occur in approximately

0.6% of live births in humans. In somatic cells chromosomal mutations may be involved in processes leading to malignancy. If, for instance, translocations occur at the site of oncogenes their expression can be changed as a consequence of their relocation (Minden 1987). Chromosome deletions and relocations can also lead to elimination of tumour suppressor genes, resulting in malignancy.

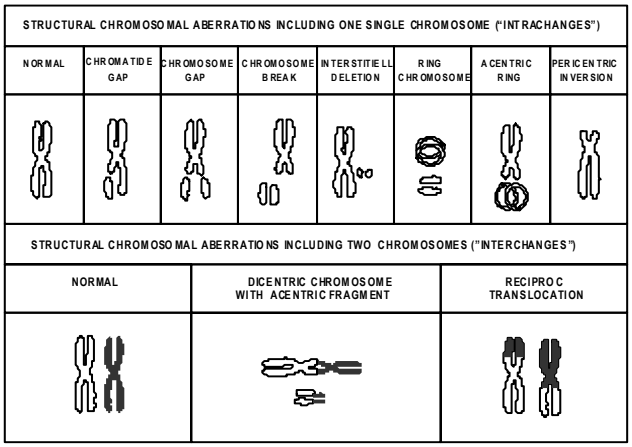


Figure 7.2.3.1. Most commonly induced chromosomal aberrations including one or two chromosomes.

7.2.3.3 Genome mutations (numerical aberrations)

In diploid cells, including mammalian and human cells, interference with normal cell division during meiosis or mitosis may produce genomic mutations, which are the result of changes in the number of chromosomes (aneuploidy). These changes are referred to as "numerical aberrations". Genomic mutations include monosomy where one of a pair of chromosomes is lost, trisomy where a chromosome is added, and polyploidy where the complete set of chromosomes is increased in number. Agents that induce aneuploidy are called "aneugenes".

Genome mutations in germ cells are detrimental to the health of the individual. In humans, well-known genomic mutations include trisomies such as Down´s (trisomi 21) and Kleinfelter´s (XXY) syndromes.
In somatic cells aneuploidy can lead to loss of heterozygosity in e.g. a tumorsuppressor gene, and possibly to development of cancer (Liu etal 1997). The most common cytogenetic aberration in breast tumours is aneuploidy (Devilee & Cornelisse 1994).

7.2.3.4 Recombination

Recombination can be defined as exchanges of genetic material between chromosomes. In germ cells high levels of recombination between homologue chromosomes occur and this is a way to increase the diversity of living organisms. However, homologous mitotic recombinations represent one mechanism by which heterozygous tumour suppressor genes become lost. The analyses of various neoplastic cell lines have provided strong evidence for this mechanism.

7.2.4 Test systems

Short-term bioassays for genotoxic effects have been extensively used as screening tools in the toxicological evaluation of complex mixtures, because they are rapid, inexpensive and sensitive indicators of a sample's potential to induce genetic damage. Since the beginning of the 1970s more than 100 short-term *in vitro tests*, employing bacteria, yeast, fungi and mammalian cells, and *in vivo* tests in plants, insects, earthworms and animals have been developed.

Table 7.2.4.1. The most commonly used guideline tests for genotoxicity.

Assays for measuring primary DNA damage
- DNA adduct formation (no guidelines)
- DNA damage and repair, unscheduled DNA synthesis (UDS) *in vitro* and *in vivo* (OECD guidelines 482 and 486)
- Mitotic recombination in *Saccharomyces cerevisiae* (OECD guideline 481)
- COMET assay *in vitro* and *in vivo* (no guideline)

Assays for measuring the induction of point (gene) mutations
- Bacterial reverse mutation assay (OECD guideline 471):
  *Salmonella/*mammalian microsome assay
  Escherichia coli WP2
- Gene mutations in mammalian cells *in vitro* (OECD guideline 476)
- Point mutations in transgenic animals (e.g. MutaMouse and Big Blue mouse/rat) (no guidelines)

Assay for measuring the induction of chromosomal aberration
- *In vitro* cytogenetic assay (OECD guideline 473)
- *In vivo* cytogenetic assay in somatic cells (OECD guideline 475), and spermatogonia (OECD guideline 483)
- Micronucleus assay *in vitro* (no guideline)
- Micronucleus assay in *vivo* (OECD guideline 474)
- Sister chromatid exchange (SCE) (OECD guideline 479)
- Fluorescent in situ hybridisation (FISH) (no guideline)

Commonly studied endpoints include DNA damage (e.g. strand breaks, DNA adduct formation, DNA repair and DNA recombinations), gene (point) mutations, chromosomal aberrations (CA), sister-chromatid exchange (SCE), micronuclei (MN) and aneuploidy. However, only a few of these test systems are so well validated that they are routinely used and international guidelines exist. Table 7.2.4.1 shows the most commonly used genotoxicity tests. The OECD guideline number is referred to in parenthesis. A few new non-guideline tests are also mentioned, which might be promising tests for future evaluations of complex mixtures for genotoxic potential.

7.2.5 *In vitro* assays

7.2.5.1 Assays measuring primary DNA Damage

DNA adducts

DNA adduct formation is most often measured by direct isolation and identification of the adduct, often using radiolabelled test compound, or by use of the ^{32}P-postlabelling technique.

DNA repair

Unscheduled DNA synthesis (UDS) measures the incorporation of DNA precursors such as tritiated thymidine (^{3}HTdR) at times other than the scheduled, S-phase, synthesis of DNA in the cell cycle. UDS indicates that the compound has damaged

DNA, and that the cell was capable of (correctly or incorrectly) repairing the DNA damage. UDS is most often measured in mammalian cells in culture, usually primary hepatocytes, but may also be measured *in vivo* (see below). DNA repair can also be measured in the comet assay (see below).

7.2.5.2 Assays detecting point mutations

Point mutations in bacteria

The *Salmonella*/mammalian microsome assay (Ames test) is by far the most widely used *in vitro* assay. The *Salmonella* assay is particularly efficient in detecting genotoxic carcinogens that produce *point mutations*, such as aromatic amines, nitro compounds, polycyclic aromatic hydrocarbons, and a number of other chemicals able to interact with DNA. Carcinogens that operate through other mechanisms, such as a number of halogenated compounds or inorganic compounds are not detected in the assay. The *Salmonella* assay has been used to measure the mutagenic activity in samples of numerous complex environmental mixtures (Houk & Waters 1996) including cigarette smoke condensate, automobile exhaust, ambient air, industrial wastes, drinking water, polluted soil and urine from people exposed to potential genotoxins. In addition, this assay has been used to test extracts from food contact materials, such as plastic (Binderup et al 2002a, Feigenbaum *et al.* 2000). In a number of studies bio directed fractionation has been used to identify single genotoxic compounds in the mixture (Marvin 2000, Dobias et al 1999, Marvin 1999, Brooks 1998). Advances in the molecular analyses of mutations in *Salmonella* have suggested that the mutation spectra produced by these complex mixtures are reflective of the predominance of a single (or a few) mutagenic chemical class (DeMarini *et al.* 1993).

Recently, new genetically modified tester strains with specific changes in genes coding for metabolic enzymes or repair genes have been developed. Also, strains with human metabolic enzymes inserted have been constructed. These strains have enhanced sensitivity for specific chemical classes (e.g. nitro- and amino aromatic compounds (Josephy *et al.* 1995), alkylating compounds (Yamada 1997) or compounds inducing oxidative damages (Josephy *et al.* 1997).

Point mutations in mammalian cells

Widely used cell lines include L5178Y mouse lymphoma cells, Chinese hamster ovary (CHO) cells and Chinese hamster V79 cells. Commonly used systems work by selecting for the loss of function of a gene product (enzyme), which is inessential for the survival of the cells in culture. Three selective systems have been widely used in mammalian cell mutagenicity tests:

- Resistance to 6-thioguanine (6TG) and 8-azaguanine resulting from lack of hypoxanthine phosphoribosyl transferase (HPRT) enzyme activity.
- Resistance to trifluorothymidine (TFT) and 5-bromodeoxyuridine (5BrdUrd) resulting from lack of thymidine kinase (TK) activity.
- Resistance to ouabaine (OUA) resulting from lack of Na^+/K^+ ATPase enzyme activity.

7.2.5.3 Cytogenetic assays

Chemicals that induce structural chromosome aberrations are termed “clastogens”, and the test systems used for their detection are called cytogenetic assays. Cytogenetic tests are based upon detection of certain chromosome changes observed in the light microscope. The most commonly used standard cytogenetic assays are chromosomal aberration analyses (CA), sister chromatid exchanges (SCE), and the micronucleus assay (MN).

Structural chromosomal aberrations (CA)

Two types of cells are routinely used, Chinese hamster ovary (CHO) cells and human lymphocytes, which are stimulated to divide *in vitro.* The assay is conducted in the absence and presence of exogenous metabolic activation. To visualise the chromosomes, the cells are arrested in metaphase by adding a spindle inhibitor (e.g. colchicine), stained and examined in the microscope. Large, visible aberrations are recorded. They can be of the "chromatide type" involving one chromatid or "chromosome type" involving both chromatids. Discontinuity in stained regions of chromosomes can be classified as "gaps" or "breaks". Deleted material may appear as fragments in metaphase preparations. Chromosomal breakage is necessary for chromosomal rearrangements. A chromosomal exchange results, when the broken ends of the same or different chromosomes rejoin in an aberrant manner. An inversion results from two breaks in the same chromosome with the broken pieces U-turned before rejoining. A translocation results from an interchange between non-homologous chromosomes. As mentioned above translocations are considered to be some of the most biological significant aberrations, but it is almost impossible to score using current cytogenetic standard techniques. CA can also be measured *in vivo* in different animal species, and have been used in several biomonitorings studies (Sorsa *et al.* 1994, Knudsen *et al.* 1999, Bogadi-Sare *et al.* 1997, Brenner 1996, Tompa *et al.* 1994, Sroczynski *et al.* 1994).

Fluorescence in situ hybridisation (FISH)

Fluorescence techniques such as FISH or "chromosome painting" (Pinkel *et al.* 1988) may significantly improve the methods currently used for studying chronic exposure to low levels of clastogenic agents in complex mixtures. Fluorescent probes have been developed that can identify specific chromosomes or part of chromosomes through *in situ* hybridisation. This technique makes it possible to determine structural chromosomal aberrations like transversions, which, as mentioned above, is almost impossible to measure by conventional cytogenetic assays. Also, aneuploidi, for which no guideline tests exist, can be detected by this method. The limitations of FISH are that only a few chromosomes at a time can be labelled because of restricted ability to differentiate multiple fluorescent signals, and that the availability to specific karyotypes is limited.

Micronucleus assay (MN)

The micronucleus can be performed *in vitro*, and international guidelines will presumably be available in the near future. The test is described below, as an *in vivo* assay.

Sister chromatid exchange (SCE)

The induction of DNA lesions by genotoxic agents leads to the formation of sister chromatid exchanges (SCE), which may be related to recombinational or postreplicational repair of DNA damage. SCE represents the interchange of DNA replication products at apparently homologous loci. The exchange process presumably involves DNA breakage and reunion, although little is known of its molecular basis. SCE are revealed by a "harlequin pattern" of differential stained chromatid segments in chromosomes from cells grown in the presence of BrdUrd (bromodeoxyuridine) for two rounds of DNA replication. After treatment with a spindle inhibitor (e.g. colchicine) to accumulate cells in a metaphase like stage (c-metaphase) cells are harvested and chromosome preparations made. After staining with fluorescence-plus-Giemsa (FPG) technique, so that one chromatid is more lightly stained than the other is, SCE can be observed by conventional light microscopy. SCE can also be measured *in vivo* in a variety of animal species, and have been used as a biomarker in human populations exposed to complex mixtures

(Sorsa *et al.* 1994, Myslak & Kosmider 1997, Karahalil *et al.* 1998, Gonsebatt, *et al.* 1995, Bogadi-Sare *et al.* 1997, Fucic *et al.* 1998).

Mitotic recombination in Saccharomyces cerevisiae
In eukaryotic cells, genetic exchange between homologous chromosomes is generally confined to meiosis. In yeast, recombinational events may also be detected during mitosis, although the spontaneous frequency is generally at least 1000 times less than the meiotic rate. Mitotic recombination can be detected in yeast both between genes and within genes. The former is called mitotic crossing-over and gives reciprocal products whereas the latter, called gene conversion, is mostly a non-reciprocal event. Crossing-over is generally assayed by the production of recessive homozygous colonies or sectors produced in a heterozygous strain, whereas gene conversion is assayed by the production of prototrophic revertants produced in an auxothrophic heteroallelic strain carrying two different defective alleles of the same gene. Mitotic crossing-over producing red and pink homozygous sectors can be assayed in D_5 and D_7, both strains being heteroallelic for complementing alleles of ade 2. D_7 also detects reverse mutations at ilv 1-92. The most commonly used strains for the detection of gene conversion are D_7 (heteroallelic at trp 5) and JD1 (heteroalleic at his 4 and trp 5). Mitotic recombination may also be measured in other fungi such as *Aspergillus nidulans*.

7.2.6 In vivo assays

Short-term *in vivo* tests have also been used to evaluate the genotoxicity of complex mixtures (DeMarini *et al.* 1989, Lewtas *et al.* 1993, Gallagher *et al.* 1993, Gallagher *et al.* 1990). In these types of tests an organic extract of the mixture - or sometimes the crude mixture in itself - is administrated to the whole animal, and, after a few days, tissues or body fluids are collected and evaluated. Indicators of genetic damage include formation of DNA adducts, DNA strand breaks, DNA repair, chromosomal aberration, SCE and detection of micronuclei (structural and numeric chromosomal aberrations). However, only a few *in vivo* genotoxicity tests are validated and currently in use:
Tests which measure structural chromosomal aberrations in somatic cells: CA and MN in bone marrow or MN in peripheral blood, and tests measuring DNA damage and repair: UDS in the liver. CA and UDS have been described as *in vitro* tests; only the micronucleus test will be mentioned here.

7.2.6.1 Micronucleus assay
Micronuclei originate from chromosomal material that has lagged in anaphase and have not travelled to the appropriate pole of the spindle to be included in the main nucleus. The fragments are included in a separate nuclear membrane at telophase, and such a structure is called a micronucleus. Micronuclei consist mainly of acentric fragments, but may also consist of entire lagging chromosomes as a result of spindle disturbances. Fluorescent probes for kinetochores have been developed (Degrassi & Tanzarella 1988) and this allows discrimination between micronuclei with chromosomal fragments and micronuclei with whole chromosomes. Therefore, the micronucleus test can also detect some aneugenes. Micronuclei can occur in any cell type of proliferating tissue. They are, however, most easily recognised in cells lacking the main nucleus, namely erythrocytes. Micronuclei can be scored using flow cytometry, and this technique significantly improves the sensitivity of the method.

7.2.6.2 COMET-assay
The application of the COMET-assay or single cell gel electrophoresis assay (SCG assay) to measure the genotoxic effect of complex environmental mixtures is increasing, and it might be a promising test for future evaluations. Singh *et al.*

(1988) introduced a microgel technique involving electrophoresis under alkaline (pH>13) conditions for detecting DNA damage in single cells. At this pH single (ssb) and double stranded (dsb) DNA breaks and alkali-labile sites (als) (e.g. apurinic sites) can be detected. Only a few cells are needed for analyses. The cells are embedded in agarose on microscopic slides, lysed and before electrophoresis placed in alkaline electrophoresis buffer to produce ssb by DNA separation/denaturation and to express als as ssb. Also, different repair enzymes can be used to improve the sensitivity of the assay (Collins *et al.* 1997). After neutralisation DNA is stained by a DNA specific dye, and the "comets" (DNA migration) are microscopically analysed. The test can be performed either *in vitro* or *in vivo*. A highly significant contribution of the COMET assay to genetic toxicology is its application to *in vivo* studies. As only a small number of cells are required for analyses literately any tissue or organ is amenable for investigation. Also, different species like earthworms (Binderup et al 2002b), mussels, fish and rodents can be used as test organisms.

7.2.6.3 Transgenic animals

During the last ten years short term *in vivo* assays to detect point mutations have been developed. Commonly used target genes are bacterial lacI (Big Blue) and lacZ (MutaMouse). The target genes are via lambda phage incorporated into genome DNA in every single cell of the mouse (or rat). Following exposure to mutagens, the target genes can be rescued from genome DNA prepared from tissues of the treated animal. Using a lambda packaging extract, the shuttle vector (target gene), present in genome DNA, can be "packed" into virulent lambda bacteriophage. The resulting virulent lambda bacteriophage can then infect *E. coli* cells producing bacteriophage plaques on a bacterial lawn. When mutations have occurred in the lacI gene (Big Blue), β-galactosidase is expressed. Presence of the chromogenic substrate X-gal in the bacterial growth medium causes formation of blue plaques, which are detected in a simple visual colour-screening assay. Accordingly, the frequency of mutations can be quantified in any tissue or cell type from which sufficient usable DNA can be recovered for analyses. Furthermore, these mutations can be sequenced so that the molecular nature of the mutation can be detected and its origin inferred.

7.2.7 Interactions between genotoxic substances

A great deal of literature exist on the genotoxic potential of complex environmental mixtures, but only relatively few studies have been performed on the genotoxic activity of mixtures of known chemical composition. Most of these studies have involved interactions between a mutagen and a non-mutagen that either enhances (co-mutagens) or inhibit (anti-mutagens, e.g. antioxidants) the potency of the mutagen. However, a few studies of mixtures of genotoxins with no interaction will be mentioned here.

Arsenic and antimony are proposed to share some toxicological features. Comparative and combined experiments with As(III) and Sb(III) were performed to gain a deeper knowledge of the mechanism of antimony genotoxicity. Both compounds induced micronuclei in human lymphocytes *in vitro.* As(III) was one order of magnitude more potent than Sb(III). The combined genotoxicity in the micronucleus assay of arsenic and antimony seemed best described by simple additivity. Neither the number of micronuclei induced by As(III) nor by Sb(III) could be suppressed by co-incubation with superoxide dismutase or catalase. This suggests that induction of oxidative stress may not be a crucial step in the mechanism of DNA damage induction by arsenic and antimony (Schaumloffel & Gebel 1998).

Four inhibitors of DNA topoisomerases: nalidixic acid, campothecin, m-amsachrine and etoposide, have been evaluated for genotoxic effects in the wing spot test of Drosophila melanogaster. Tested alone only campthothecin and etoposide was significantly genotoxic in this assay. A significant proportion of the total spot induction was due to mitotic recombination. On the other hand, the co-treatment of each topoisomerase inhibitor with the alkylating agent ethyl methansulfonate (EMS) indicate that, while nalidixin acid, m-amsachrine and etoposide show a tendency of antagonistic effect, campothecin shows an additive effect, suggesting mechanistic differences between the activity of the four inhibitors of DNA topoisomerases (Torres *et al.* 1998).

In some complex mixtures especially single class mixtures like PAH containing mixtures, additive interactions may be assumed. Although both synergistic and antagonistic effects have been demonstrated in binary mixtures of different PAH (Hermann 1981, Hughes & Phillips 1990) (se also section on carcinogenesis) linear correlation between mutagenicity in *Salmonella* and PAH content in city air have been demonstrated at lower PAH concentrations, but at high PAH concentrations the mutagenicity increased much more than the PAH content (Nielsen *et al.* 1999). Booth *et al.* (1998) demonstrated linear correlation of DNA adducts in mouse skin *in vivo* with PAH content and mutagenicity in *Salmonella* in a range of petroleum products.

7.2.7.1 Toxicokinetic interactions

Bioavailability

If a substance is not absorbed or cannot pass biological barriers it will not react with DNA. Most chemical compounds are absorbed via a passive diffusion-driven process and interaction with such compounds will normally not be expected. However, some solvents like DMSO are known to facilitate the cellular uptake of some chemical compounds. Interaction can be expected when active transport processes or a specific transporter is involved. An example of the latter is the absorption of cobalt. Iron is known to interfere with the absorption of cobalt in the gastrointestinal tract. Iron deficiency increases the absorption of cobalt, thus, simultaneous administration of ion reduces cobalt absorption (Elinder & Friberg 1986). Also, amino acids and proteins in the diet reduce the absorption of cobalt, since both amino acids and sulfhydryl groups complex with cobalt ions. It has also been shown that the genotoxic effect of cobalt is reduced with addition of the amino acid adenine (Binderup 1993, Binderup 1999), these antagonistic effect might be due to a decrease in the absorption of the cobalt-adenine complex compared to the cobalt ion.

Biotransformation

Many genotoxic compounds must undergo biotransformation before they can react with DNA, and some observed species differences in susceptibility to mutagens/carcinogens have a metabolic basis. Also individual susceptibility among humans can be due to differences in metabolism.

Cytochrome P-450 enzymes carry out many of the reactions that convert stable promutagens to electrophilic, reactive agents. Multiple cyt. P-450 isozymes exist with different substrate specificities or differences in their distribution among organs, species, and individuals. However most chemicals are enzymatically deactivated in the organism, while rather non-polar, lipid-soluble compounds are converted by phase I enzymes (eg oxidised by cyt. P450) to more polar and water soluble compounds which are more readily excreted. The oxidation product may be further metabolised by phase II enzymes (e.g., by hydroxylation, sulphuronidation or glucuronidation). Competition between metabolic activation and detoxification processes can strongly influence the genotoxic effect of complex mixtures, as some

chemicals in the mixture can activate different enzymes, and thereby modify the genotoxic response of other compounds by activation/deactivation. Some examples are given below.

Activation of the hepatocarcinogen 2,6 dinitrotoluene (DNT) is a process involving both hepatic and intestinal enzymes (Chadwick 1995). Pre-treatment of Fischer 344 rats with Aroclor 1254 or creosote (a complex mixture of PAH, phenols and heterocyclic compounds), potentiate the genotoxic effect of DNT, measured as hepatic DNA adduct formation. Also a higher level of DNT related mutagenic metabolites were excreted in urine after pre-treatment with either of the two complex mixtures. Using the ^{32}P-postlabeling technique three of fourDNT derived DNA adducts were significantly increased in rats treated with creosote compared to animals treated with DNT alone. Data from the study indicated that the creosote-DNT interaction resulted from altered enzyme activity in the intestinal tract as well as in the liver. Optimum bioactivation of DNT requires:

- reduced nitroreductase (NR) activity in the small intestine (NR deactivate DNT in the small intestine before it is absorbed, therefore reduced activity permits more unaltered DNT to reach the liver for biactivation of hepatic enzymes)
- elevated caecal ß-glucuronidase activity (2,6-dinitrobenzoyl alcohol glucuronide is a key intermediate in the bioactivation of DNT, while elevated ß-glucuronidase activity will release more DNT metabolites for further activation).
- elevated hepatic cyt-P450 isozymes (Aroclor 1254 is a well known inducer of different cyt-P450 isozymes and creosote is reported to induce a 50 fold increase in cyt-P450 activity (Schoor *et al.* 1991).

Combinations of arylamines and organophosphate isomers have, for instance, been studied in the BHK cell transformation system. A supraadditive response was generally obtained, in spite that the compounds would be expected to act in a simple additive way. It was concluded that the potentiation most probably was caused by competitive inhibition of cellular detoxification processes (Ashby & Styles 1980). Pre-treatment of Chinese hamster V79 cells with N-acetylcystein increases the intracellular level of glutathione, and this implies increased levels of mutagenicity of MNNG (N-methyl-N-nitro-N-nitrosoguanidine), which is activated by intracellular thiol or amino groups (Romert & Jenssen 1987). More often, genotoxic effects are decreased by increases in the intracellular levels of glutathione. The mutagenic effects of benzo[*a*]pyrene is, for instance, decreased by glutathione-S-transferase mediated conjugation with glutathione. Rat hepatoma cells (H4IIE) are able to detoxify benzo[*a*]pyrene, but MCF-7 cells (human mammary carcinoma cells) have been shown to fail to form the conjugates and they do not detoxify benzo[*a*]pyrene (Jenssen 1986). Depletion of glutathione does most probably preferentially serve as a basis for synergistic effects concerning genotoxic compounds.

Peroxisome proliferators constitute structurally disparate groups of rodent hepatocarcinogens. It is generally accepted that these compounds are epigenetic carcinogens. They are known to induce various isoforms of cytochrome P-450 (CYP2B1/2 and CYP4A1) and at the same time depress GST activities. Cyclophosphamide is an indirect acting mutagen, which requires activation of CYP2B1/2. The reactive electrophilic metabolite is subsequently detoxified by glutathione S-transferase. The peroxisome proliferator nafenopin potentiated the cytotoxicity and genotoxicity of cyclophosphamide both in the liver and bone marrow (Voskoboinik 1997). Although the mechanisms of these synergistic effects are not fully understood, there is evidence that the modulation of

cyclophosphamide-metabolising enzymes by nafenopin was enough to potentiate the toxicity of cyclophosphamide.

7.2.7.2 Toxicodynamic interactions

DNA interaction and binding

As mentioned earlier reactive electrophilic chemicals or compounds, which are metabolically activated to electrophiles, can react with DNA and cause DNA damage, which, if the damage is misrepaired, can lead to mutations. Because different electrophiles will attack different positions in DNA additive or synergistic effects are most likely when the reactive species behave very similarly. This can be exemplified by a study with quite similar "cooked-food" mutagens, which showed that the combination effect of these mutagens was additive at high doses but synergistic at lower doses (Ito *et al.* 1991).

However, the DNA binding effect of genotoxic chemical compounds can be enhanced when other compounds alter the DNA conformation. Sakai (1994) has shown that the DNA binding of the genotoxic and carcinogenic 1-nitropyrene (1-NP) was enhanced by zinc acetate. It was suggested that the enhancing effect was due to alteration of the DNA conformation from the B-form to the Z-form making the binding of 1-NP easier. The main components of hard metals cobalt (Co) and tungsten carbide (WC) and Co-WC mixtures have been investigated for genotoxic effect in several experiments. In one *in vitro* study (Van Goethem *et al.* 1997) with human leukocytes Co-WC showed synergistic effect at the highest dose tested measured as DNA strand breaks in the COMET-assay. This might suggest that the carbide allowed some uncoiling of the chromatin loops or induced the formation of slowly migrating DNA fragments (Anard *et al.* 1997). By uncoiling the chromatin, WC might amplify the clastogenic effect of cobalt in the mixture.

Classes of genotoxins that interact with DNA by intercalation may have altered genotoxic responses in cells that contain pre-existing adducts, which can severely distort normal DNA conformation. Results presented by (Said *et al.* 1999) suggest that consecutive exposure to genotoxins may not always give rise to additive effects, especially if the mixture contains different classes of genotoxins. Aflatoxin B_1-8,9-epoxide (AFB_1-8,9-epoxide) and N-acetoxy-acetylaminofluorene (N-AcO-AAF), both direct acting mutagens, were tested in the *Salmonella*/mammalian microsome assay in the strains TA98 sensitive to (N-AcO-AAF) and TA100, sensitive to AFB_1-8,9-epoxide. Pre-treatment of the frameshift strain with the basepair-substitution mutagen AFB_1-8,9-epoxide enhanced the mutagenicity induced by subsequent exposure to the frameshift mutagen N-AcO-AAF ~2-3times above theoretical values for aditivity. Pre-treatment of base substitution strain TA100 with N-AcO-AAF inhibited the mutagenicity following exposure of AFB_1-8,9-epoxide by a factor 3 below the theoretical additive value. The mechanism behind these interactions is still not clear. One possible explanation for the inhibitory effect of N-AcO-AAF on the mutagenicity of AFB_1-8,9-epoxide in TA100 could be, that prior adduction of *Salmonella* genomic DNA by N-AcO-AAF reduced the number of binding sites available for reaction with AFB1-8,9-epoxide. This could be due to steric interference and/or conformational alterations in the DNA polymer, which precluded reaction. Such an interaction was described by Ross *et al.* (1999). Conversely it was found that initial modification of DNA by AFB_1-8,9-epoxide did not alter subsequent binding of N-AcO-AAF.

DNA repair

In general, two fundamental reactions are involved in cellular responses to DNA damage: repair of DNA damage and tolerance to DNA damage. Most DNA alterations are quickly corrected by a variety of repair processes. DNA repair may be defined as those cellular responses associated with the restoration of normal

nucleotide sequences and stereochemistry of DNA. The major forms of DNA repair include direct reversal of DNA damage excision repair and post replication repair.

In the simplest form of DNA-repair, *direct reversal*, one enzyme catalyzes a single reaction, such as *photo reactivation* of pyrimidine dimers induced by UV irradiation. Two different types of excision repair are considered. *Base excision repair* removes smaller types of base damage such as lesions caused by monofunctional alkylating agents e.g. 7-methylguanine and 3-methyladenine. *Nucleotide excision repair* is a more versatile repair pathway, which operates in cellular responses to a large number of various lesions (e.g. bulky DNA-adducts, like DNA-PAH adducts, and ionizing radiation). *Post replication repair*: More complex damage, such as DNA-DNA interstrand crosslinks and double-strand breaks, may require repair of both DNA strands. It appears that interstrand-crosslinks are repaired by a combination of nucleotide excision and recombinational/post-replication repair. This repair pathway involves interchanges between alleles and the mechanism is rather a mechanism for *tolerance* to DNA damage than for *repair* of DNA damage, as this repair often results in misrepair of the lesion.

Modulation of DNA repair

Numerous possibilities exists for chemicals to enhance or inhibit the genotoxic effects of other substances through modulation of DNA repair processes, since most DNA alterations can be repaired by different repair mechanisms, which can be saturated, limited or subject to mistakes. The reparability of DNA lesions in mammalian cells depends moreover upon the type of lesion, the localisation of the lesion in the genome, and the functional state of the DNA in question (Hanawalt 1986).

Modulation of the genotoxic response of mammalian cells has in many cases been shown to be mediated by influence on different repair systems. Brown *et al.* (1979) have, for example, measured DNA excision repair in cultured human fibroblasts after single or dual treatments with ultraviolet radiation, 4-nitroquinoline 1-oxide (4NQO), or N-acetoxy-2-acetylaminofluorene (AAAF). Three approaches were used to monitor repair: unscheduled DNA synthesis, measured by autoradiography; repair replication, measured by the incorporation of a density-labelled DNA precursor into repaired regions and excision of ultraviolet endonuclease-sensitive sites. When a single repair-saturating dose of one of the three carcinogens was administered, little stimulation of unscheduled DNA synthesis or repair replication could be observed by additional treatment with one of the other carcinogens. In no instance was total additivity of repair observed. These observations were confirmed by showing that the excision of endonuclease-sensitive sites produced by ultraviolet damage (i.e., pyrimidine dimers) was inhibited by exposure to 4-nitroquinoline 1-oxide and N-acetoxy-2-acetylaminofluorene. The data indicate that the repair of lesions induced by these substances may have common rate-limiting steps, a conclusion previously indicated by the repair deficiency in xeroderma pigmentosum cells in which a single mutation eliminates the repair of damage caused by each of these agents. Ahmed & Setlow (1981) have later measured the excision repair of DNA damage by the photolysis of bromodeoxy-uridine incorporated in the DNA during repair in normal human and xeroderma pigmentosum fibroblasts treated with a combination of the AAAF and 4NQO. Repair was additive in both types of cells treated with AAAF plus 4NQO, but the results indicated that there are different rate limiting steps for removal of 4NQO and AAAF lesions.

Nickel compounds are carcinogenic to humans and animals. However, the mechanism leading to tumour formation is still not fully understood since the mutagenic potential is rather weak. Nickel(II) is mostly non-mutagenic in bacterial test systems and only weakly mutagenic in mammalian cell lines. In contrast to the weak mutagenic activity Ni(II) enhance cytotoxicity and mutagenicity of several other DNA damaging agents. In mammalian cells in culture Ni(II) enhanced the UV-induced cytoxicity, mutagenicity and SCE in Chinese hamster V79 cells (Christie 1989). In combination with benzo(a)pyrene, nickel(II) enhances the frequency of mutations and cell transformation in Syrian hamster embryo cells (Rivedal & Sanner 1980). A possible mechanism for the synergistic effect of Ni(II) could be interference with excision repair processes. Hartwig *et al.* (1994) used UVC light to study the interaction of Ni(II) with DNA repair. By different techniques they were able to show that non-cytotoxic doses of Ni(II) delayed the incision step in nucleotide excision repair in mammalian cells after low, biological relevant UV doses. The interference with DNA repair is partly reversible by the addition of magnesium(II), providing further evidence that the competition with essential metal ion may be an important mechanism for the toxic action of Ni(II). Similar interactions with DNA repair of UV-induced DNA damage have been observed with other carcinogenic and/or mutagenic metal ions.

Co(II) is weakly mutagenic at the HPRT locus and enhances the frequency of SCE in Chinese hamster V79 cells. Additionally, at both endpoints the metal ion enhanced the genotoxicity of UV light. Analyses of the kinetics of strand-break induction and closure after UV irradiation by nucleotide sedimentation reveals an accumulation of strand breaks in the presence of Co(II). This indicates that either the polymerisation or ligation step in excision is affected (Hartwig 1991). It has also been shown that Co(II), at non-cytotoxic doses, inhibits both the incision and polymerisation step of the repair of UV induced DNA lesions, as measured by the alkaline unwinding technique in human fibroblasts (Kasten *et al.* 1997). It was demonstrated by competition experiments that one possible mechanism is the exchange of essential magnesium (II) ions by cobalt (II), since the cobalt(II) induced inhibition of the polymerisation step was completely reversed by the addition of magnesium(II).

Using the COMET assay on isolated human lymphocytes De Boeck et al (1998) could demonstrate that cobalt metal was able to inhibit the repair of methylmethansulfonate (MMS) induced DNA damage. The majority of the MMS induced lesions are processed via the base excision-repair pathway. After recognition and excision of the MMS-alkylated bases, an apurinic site (AP) occurs as intermediate. When cobalt inhibits the action of the polymerase, the persisting alkali labile AP sites will give rise to single strand breaks, which will result in DNA fragmentation /migration in the COMET assay.

An analysis of DNA strand breaks by alkaline elution indicates that DNA repair of Chinese hamster ovary cells treated with MMS was inhibited by sodium arsenite (Lee-Chen 1993). The enhancing effect was mainly due to the inhibition of the excision of alkali-labile sites.

The above-mentioned investigations indicate that interaction with DNA repair might be a common mechanism of metal genotoxicity.

It is important to note that large differences exist in the efficiency of various DNA repair systems between different types of mammalian cells, e.g. between rodent cells and human cells. Chinese hamster ovary (CHO) cells clearly differ from human cells concerning the relative resistance to methylating and ethylating agents, and the persistence of the chromosomal aberrations induced by such agents. CHO

cells are deficient in O^6 alkyl guanine-DNA-alkyl-transferase, but the differences in resistance to ethylating substances may moreover be due to a more efficient excision repair system in human cells (Galloway 1994).

DNA replication

Changes in the fidelity of the replication of DNA are another source to combinational effects concerning genotoxic compounds. Errors during DNA replication may, for example, be caused by imbalances of the intracellular DNA precursor pool. In this case, the repair system is unable to function because the right nucleotides are missing. Exposure to hydroxyurea inhibits the deoxyribonucleotide reductase thereby giving imbalance of the nucleotide pool. A dramatic enhancement of the mutagenic effect of alkylating agents has, for example, been observed after co-exposure with hydroxyurea (Walum *et al.* 1990).

Oxidative damage

In general, metal genotoxicity appears to follow two predominant modes of action, i.e. interaction with DNA repair processes (described above) and induction of oxidative damage. Co(II) is known as a transition metal and can therefore, after reaction with cellular H_2O_2, generate active oxygen species (AOS) in a Fenton-like reaction.

Hard metals (WC-Co) are made of a mixture of cobalt metal (5-10%) and wolfram-carbide particles (WC>80%). Excessive inhalation of WC-Co is associated with the occurrence of different lung diseases including an excess of lung cancers. In human peripheral lymphocytes Co powder and WC-Co both induced DNA single strand breaks (ssb) measured by the alkaline COMET and alkaline elution assay (Anard *et al.* 1997). On the basis of equivalent cobalt-WC content, WC-Co produced significantly more ssb than Co. WC alone did not produce ssb. There is strong experimental evidence (Lison *et al.* 1995) that the interactive (geno)toxicity of WC-Co is mediated by AOS produced by the association of Co and WC but not by Co or WC alone. Anard *et al.* (1997) showed that 1 M formate, a hydroxyl scavenger had a protective effect against the production of ssb by WC-Co particles, which is consistent with the implication of an increased production of hydroxyl radicals when Co is mixed with WC particles.

Di-(ethylhexyl)phthalate) (DEHP) is a plasticizer used in the production of PVC plastics products. DEHP is assumed to be a non-genotoxic carcinogen, acting as a peroxisome proliferator. DEHP is not genotoxic in *Drosophila melanogaster*, on the other hand DEHP enhances the genotoxic effect (acts as a co-mutagen) of N-nitrosodimethylamine (NDMA) in this test organism measured as DNA-dsb. It was assumed that the co-mutagenic effect of DEHP was caused by oxygen radicals generated by DEHP (Kawai 1998).

Cell cycle regulation

Some clastogenic agents, e.g. ionising radiation and various cytostatic drugs such as bleomycin, are able to induce double strand breaks at any time in the cell cycle. When the cells are exposed prior to DNA synthesis phase (S-phase) the aberrations are of chromosome-type. When the cells are exposed after the S-phase, the aberrations are of chromatid-type. Most clastogens, however, are S-phase dependant. The breaks will manifest as chromatid-type aberrations in the subsequent metaphase. Many cytotoxic chemicals do prolong the cell cycle time. This is a potential basis for antagonistic effects, since the longer the period between exposure and DNA synthesis, the more chance the cell has to repair the damage.

7.2.8 Conclusion

As shown in this review combined effects can be due to interaction on various biological processes of different chemicals that are not necessarily genotoxic as such. Interactions of simple mixtures (binary or tertiary) have been reviewed in order to illustrate possible mechanisms of combined effects. However, humans are exposed to a broad range of more complex chemical mixtures, mostly of unknown composition, and interactions of such mixtures are very difficult to predict. Risk assessment associated with complex mixtures is difficult if not impossible without detailed chemical characterisation. However, the varieties of chemical agents in a complex mixture (e.g. automobile exhaust, polluted water or soil or extracts from food packaging material) could be countless. Therefore, extensive chemical analyses of the micro-quantities and costly bioassays on the individual agents seem to be a waste of resources, because the biological effects of the mixtures are often quite different from the predicted effects of the individual agent tested singularly. Biologically based characterisation of a complex mixture, however, allows direct measurement of a specific toxicological endpoint in a biological system. Several of the above mentioned studies illustrate that bioassays are useful for detecting interactions of binary mixtures. Bioassays have also been used to interpret the interactions of components of complex mixtures and are a valuable tool for risk assessment especially in combination with chemical analyses and fractionation (see section on bioassay-directed fractionation). This approach have been used for risk assessment of many complex environmental mixtures (Marvin *et al.* 1999, Marvin *et al.* 2000, Brooks, *et al.* 1998, Dobias *et al.* 1999 , Casellas *et al.* 1995, Houk & Waters 1996).

Genotoxicity might be the most frequently studied effect of complex mixtures, and many of the above-mentioned test-systems have been used for screening potential genotoxic effect of complex environmental mixtures. The *Salmonella*/mammalian microsome assay is applied most often. This test system has also been used to test extracts from food contact materials (Binderup *et al.* 2002a). Although some of the existing short-term genotoxicity tests are quit fast and easy to perform there is a need for even faster and easier screenings tests. Some "high-throughput" genotoxicity tests have been used as screening tests for genotoxins in complex mixtures: The UMU/SOS test (Hamer *et al.* 2000) Mutatox (Jarvis *et al.* 1998, Hauser *et al.* 1997) and Vitotox (Verschaeve *et al.* 1999). However, these test systems need further validation.

Due to the potential differences in the biological processes in *vitro* and *in vivo,* combined effects demonstrated *in vitro* should be confirmed *in vivo*. Of the tests systems mentioned above the COMET-assay *in vivo* and genotoxicity tests in transgenic animals might be promising *in vivo* tests for future evaluation of complex mixtures.

Also, biological monitoring is a valuable tool for genetic risk assessment of complex mixtures. CA, SCE, MN and UDS are some of the test systems conventionally used in biological monitoring studies. For future risk assessment research on complex mixtures new molecular technologies have enabled direct investigation of the effect of exposures to genetic toxicants on the structure and function of DNA. Key mutational events in cancer causation (e.g., mutation in the tumour suppresser gene *p53*) are rapidly being discovered. These findings present unique aids in cancer diagnostic, useful biomarkers for cancer epidemiology, and tools for the development of new biologically based risk assessment models.

7.3 Carcinogenicity

Prepared by Lars Ove Dragsted

7.3.1 Introduction

Cancer has been known for more than 50 years to be the result of combinational toxicology. In the nineteen forties Berenblum and co-workers published their famous papers showing that chemical carcinogenesis can be divided into two distinct processes, initiation and what was later called promotion (Berenblum, 1941a; Berenblum and Shubik, 1947). Moreover, very different compounds were shown to affect either process. But even some years earlier, Twort and Twort (Twort and Twort, 1939) had shown that the carcinogenic effect of polycyclic aromatic hydrocarbons (PAHs) was dependent upon the vehicle by which they were applied, thereby indicating the possible existence of co-carcinogenesis. In fact, the first combination effect in carcinogenesis was noted as early as 1924 by Deelman, who found that scarification of the skin enhanced mineral oil carcinogenesis in the mouse skin model (Deelman, 1924). Early observations on anticarcinogenesis date back to 1925, when Nakahara found that oleic acid injections decreased spontaneous and grafted mammary tumours in mice (Nakahara, 1925).
Several reviews, reports and international consensus documents have been authored through the years on the issue of carcinogen mechanisms and interactions. Some recent publications from international bodies are (Joint WCRF and AICR study group, 1997; Greenwald and Kelloff, 1996; Kelloff *et al.*, 1996; Wattenberg, 1996).

7.3.2 Combination effects in initiation

7.3.2.1 Initiation
Initiation is believed to be caused by changes in the cellular genetic material causing changes in the response of a cell to the regulation of cell turnover, including division and apoptosis. Clearly, compounds causing mutations or gene rearrangements in a target cell will also be potential tumour initiators. Initiation causes an altered response to external stimuli resulting in cell growth or apoptosis. This makes the initiated cell vulnerable to abnormal division or to escape of signals for programmed cell death, and these abnormal responses can be affected by a range of internal or external stimuli including the influence of chemical, physical and biological agents (see tumour promotion).

No formal test systems exist to test for cancer initiation. The most widely reported systems are the initiation-promotion protocols for mouse skin and rat liver tumour formation. In the mouse skin test, the test compound is applied to the shaved back of a suitable mouse strain such as the Swiss or NMRI mouse or even the hyper-sensitive Sencar mouse, and a tumour promoter is subsequently applied twice a week thereafter until tumours are scored at around 10 weeks or later. Such a system has successfully been applied to show the initiating effects of the weak carcinogen urethane (Salaman and Roe, 1953). The rat liver system can be performed in several variants (Autrup and Dragsted, 1987), which will not be detailed here. Briefly the test compound is given orally or intraperitoneally, and liver growth is induced by partial hepatectomy and/or by treatment with one of several chemicals increasing liver cell turnover.

Since no compounds are known to cause mutations *in vivo* without being initiating carcinogens, the OECD-guideline tests for these effects (see section on mutagenesis) could be used to identify some initiating carcinogens.

More recently, the newborn mouse and rat assays have become popular. The animals are treated with the test compound at the age of one day and one or two weeks intraperitoneally, and tumours are scored at about six to eight weeks later. Since these assays use the natural growth of the animals to enhance the effects of the test compounds they may be presumed to be extremely sensitive to tumour initiators. However they are not operationally defined as test systems to identify initiating compounds.

Several initiators may work in concert to elicit a response, which is stronger or weaker than that of the expected (additive) effect. A stronger response has been observed with mixtures of heterocyclic aromatic amines (HAAs) from fried meat in rat liver tumourigenesis (Hasegawa *et al.*, 1996a; Hasegawa *et al.*, 1996b), whereas both stronger (synergistic) and weaker (antagonistic) responses have been observed with PAH mixtures (Iversen, 1994; Warshawsky *et al.*, 1993; Horton and Christian, 1974). Such modulated responses can be caused by several mechanisms. The most straightforward is interaction at the level of uptake, where compounds may compete during absorption. A similar mechanism is competition at the level of enzymatic metabolism and activation. Competition for activating enzymes may direct a larger proportion of the dose to detoxication pathways and vice versa. Moreover, there may be threshold doses for enzyme induction leading to increased activation or deactivation. Such mechanisms can also lead to non-linearity of the dose-response curves for individual compounds.

Initiators acting by different genetic mechanisms may also act synergistically. This is often used for chemotherapy, where genotoxic compounds leading to mutation act in synergy with spindle poisons and topoisomerase inhibitors (Fukuda *et al.*, 1996; Raymond *et al.*, 1996).

Formal guideline tests for initiation do not exist, but a range of models has been used in the scientific literature and could be used to inspire the setup of formal tests for initiation and co-initiation. Genotoxic compounds (see chapter on mutagenesis) are potential tumour initiators, and when such compounds are found also to be carcinogenic they are often described as genotoxic carcinogens.

7.3.2.2 Co-carcinogenesis (co-initiation)

Co-carcinogenesis is defined as a mechanism by which a non-carcinogen increases the action of a carcinogen, most often an initiating carcinogen. No formal test systems exist for this effect. Co-carcinogenesis can be affected by at least three distinct mechanisms. One is by compounds, which increase the penetration of carcinogenes through epithelial barriers. Examples include organic solvents, which help PAHs through the skin barrier, and fatty diets, which increase the uptake of unpolar substances through the gut wall. Dimethylsulphoxide (DMSO) is well known as a solvent which destroys the skin barrier and increases the penetration of toxic compounds, and it has also been shown to increase the tumourigenic response to benzo[*a*]pyrene, but the solvent has several effects in skin, and DMSO can therefore be both increase and decrease carcinogenesis (Jacoby and Weiss, 1986). The DMSO-induced decrease seems to be mainly related to the promotion phase of carcinogenesis, however.

Co-carcinogenesis can also be elicited by compounds, which increase the activation or the impact of genetic damage of the initiator. Benzo[*e*]pyrene is a well-known example, acing as a co-carcinogen with benzo[a]pyrene by increasing

its activation and DNA-binding (Lau and Baird, 1992). If mixtures of polycyclic aromatic hydrocarbons (e.g. tars and mineral oil residues) are applied together with benzo[*a*]pyrene they generally reduce its carcinogenic potency by decreasing the fraction binding to DNA (Springer *et al.*, 1989). As mentioned above, compounds, which induce the relevant activating enzymes for a given carcinogen, can be cocarconogenic. Likewise can co-carcinogens be compounds, which deplete natural scavengers of electrophiles such as glutathione depleters or antioxidant depleters, or compounds, which inhibit the ability of DNA-repair and functionality. 5-Azacytidine an analog of cytidine which is incapable of being methylated increases benzo[*a*]pyrene mediated initiation by increasing the expression of repaired genes thereby effectively increasing the expression of mutated genes (Denda *et al.*, 1985).

Finally, co-carcinogens can act by increasing cell turnover. Compounds with irritant actions on the tissue cause increased cell turnover thereby increasing the number of cells undergoing division and decreasing the time for DNA repair between cell cycles. Dividing cells are much more vulnerable to mutation than resting cells. The classical effect of skin co-carcinogenesis by scarification and liver co-carcinogenesis by partial hepatectomy are caused by this mechanism (Mottram, 1944; Deelman, 1924). Among chemical and physical agents acting as co-carcinogens by this mechanism are phorbol esters, catechol and asbestos (Kimizuka *et al.*, 1987; Van Duuren *et al.*, 1986). The co-carcinogenic action on formaldehyde carcinogenesis in the nasal epithelium of the rat by corrosive hydrochloric acid fumes is a classical example from carcinogenesis testing (Sellakumar *et al.*, 1985). The increased risk of breast and possibly colorectal cancers with the median height (growth during adolescence) of a population may also be caused partly by this mechanism (Joint WCRF and AICR study group, 1997).

In conclusion, several combination effects exist in the earliest phase of carcinogenesis. Some of these effects are specific in the sense that a certain co-carcinogen will only have its action with one group of carcinogens, whereas the effect may be nil or even opposite with other carcinogens. This will be the case with enzyme inducers and other compounds affecting specific mechanisms of distribution, activation, and repair. The example of synergy between benzopyrenes and tars is a good example. Other effects are of a more general nature, e.g. co-carcinogens that increase the penetration of skin such as detergents and defatting solvents, and solvents such as DMSO, which penetrate both polar and non-polar barriers. Another group of compounds with a more general effect on co-carcinogenesis may be skin and lung irritants such as corrosives, particles and fumes. The effect of such compounds is most often weak, but in combined occupational or environmental exposures they may still be significant, albeit difficult to measure.

7.3.3 Combination effects in promotion

Promotion has been defined for several years as an operational term, i.e. a tumour promoter is a compound that increases the response in an experimental initiation-promotion protocol when dosed repeatedly after the initiator. Moreover, the effect of the promoter was found to be reversible as opposed to the effect of the initiator. The traditional test systems already outlined above under initiation, the mouse skin assay and the rat liver assay, have been the most widely used assays for tumour promotion, but there exist no formal outlines for how to perform them (Autrup and Dragsted, 1987).

In a more mechanistic sense, promotion can be viewed as any process, which gives the initiated cell a growth advantage over normal cells. If initiation is caused by a mutation leading to partial loss of a protein involved in termination of the cell cycle, almost any cell growth stimulus will lead to more cycles of cell growth in initiated cells than in neighbouring normal cells. In this sense tumour promotion is quite similar to co-carcinogenesis and some authors have reviewed these effects together (DiGiovanni, 1991; Stara *et al.*, 1983).

Since the total apparatus involved in cell cycle control has not been mapped yet, it is very likely that compounds acting by different mechanisms to elicit cell growth stimuli to the same target tissue may have potentiating effects on each other. However, the numbers of examples are relatively few. In the early 1980ies a range of studies were performed by Slaga and co-workers showing that tumour promotion in the classical operational sense can be subdivided into phase 1 and phase 2 promotion. The first of these phases was found to be partially irreversible and very similar to co-initiation, whereas the second phase was truly reversible (Slaga, 1983; Slaga, 1984). For example weak tumour promoters like the pro-oxidant agent, hydrogen peroxide, and the calcium ionophore, A23187, are most effective at stage I and almost unable to stimulate stage II, whereas the experimental tumour promoter 12-tetradecanoylphorbol-13-acetate (TPA) is highly effective at both stages (Slaga, 1984).

The evaluation of combination effects and reversibility in carcinogenesis has been complicated by the early termination of most experiments after the induction of benign neoplasms. There may be no correlation between the number of benign lesion and the observable malignancy at a later time point. This has been observed both in the mouse skin model and in the rat liver. Combination effects at the promotion stage should therefore preferably be observed as increased malignancy. This decreases the number of examples drastically but the very early studies with croton oil (containing TPA) are still valid since malignant tumours were often reported in these studies (Berenblum, 1941b). Examples of possible combination effects in promotion may therefore be taken more appropriately from epidemiological evidence. It has been shown that long-term smoking leads to major histological changes and loss of ciliated cells (Verra *et al.*, 1995; Kaidoglou *et al.*, 1991) indicating a strong effect on the turn-over of many cell types in the bronchial tissue. Moreover, the lung tumour risk from smoking seems to be partially reversible upon cessation (Halpern *et al.*, 1993), indicating that promotion both in a mechanistically sense and in an operational sense is a strong element in smoking-induced bronchogenic carcinoma. In a case-control study evidence was presented for a strong effect even from intermittent smoking cessation (Becher *et al.*, 1991), an observation also seen in experimental skin initiation-promotion studies, where too long intervals between treatments with promoters decreases or abolishes their action (Boutwell, 1964). Such decreases are not caused by DNA repair since the level of adducts is usually not affected by smoking cessation (Eder, 1999). The synergistic effects of smoking and the exposure to several kinds of fibre, including asbestos, have been known for some time. This synergism may be caused by a combination effect at the promotional level (Kimizuka *et al.*, 1987). The combined effects of smoking and alcohol (Longnecker and Enger, 1996) on oesophagus cancer as well as aflatoxin and hepatitis on liver cancer (Yu *et al.*, 1996) may be other examples. Aflatoxin and alcohol are also known to synergistically increase the risk of liver cancer but in this case alcohol works partly by increasing the number of aflatoxin-DNA adducts, i.e. as a co-initiator (Yu *et al.*, 1996).

In conclusion, no particular toxic effect and no specific group of chemical compounds can be pointed out as enhancers of the action of tumour promoters. On the other hand is it clear from the examples mentioned above that promoters may

act in synergy when they elicit their effect by different mechanisms. As in the case of initiation, no formal guidelines exist for testing of promotion. The identification of tumour promoters is complicated by the fact that they may be organ specific and, possibly, initiator specific. However, good models seem to exist for mouse skin, rat liver and rat bladder (Autrup and Dragsted, 1987).

7.3.4 Combination effect at later stages

7.3.4.1 Conversion

It has been shown by Hennings et al. (Hennings, 1991) that the tumour response after initiation and promotion may be enhanced further in the mouse skin by subsequent treatments with a direct-acting genotoxic compound. Hennings termed this effect, conversion. Similar results were observed in rat liver carcinogenesis by Scherer and co-workers (Scherer *et al.*, 1984; Scherer, 1984), and the effect is also known from *in vitro* transformation of "normal" cells to malignant cells (see section on mutagenesis). Thus, two terms, conversion and transformation may be used for this effect. No formal test systems exist for conversion testing although the methods of Hennings and of Scherer cited above may be generally applicable. The genotoxins active in conversion or transformation may increase the risk of cells loosing the second allele of the gene targeted by the initiation treatment. This risk is much larger after promotion since tumour promoters increase dramatically the pool of initiated (daughter) cells with identical genetic lesions. It is therefore likely that many genotoxic compounds are not only initiators but also converters leading to the formation of "the first" malignant cell in the tumourigenic process.
No studies have been concerned with interactions at this stage of carcinogenesis, but most of the observations related to initiation may be relevant here as well, i.e. co-initiation would be similar to co-conversion. In humans the enhancing effects of beta-carotene on smoking-induced cancers of the lung at a very late stage (Omenn *et al.*, 1996; ATBC cancer prevention study group, 1994) may be an example of interaction at the conversion level, but the mechanism behind the effect is still obscure. No formal test guidelines for converters exist, but the mouse skin and rat liver models might be used to develop such guidelines.

7.3.5 Anticarcinogenesis

Anticarcinogens may be defined as compounds, which decrease the response of carcinogens. Their effects can be elicited at any stage during carcinogenesis by counteracting the effects of the carcinogenic treatments. Anti-initiators are often termed 'blocking agents' since they may prevent carcinogenesis altogether (Wattenberg, 1980). A large number of different assays have been applied to identify anticarcinogens, but few, if any, have been formally validated, so no formal test system exists. They can act as enzyme inducers as described above in section 3.3.2.1, or they may specifically induce detoxifying (Phase II) enzymes only. An *in vitro* assay has been proposed for the identification of such anticarcinogens (Talalay *et al.*, 1988). They may also decrease the absorption of carcinogens by affinity binding in the gut. An example is the sequestering of aflatoxin in the trout gut by chlorophyllin (Breinholt *et al.*, 1999). Finally they may protect DNA by as yet undefined mechanisms. An example here is the preventive actions of many polyphenols on carcinogen-DNA binding (Webster *et al.*, 1996; Giri and Lu, 1995; Huber *et al.*, 1997; Malaveille *et al.*, 1998). One of the most studied effect of anticarcinogens is the scavenging of activated, DNA-binding carcinogens, e.g. the binding by ellagic acid of benzo[*a*]pyrenediolepoxides (Barch *et al.*, 1996; Dixit *et al.*, 1985) and the scavenging of radicals by antioxidants and spin-trapping agents (Poulsen *et al.*, 1998).

Several inhibitors of tumour promotion are described in the literature but their mechanisms of action are not always understood. Many antioxidants may both enhance or inhibit promotion, but they seem most often to be inhibitors. Non-steroidal anti-inflammatory agents (NSAIDs) have an antagonistic action on colon carcinogenesis by inhibiting cyclooxygenase which forms cell-growth stimulating prostaglandins (Reddy *et al.*, 1996; Thun *et al.*, 1991), and certain terpenoids may inhibit carcinogenesis by inhibiting farnesyl transferase so that the *ras* protein can not be inserted correctly in the membrane of malignant cells (Gelb *et al.*, 1995). Some early defined anticarcinogens were inhibitors of ornithine decarboxylase which is important for the formation of polyamine cytoskeleton components needed during cell division, and many others had hormonal actions (Slaga *et al.*, 1980; Yuspa *et al.*, 1976).

Some anticarcinogens have been shown to have an effect very late in carcinogenesis, even after the appearance of tumours, notably beta-carotene on oral cancers in hamsters (Schwartz and Shklar, 1987) and also on the appearance of oral plaques in human smokers (Sankaranarayanan *et al.*, 1997; Garewal, 1995). However, the anticarcinogenic action of beta-carotene against a minor cancer in smokers cannot be outweighed by its negative actions on their major cancer of the lung. Similarly, are potentiating actions known for several anticarcinogens, and much more research is necessary in order to sort out why. In some cases it may simply be a question of dose, with lower doses being protective, and higher doses leading to damage. In others it may be that anticarcinogens act by provoking natural defence mechanisms in the body, in other words, some toxicity is necessary for the beneficial effects to appear.

In conclusion many anticarcinogens are known in the scientific literature, but we are only beginning to understand their mechanisms. The only anticarcinogens for which there is some international consensus on their beneficial effect are the NSAIDs, for which the conclusions of an international work group under IARC are positive (IARC working group, 1997). For other compounds, including carotenoids and retinol, IARC has not been able to conclude that there is evidence for a positive effect in humans (IARC working group, 1998a; IARC working group, 1998b).

7.3.6 Conclusions: Over-all effects and complex mixtures

Carcinogenesis is often described as a multi-step process. The fact that we know that different chemical, physical or biological agents may affect either step makes it clear that cancer is most often the result of combined toxic effects. The interaction of carcinogens affecting different steps in carcinogenesis has been known for half a decade to cause synergistic effects, strongly increasing the tumour response. Thus, initiators, promoters, converters, and co-carcinogens all act in concert to potentiate the final tumour outcome. Anticarcinogens may prevent or inhibit cancer at either step, and are also known to potentiate each other in some cases. What is clear from the review above is that potentiation can also be caused by compounds affecting the same step by different mechanisms. The possibilities for combinational effects in carcinogenesis are therefore many, and new models for short-term induction of tumours in animals are developed all the time leading to more and more reduced tumour induction times. The interpretation of these models is, however, not straight forward, and the long-term animal models still act as reference assays for the observed effects.

7.4 Reproductive toxicity

Prepared by Otto Meyer, Alireza Hossaini and Majken Dalgaard

7.4.1 Introduction

Reproduction is the term used to describe the biological processes, which ensures the continuation of species. Through this process the existing genetic material is passed on to the next generation. The reproductive cycle does not consist simply of conception, pregnancy and birth. It actually begins with the formation of the primitive germ cells in the parents at the embryo-foetal stage and does not end until sexual maturity has been reached. A disturbance of the reproductive cycle can, depending upon type or timing, prevent or inhibit reproduction or result in developmental defects in the offspring. Developmental toxicants can affect growth, development or acquisition of normal organ function between conception and puberty. Exposure to such compounds may lead to death (*in utero* or postnatal), structural defects, growth retardation, and functional deficits or childhood cancer.

In developmental and reproductive toxicology the following potential effects caused by exposure to chemical mixtures have been considered:

- Impairment of male and female reproductive functions or capacity, i.e. adverse effects on libido, sexual behaviour, aspects of spermatogenesis or oogenesis, hormonal activities or physiological responses which would interfere with the capacity to fertilise, with the fertilisation itself or the development of the fertilised ovum up to and including implantation.
- Induction of harmful effects on the progeny in the widest sense that is effects interfering with the normal development, both before and after birth up to puberty. Both morphological malformation(s) and functional disturbances (e.g. hormonal, neurological) are included.

The above-mentioned developmental effects are considered to be non-inheritable effects. This should be taken with some reservation, as the data very often do not offer the possibility to distinguish or identify the cause(s) of the developmental effect. Thus, for 65-70% of developmental defects in man the causes of the pathogenesis are not known.

Many different experimental methods are in use for the assessment of reproductive toxicity of chemicals (Table 7.4.1.1). When the results of testing single compounds are used in the risk assessment of human exposure to chemicals in complex mixtures there are a number points to consider. These points include the inherent differences in the experimental conditions e.g. laboratory animal species, strain or stock, diet and dosing. In addition, time-effect relationships like the relative time-courses of action of the single agents and the time courses of action of the agents relative to the timing of developmental events occurring during gestation must also be considered when interpreting interactive effect on e.g. the development.

Table 7.4.1.1 Overview of in vivo tests for reproductive toxicity testing

Test	Exposure period	Endpoints in offspring	Guideline(s)
Generation studies	Continuously over one, two or several generations	Growth, development and viability Histopathology of sex organs, brain and target organs Fertility Proposal: oestrus cyclicity and sperm quality	OECD TG 415 One-generation Study OECD TG 416 Two-generation Study
Prenatal Developmental Toxicity Study (Teratology study)	Usually during organogenesis Proposal: from implantation to the day before birth	Resorptions Fetal growth Morphological variations and malformations.	OECD TG 414
Developmental Neurotoxicity Study (Behavioral teratology studies)	During pregnancy and lactation	Birth and pregnancy length Physical and functional maturation Behavioral changes due to CNS and PNS effects Brain weights and neuropathology	OECD TG 426 Developmental Neurotoxicity Study (draft 1999)
Reproduction/ Developmental toxicity screening test	At least three dose levels from 2 weeks prior to mating until day 4 postnatal	Fertility Pregnancy length and birth F**o**etal and pup growth and survival until day 3	OECD TG 421 and 422

Modified from Hass et al 1994

This chapter illustrates the consequences of exposure to chemical mixtures on reproduction based upon essential, relevant publications and as such it should not be considered as a bibliographic review of the subject. Concerning the effects presented above, the literature search does not consider congenital cancer and behavioural effects, including sexual behaviour. The main focus is on examples of interactions *in vivo*. However, a brief introduction of *in vitro* techniques used in the study of reproductive effects is included. One group of potential reproductive and developmental toxicants, the endocrine disrupting chemicals (EDCs) are considered in the next chapter (7.5.1) because exposure to such compounds may also affect other targets than the reproductive system.

7.4.2 *In vitro* studies for testing interaction of teratogenic compounds

During the last 30 years more than 20 *in vitro* test systems have been used in the study of teratogenic effects. They can be divided into two categories, based on the involvement of morphogenesis. Morphogenetic tests are based on the use of isolated embryos, organs or regenerating tissues, whereas non-morphogenetic tests are based on cell cultures. In general, morphogenetic tests are sensitive and provide information on the effects on the morphology of the foetus. However, the test protocols are complex and not well suited for screening large numbers of chemicals. In general, the results of *in vitro* testing within the field of reproduction and development are difficult to interpret in terms of risk assessment due to the complex nature of these processes. Two individuals - the mother and foetus - are involved and effects on both have to be considered. In the maternal body, most chemicals are metabolised, and the foetus is protected to some extend by the placenta. However, with the exception of compounds with a very high molecular weight (over 600 dalton) or compounds, which are highly electronegative or electropositive, almost all chemical substances may pass from the mother's circulation to that of the foetus. This means that *in vitro* studies can only indicate either a possible effect or lack of effect in the whole animal.

This complexity is illustrated by studies on 5-fluoracil (5-FU). 5-FU is a known teratogen in the rat. It acts primarily by disrupting the balance of the intracellular nucleotide pools, leading to arrest of DNA synthesis and cell replication. *In vitro*

studies using leukaemic cells have shown that 5-FU toxicity could be ameliorated by nucleoside (thymidine or deoxycytidine) supplementation (Elstein *et al.* 1997). Other reports, too, have indicated a protective role of deoxyribonucleotides against this class of teratogens. However, an *in vivo* developmental study showed that supplementation of deoxycytidine and deoxyguonosine had no protective effect on 5-FU induced toxicity. On the contrary, the adverse effect of 5-FU was found to be potentiated (Lau *et al.* 1997).

The developmental effect of combinations of methanol and formic acid was studied in 9-day-old rat embryos (Whole Embryo Culture, WBC). Methanol has shown teratogenicity in rodents, and *in vitro* studies have demonstrated that embryonic development was adversely affected under conditions where methanol was not metabolised to formate to any appreciable extent. After the embryotoxicity of the individual substances had been established in previous studies, concentrations of methanol and formate were chosen which would produce similar effects using the parameter "Developmental Score" (DEVSC), and isoboles were plotted joining the equivalently toxic doses. (Andrews *et al.* 1998). The results showed that low concentrations of formate together with various concentrations of methanol did not significantly change the DEVSC from that expected from methanol alone, while high concentrations of formate resulted in significant reductions of embryonic DEVSC. The authors suggest that the result was indicative of an antagonistic interaction. They cite a publication in which *in vivo* studies indicate that formate enters the metabolic pathway shared by it and 2-methoxyethanol (ME) or a metabolite of ME thereby rendering protection of the embryo as a result of a competitive inhibition. This could indicate that the interaction shown in the present *in vitro* study was a result of a complex dissimilar action.

7.4.3 Examples of interaction of reproductive toxicants *in vivo*

The selection of chemical mixtures as they appear in the environment or in foods can be used as one approach to test chemical combinations in animal experiments. An alternative approach is to select chemical mixtures containing agents with equal target organ, similar modes of actions or similar chemical structure. In addition, therapeutic use of antidotes as protectors of known toxicants are well described.

The first approach can be illustrated by a study on exposure of rodents to chemical mixtures representative of groundwater contamination (Heindel *et al.* 1994). The potential reproductive and developmental consequences of exposure to pesticides and fertilisers were evaluated during a continuous breeding study in CD-1 mice, and during a developmental study in pregnant Sprague-Dawley rats. The animals were exposed to two pesticide mixtures representing the groundwater contamination in California and Iowa at dose levels of 1, 10, and 100 times the concentrations of the pesticides in the water. No detectable reproductive, general or developmental toxicity of the pesticides mixtures was observed at any of the dose levels. The authors specified that the purpose of the study was to evaluate the health effects of realistic concentration of human exposure. Thus, the concentrations tested were probably considerably lower than the maximally tolerated doses. Consequently, the low but realistic doses used in this study did not induce any interaction or combined effect.

In a 5x5x5 full-factorial design, three compounds, commonly found at hazardous waste sites, were combined using 5 dosages of each agent. Trichloroethylene (TCE), di-(2-ethylhexyl)phthalate (DEHP) and heptachlor (HEPT) were administered to rats by oral gavage from days 6-15 during gestation**.** The selection of dose levels was based on data from single-agent studies, and linear regression

analysis was used to predict the high dose for each agent causing a pup weight loss of 2 g on postnatal days 6-8. The other doses were equally spaced between the top dose and the 0-level. Combination of TCE and DEHP caused synergism, appearing as a negative effect on the parameters: maternal weight gain, prenatal loss, and full litter resorption and pup weights on day 6. Combination of DEHP and HEPT had a synergistic effect on maternal death and antagonised main effects on the parameters: maternal weight gain, full litter loss, pup weights on day 1 and pup weights on day 6**.** However, the authors discussed that this antagonistic effect may be due to a ceiling effect of combining high responses or due to biased exclusion of susceptible dams as a consequence of the synergistic interaction of these agents towards maternal mortality. HEPT potentiated the effect of TCE and DEHP on prenatal loss and full litter resorption. TCE-HEPT interaction was antagonistic concerning adverse effects on full litter loss (Narotsky *et al.* 1995). The authors conclude that assumption of additive toxicity may be inadequate in some risk assessment situations. Thus, at the doses used in this study several mechanisms of interaction are involved, when these compounds are administered simultaneously. However, the mechanism of action was not elucidated and therefore it is not possible to classify the kind of interaction as being either complex similar or dissimilar action. However, if lower doses close to NOAEL were used the assumption of additivity would have been more appropriate.

The second approach is illustrated by a study on interaction between chemicals with similar chemical structure. Halogenated aromatic hydrocarbons are among the most persistent chemicals in the environment. They have been detected in blood, milk and adipose tissue of wild life animals and humans. When TCCD (20 µg /kg bw) was administrated to pregnant C57BL/6J mice, 62% of the foetuses developed cleft palate, without any observable maternal toxicity. Aroclor 1254 (244mg/kg bw), which is a commercial mixture of polychlorinated biphenyls (PCB), did not induce cleft palate. Co-treatment of the pregnant mice with Aroclor 1254 (244 mg/kg bw) and TCDD (20 µg/kg bw) resulted in an 8.2% incidence of cleft palate. These results demonstrate that Aroclor 1254 antagonised 2,3,7,8-TCDD-mediated teratogenicity in this strain of mice (Haake *et al.* 1987). Data is insufficient to evaluate the mechanism of action of the chemicals. However, we suggest that the interaction demonstrated here may indicate a result of complex dissimilar action, as aroclor 1254 is a known inducer of liver enzymes thereby increasing the metabolism of TCDD.

Another example of an antagonistic effect is the interaction between cadmium and selenium. Cadmium is a well-known toxicant of the male reproductive system. Wlodarczyk *et al.* (1995) tested the protective capacity of selenium in acute cadmium intoxication in male golden hamsters. Selenium alone caused a significant increase in epididymis weight after 1 and 4 weeks of dosing, whereas cadmium alone decreased the weight of testes, epididymis and accessory sex organs compared to the control group. In addition, a decrease in sperm number and azospermia was observed. Selenium completely reversed the damaging effect of cadmium on the male reproductive system, when the agents were mixed in a ratio of 1:1 at a dose-level of 0.5 mg/kg. This kind of interaction is interpreted as complex dissimilar action.

Mixtures of 2,4-dichlorophenoxyacetic, 2,4,5-trichlorophenoxyacetic and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin were examined in mice for reproductive disorders and sister chromatid exchange in bone marrow cells. Fertility, sperm number, motility and morphology were among the evaluated parameters. No significant dose-related effects were observed (Lamb *et al.* 1981). The doses used in this study did not induce any combined effect.

The additivity of responses is illustrated by an investigation of the interaction between the polychlorinated aromatic hydrocarbons, TCDD and TCDF, which cause similar foetal anomalies in mice (Weber *et al.* 1985). In this teratology study, pregnant mice were exposed to the chemicals separately and as mixtures at several dose levels. The results indicated an additive effect of the two chemicals (Weber *et al.* 1985). Since, the same mechanism and the same target organs are involved the effect is defined as a simple similar action.

The concept of potentiation is another aspect of interaction between chemicals. TCDD is a ligand of the Aryl hydrocarbon (Ah)-receptor, and in the foetus this receptor has been localized especially to the maxillary region. TCDD alone induces cleft palate in mice. β-Naphthoflavone is a non-teratogen and another ligand of the Ah-receptor. When β-naphthoflavone (80-100 mg/kg) was co-administered with TCDD (25 mg/kg) simultaneously or 8 hours before, the effect of TCDD was potentiated, as the frequency of cleft palate was increased significantly. However, this was not observed when β-naphthoflavone was administered 24 hours before or after TCDD exposure, indicating the importance of the time-effect relationship in developmental studies (Hassoun & Dencker 1982). TCDD and β-naphthoflavone are both ligands of the Ah-receptor. It is our suggestion that β-naphthoflavone may induce the expression of the Ah-receptor thereby increasing the number of receptors. This effect is time dependent and seems to disappear between 8 to 24 hours. The results of the interaction could be explained by a complex similar action.

A developmental study evaluated the effect of deoxycytidine (d-cyt) and deoxyguanosine (d-guo) supplementation on 5-FU-induced toxicity. On gestational day 14, pregnant rats received the agents either alone or in combination. Animals were sacrificed on gestational day 21, weighed, and examined for skeletal abnormalities. Administration of d-cyt or d-guo alone did not affect foetal outcomes. 5-FU alone reduced foetal weight and produced skeletal alterations. Pre-treatment with either d-cyt or d-guo did not protect the rat from 5-FU malformations as expected from the *in vitro* study. In contrast, the combined treatment led to a potentiation of 5-FU, which resulted in a more severe foetal weight deficit and an increased incidence and severity of skeletal abnormalities (Lau *et al.* 1997). As this study only is published in an abstract the results are not described in details and we have no information about dose levels in relation to NOAEL. The results indicate that the interaction could be classified as a potentiation. However, it is impossible to say whether the effect represent a true potentiation as the relation of the one dose level to NOAEL is not known and as only one dose level has been used. In addition, the mode of action cannot be evaluated.

An example of a study on interactions of chemicals with different modes of actions, but with equal target organ has been performed by Birnbaum *et al.* (1986). They investigated a mixture of glucocorticoids and TCDD in C57/6N mice. Glucocorticoids cause cleft palate in sensitive mouse strains by interfering with the proliferation of mesenchymal cells in the palatal shelves. TCDD also caused cleft palate, but its effect involved the epithelial cells. TCDD (3 µg/kg bw) did not, however, induce cleft palate in this study, whereas hydrocortisone resulted in cleft palate in a dose-related manner (25, 50, 100 mg/kg bw). Combination of all doses of hydrocortisone with TCDD (3 µg/kg) resulted in a 100% incidence of cleft palate, which was reported as a synergistic effect. This effect was 25 times higher than the incidence of cleft palate induced by hydrocortisone alone. Morphological assessment of the palatal shelves late on gestation day 14 showed that the embryos exposed to the combination of TCDD and hydrocortisone resembled the embryos given hydrocortisone alone (Birnbaum *et al.* 1986). A similar study on the

interaction of TCDD and retinoic acid also revealed a synergistic effect on the percent of mouse foetuses with cleft palate (Birnbaum *et al.* 1989). The action is interpreted as a dissimilar action i.e. same target organ but different mode of action.

Besides laboratory animal models to clarify human exposure, models representing wild life exposure are used in order to evaluate the ecological consequences of chemical mixtures in the environment. Length and weight of Zebra fish were used as growth parameters in a combination study of 3,4-dichloroaniline and lindane. When testing the mixtures in a complete life cycle model a significant reduction in length and weight of fish was observed. None of the chemicals tested individually showed any effect on growth not even in much higher doses than in the mixture (Ensenbach *et al.* 1997). This effect reflects a synergistic interaction between the two chemicals on growth. However, the study did not include investigation of the mode of action.

7.4.4 Evaluation of the *in vivo* studies

One of the major problems when evaluating the literature regarding combination toxicology is the lack of a consistent use of terms and definitions. On the surface these definitions appear to be relatively straightforward. However, there is not uniform agreement in the use of terms like synergism and potentiation. For instance, the interaction between TCDD and glycocorticoids was described as a synergistic effect (Birnbaum *et al.* 1986). However, according to other definitions this effect is better defined as a potentiation. TCDD alone at this concentration did not induce cleft palate, but increased the effect of glycocorticoids. If TCDD induced cleft palate at the selected dose, the interaction would be defined as synergistic. Another problem is that some authors do not define the interactions they observe. In some cases the term 'cumulative' has been applied in order to define the phenomenon observed, when an effect of two or more chemicals were observed at their individual No Observed Effect Level (NOEL).

Another important issue when investigating chemical interaction is the selection of appropriate dose levels. It is our impression that in most studies of mixtures the low observed effect level (LOEL) is used as a minimum dose. The advantage of using LOEL is that one has the opportunity to characterise the observed interaction. The problem is the extrapolation to the actual human exposure level. However, selecting dose levels, corresponding to human exposure has the limitation that the sensitivity of the study in most cases will be too low to identify any effect i.e. a precise description of the toxicological effect and the possible interaction of the tested compounds.

Time of exposure according to 'critical windows' is a significant issue in reproductive and developmental combination toxicology. As the chemical agents often have different modes of action, the exposed animals may also have different periods of sensitivity during embryo and foetal life. Should the two or more chemicals be administrated at the same time or by the same route? Exposing animals to a chemical before pregnancy may antagonise the effect of another toxicant given during gestation. Exposing animals to two chemicals within an 8-hr interval or at the same time was shown to result in an interaction between the chemicals. However, if the order of exposure of the two chemicals was reversed, no interaction was observed (Hassoun & Dencker 1982).

In a literature review on the effect of interaction of chemicals on reproductive and developmental toxicology *in vivo*, Nelson (1994) has gone through and evaluated 160 published studies. The conclusions were that:

Thirty percent of the papers reported no interaction (including additive effects), 35% reported antagonistic effects, and the rest of the papers reported synergistic or rarely potentiative effects. Additivity was defined as 100 ± 20 % of the combined result of the single-agent effects.

The percent response to combined chemicals showing synergism, according to the definition of the author, was in the range 120-5300 % with a mean ± SD of 360 ± 589 (n=215 combinations). All dose combinations from the table by Nelson was included in the calculation. Only 11 combinations gave rise to effects exceeding 1000 %. Almost all of these (n=10) were studies on retinoic acid in combination with either TCDD or zinc or protein deficiency *i.e.* studies highly related to nutrition.

The quality of the papers was highly variable (in general: few animals, few dose levels, maternal toxicity not considered, time-effect relationships not often examined, inconsistent use of terms). 'Potentiation' was the term misused most often; frequently the more appropriate term would have been synergism.

7.4.5 Conclusions

Combined exposure of two or more chemical compounds has shown that interaction may or may not occur, and that it is impossible predicting the outcome based on experimental conditions alone. However, the published data suggest that the prevailing outcome of exposure to mixtures deduced from *in vivo* experiments is either an antagonistic effect or no interactive effect, including an additive effect. Frequently, one type of interaction was noted at some doses, while another type of interaction was noted at other doses. An evaluation of the results suggests that low doses of combinations often produced either no effect or additive effects, whereas higher doses produced antagonistic or synergistic effects.

In addition, combinations of chemicals with common features such as similar structure or similar type of effect on a common target organ probably are more likely to result in an - at least additive - effect. In such cases, additivity should be considered in the assessment of the exposure to mixtures. However, when assessing simultaneous exposure to two or more chemicals in general, the consideration of consequences of a possible interaction or an additive effect should be considered on a case-by-case basis.

7.5 Endocrine disrupting chemicals

Prepared by Anne Marie Vinggaard

7.5.1 Introduction

Abnormal sexual development in wildlife, the worldwide rise in the incidence of testicular cancer, developmental disorders of the male reproductive tract and the continuing rise in the incidence of female breast cancer are causing widespread concern. It has been suggested that these effects might be related to environmental chemicals, which are able to mimic endogenous estrogens. The list of chemicals reported to show estrogenic activity has grown considerably during the last years

and now includes compounds as diverse as o,p'-DDT, methoxychlor, non-coplanar PCBs, bisphenol A and alkoxyphenols.

Although there is evidence to suggest that some of these chemicals cause adverse effects in wildlife, attempts to establish a link between human health risks and exposure to estrogenic chemicals are complicated by the fact that many of the suspected chemicals have very low estrogenic potencies when compared with 17ß-estradiol. Most of the chemicals are weak agonists or antagonists of the steroid receptors being effective at concentrations in the μM range *in vitro* and thus are several thousand folds less potent than the natural steroid hormones. It is therefore difficult to explain possible health risks simply on the basis of toxicity from exposure to the single compounds. However, human populations are exposed to mixtures of xenoestrogens and it is conceivable that environmental contaminants might interact with each other in a way that enhances their estrogenicity. Therefore, studies on interaction effects of chemicals are a very important issue within the field of endocrine disruption.

7.5.2 Examples of studies on interaction of endocrine disrupting chemicals (EDCs)

Even though chemicals are usually found as mixtures in the environment, the hormonal activity of mixtures has been investigated only to a limited extent and the systematic analysis of combined effects of xenoestrogens has only recently begun. The main focus so far of the effects of mixtures of EDCs has been on the estrogenic activity of mixtures in cell cultures. It has to be kept in mind, though, that the ability of certain chemicals to bind to and activate the estrogen receptor is only one of the mechanisms, by which chemicals may interfere with the development of the reproductive system. A number of other relevant *in vitro* systems including assays for other hormone receptors or steroidogenic enzymes still remain to be tested with mixtures of EDCs. In addition, the field of endocrine disruption is naturally related to reproduction toxicology, but reports on interaction of chemicals with the estrogen receptor is also belonging to the field of cancer research, as for instance breast and prostate cancer.

The following is a summary of most of the studies on interaction between EDCs, which have been reported, in recent years.

As early as 1994 Soto *et al.* demonstrated that a mixture of ten pesticides, at concentrations that by themselves were inactive, enhanced the proliferation of MCF7 human breast cancer cells by an effect which was classified as cumulative by the authors. The involved pesticides were: endosulfan α and β, toxaphene, dieldrin, tetrachlorbiphenyl, hexachlorbiphenyl, p,p'-DDT, -DDD, -DDE and methoxychlor.
No dose-response curves were made and the study is an example of the application of intuitively effect summation as described later. Thus, these data cannot be used to analyse the mechanism of interaction, if any.

Later Sumpter and Jobling (1995) made preliminary experiments showing that vitellogenin induction in cultured rainbow trout hepatocytes was 'enhanced' by a combination of the following chemicals: 1 μM concentrations of nonylphenol, octylphenol, o,p'-DDT, Arochlor 1221 and bisphenol A. Dose response curves of the individual compounds were made, but no detailed analysis of the mechanism of interaction was performed.

An example of mixtures of EDCs showing synergistic activity is a study employing the use of turtle eggs (Bergeron *et al.* 1994). The sex of turtle eggs is temperature

dependent; eggs incubated at 26°C or 31°C develops into males or females, respectively. Male-determined turtle eggs can be sex-reversed by applying estradiol or estrogenic hydroxylated polychlorinated biphenyls (PCBs) to the eggs. In fact, two of the hydroxy-PCBs (2',4',6'-trichloro-4-biphenylol and 2',3', 4',6'-tetrachloro-4-biphenylol) in combination were effective in sex reversal at concentrations that had little or no activity individually. Kortenkamp and Altenburger (1998) reevaluated this study in detail. They applied the isobole method, and concluded that the authors had correctly concluded that the two compounds acted synergistically in affecting sex determination in turtle eggs.

These reports were followed by a paper reporting a dramatic synergistic effect on estrogen receptor activation with combinations of environmental chemicals (Arnold *et al.*1996), a paper that caused a great scientific, regulatory and public concern around the world. Combinations of two weak environmental estrogens, such as dieldrin, endosulfan or toxaphene (tech.mixt.) were tested in a yeast estrogen system (YES) containing the human estrogen receptor and were reported to act synergistically. The combinations were around 150-1500 times as potent in estrogen receptor-mediated transactivation than any of the chemicals alone.

In the time after, several laboratories tried to reproduce the results without success. Most remarkably was a report from a group of ten well-estimated scientists, who tested the potential synergistic activity of dieldrin and toxaphene in ten different *in vitro* or *in vivo* estrogen receptor assays (Ramamoorthy *et al.* 1997). The combined activities of the two compounds were found to be essentially additive. The compounds alone (up to 60 μmol/kg) or in equimolar concentrations were investigated in 21-day old B6C3F1 mouse uterus and none of the treatments either alone or in combination affected the uterine wet weight, peroxidase activity, or progesterone receptor binding. In addition, the interaction of the other mixtures of pesticides reported by Arnold (1996) was reinvestigated in two different yeast-based assays and the results could not be confirmed. The story ended by a retraction of the original paper by Arnold (McLachlan, 1997).

However, in 1997 a few other reports on synergistically interacting EDCs appeared. Different combinations of the weak estrogenic compounds benzylbutylphtalate, bisphenol A, p,p-DDE and 2,2',3,3',6,6'-hexachlorobiphenyl were investigated in the MCF7 cell proliferation assay (Soto *et al.* 1997). Five different combinations of three or four of these compounds induced what the authors called a cumulative proliferation response. In this case no dose-response curves of the single compounds were made and the conclusions were based on intuitive effect summation.

Later Arnold *et al.* (1997) reported again on the synergistic interaction of toxaphene, dieldrin and chlordane, this time with competitive binding to the alligator and human estrogen receptor as the endpoint. Neither 220 nM chlordane, 630 nM dieldrin nor 200 nM toxaphene gave any binding response by themselves, but in combination a response was observed. It was suggested that the estrogen receptor might contain more than one site for binding environmental chemicals.

In contrast to these papers reporting synergism, endosulfan and dieldrin showed no synergism in different estrogen receptor assays (Wade *et al.*, 1997). Interaction was tested with the estrogen receptor in or extracted from mammalian cells and no synergism was observed. Furthermore, the compounds did not stimulate any uterotrophic activity *in vivo* nor had any effect on pituitary prolactin or other endocrine-related endpoints in immature female rats.

Furthermore, the estrogenic effects of simple mixtures of benzylbutylphtalate, dibutylphtalate, and 17β-estradiol were tested in the yeast estrogen screen and the activities were found to be approximately additive (Harris *et al.* 1997).

The complexity in evaluating reports on combination toxicology is illustrated by a recent report on the interacting effects of phytoestrogens on the response to 17β-estradiol (Wang and Kurzer, 1998). At low concentrations, genestein and coumestrol significantly enhanced estradiol-induced DNA synthesis, whereas at high concentrations, inhibition was observed. Furthermore, differing effects were observed with the other phytoestrogens. Thus, the type of interaction depended on the concentration of the chemicals. This study deviates from many of the above-mentioned studies in being performed very thoroughly including dose-response curves of the single compounds. These data could therefore form the basis for a more detailed analysis of interaction effects.

Kortenkamp and Altenburger (1998) have evaluated a study by Gaido *et al.* (1997) applying the isobole method. The study focussed on binary mixtures of the hydroxylated polychlorinated biphenyls, 2,4,6-trichloro-4-biphenylol and 2,3,4,5-tetrachloro-4-biphenylol, which had been analysed by Arnold *et al.* (1996). The transcriptional activation of human estrogen receptors in human hepatoma cells (HepG2) was determined. The cells were transfected with a reporter plasmid containing an estrogen-responsive promoter linked to the luciferase gene. The estrogen receptor was expressed from a second plasmid, referred to as estrogen receptor expression plasmid. In order to study the influence of receptor concentration on response, the cells were transformed with varying amounts of this expression receptor plasmid (270, 27, and 2.7 ng plasmid per well). Considering the experiments in which 27 ng plasmid was used, the evaluation resulted in a data point for the mixture of the two hydroxylated polychlorinated biphenyls which lied close to the additivity line. Thus, the hydroxy-PCBs acted additively which support the conclusion made by the authors. In contrast, the results of experiments in which 270 ng expression plasmid per well were used for cell transformations indicate that the combination effect of the same binary mixture is synergistic. With the amount of expression plasmid further lowered to only 2.7 ng/well a strong antagonism between the two agents becomes apparent.

The work by Gaido *et al.* is interesting in that it shows how the type of combination effect can change as the concentration at the target site, the estrogen receptor, is varied. It is too early to suggest any explanations for this phenomenon, but it would be worthwhile to pursue studies of this kind by measuring the effects of combinations of the two hydroxy-PCBs at different mixture ratios.

Estrogenic activity in 2-, 3- and 4-component mixtures of o,p'-DDT, genistein, 4-nonylphenol, and 4-n-octylphenol were thoroughly evaluated in a yeast estrogen screen (Payne *et al.*, 2000). The models of dose-addition and response addition were applied for calculation of the mixture effects and the conclusion was the responses did not deviate from expected additivity. Both models were considered as useful tools for evaluating the effects.

Another study from the same group investigated the impact of bisphenol A and o,p'-DDT on the estradiol-induced estrogenic response in yeast cells (Rajapakse *et al.* 2001). It was concluded that the combined effects did not deviate from additivity and that the assumption that weak xenoestrogens are generally unable to create an impact upon the already strong effects of endogenous steroidal estrogens was not supported.

Finally, Gray has very recently published an abstract saying that the combined effect of vinclozolin and procymidone in an *in vivo* Hershberger test was found to be additive.

Furthermore, Safe (1998) has suggested using the TEF approach for estimating total intakes of potential dietary and environmental estrogens

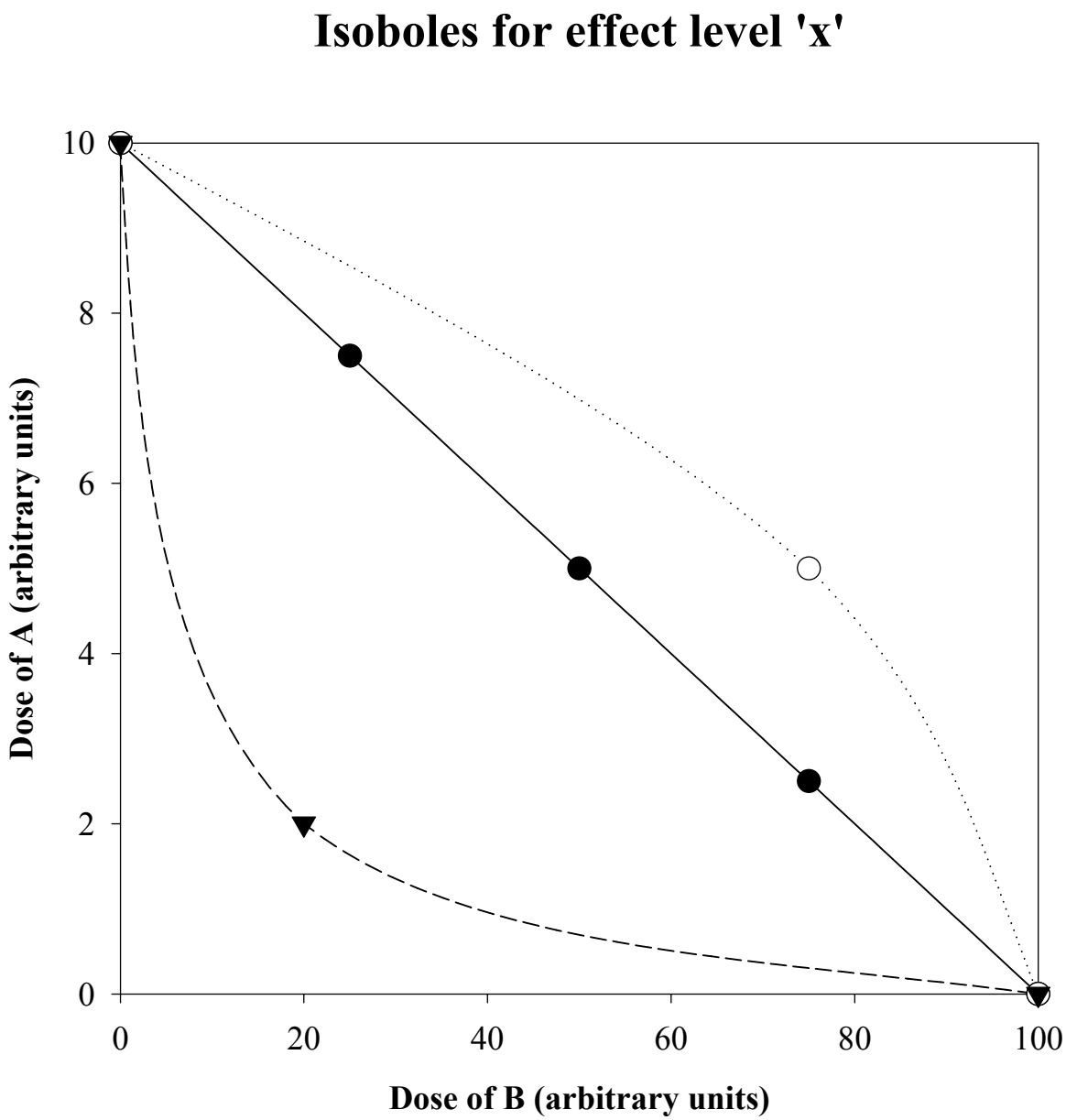
7.5.3 Comments on studies dealing with interactions of EDCs

In exploring the combined effect of estrogenic compounds many authors (Soto *et al.*, 1994; Sumpter and Jobling, 1995; Arnold *et al.*, 1997; Soto *et al.*, 1997) have adopted an experimental design where the single agents as well as the mixture were tested at only one dose level. This approach is intuitively appealing but is regarded as inappropiate in most cases because it only allows assessments of combination effects, which are based on expectations of effect summation. However, the application of the method of effect summation is limited to combinations of agents, which produce linear dose-response curves that pass through the origin of the dose-effect plot. Linear dose-response curves are rarely seen in toxicology (Kortenkamp and Altenburger, 1998). They may occur in the low-dose range of otherwise non-linear curves such as for carcinogens. In most of the above-mentioned studies linear dose-response relationships have not been demonstrated.

Kortenkamp and Altenburger (1999) have recently reevaluated studies of combination effects within the field of EDCs. They concluded that the debate in the estrogen field has taken no account of the concepts for assessing combination effects that have evolved during the last 100 years in pharmacology and toxicology. Furthermore, they have demonstrated that some studies, which purportedly showed absence of synergy, have in fact overlooked synergistic effects. There were also unidentified antagonisms, but the overwhelming majority of studies were inconclusive. These problems were traced to an undue focus on measuring effects of mixtures at only one dose level and to a general unawareness of the necessity to use criteria that define clearly what constitutes synergism, additivity or antagonism.

The emphasis on synergisms has diverted attention away from the possible implications of seemingly less spectacular additive combination effects. Silva *et al.* (2002) have provided support for an additive effect of estrogenic compounds in vitro. They tested mixtures of eight estrogenic compounds, including hydroxylated PCBs, benzophenones, parabenes, bisphenol A, and genistein, in a recombinant yeast estrogen screen (YES). To ensure that no chemical contributed disproportionately to the overall combination effect, a mixture was prepared at a mixture ratio proportional to the potency of each individual component. The performance of four approaches for the calculation of additive combination effects (concentration addition, toxicity equivalency factors, effect summation and independent action) was compared. The authors concluded that concentration addition and its application, the toxicity equivalency factor approach, were valid methods for the calculation of additive mixture effects. There were excellent agreement between prediction and observation. Use of the concepts of independent action and effect summation led to clear underestimations of the experimentally observed responses. Substantial mixture effects were reported even though each chemical was present at levels well below its no-observed-effect-concentration (NOEC) or EC_{01}. The authors concluded that estrogenic agents are able to act together to produce significant effect when combined at concentrations below their NOECs.

Most of the work made within this field so far is based on *in vitro* experiments, which suffer from the well-known limitations (lack of consideration of absorption, metabolism, and excretion). Very few *in vivo* experiments on mixtures on EDCs have been performed and such experiments will be one of the future challenges within the field of endocrine disruption. Whether it will be possible to confirm the above-mentioned interactions of EDCs *in vivo*, future experiments will show.



However, it has been shown that the kind of combination effect can vary in the same experimental system depending on the endpoint chosen for analysis

(Altenburger etal. 1993). Agents that act synergistically at the molecular level (e.g. receptor binding) may display additivity with endpoints at more complex functional levels such as cell growth or cell death (Grimme *et al.* 1996). For this reason it is important to carry out analysis of combination effects of estrogenic compounds at different levels of molecular complexity.

7.5.4 The isobole method – a practical approach

Two main reference models for defining expected effects of mixtures of agents have emerged: the models of concentration addition and response addition. In the following discussion the model of concentration addition is used. It is by far the most frequently employed reference model and has sound pharmacological foundations. Unlike the model of response addition, it is easily applied to the non-fractional effects yielded by assays employed to quantify estrogenic effects. The basic idea behind the concept of concentration addition is to compare doses, which produce effects of equal strength (iso-effective doses). The isobole method, which is derived from the method of concentration addition and has the advantage of being illustrative, is widely applied. One of the strengths of the method of isoboles is that it can be used to analyse combinations of agents, irrespective of the shape of their dose-response curves (Kortenkamp and Altenburger, 1998). It is possible to assess mixtures of agents with dissimilar dose-response curves, even when the maximal effects are not identical.

An analysis using the isobole method is carried out by constructing graphs that show curves describing combinations of two compounds A and B which produce the same specified effect (isoboles) (see chapter 2.4.3). The axes of the graph represent doses of the two compounds on a linear scale. A line joining the iso-effective doses A and B of the single agents predicts the combination of A and B, which will yield the same effect, provided the interaction between A and B is additive. In this case the relationship can be expressed:

$$d_A/D_A + d_B/D_B = 1$$

where d_A and d_B are the doses/concentrations of A and B in a mixture that produces a specified effect and D_A and D_B are the doses/concentrations of the single agents which on their own elicit the same effect.

If datapoints lie below the additivity isobole then $d_A/D_A + d_B/D_B < 1$, indicating synergism. With antagonism concave-down isoboles above the additivity line appears, as $d_A/D_A + d_B/D_B > 1$. Note that the position of isoboles varies depending on the effect level chosen for analysis.
The method can also be applied to mixtures where only one of the two agents produces the effect under consideration. The iso-effective dose of the agent lacking the effect of interest can be regarded as infinitely large, so that the resulting additivity isobole runs parallel to the respective dose axis.
Combination of three agents can be analysed by constructing three-dimensional isobolar surfaces, and combinations of more than three compounds can be assessed more easily by using a generalisation of the above-mentioned equation:

$$d_A/D_A + d_B/D_B + d_C/D_C + d_D/D_D + \ldots\ldots = 1$$

Using this approach, valid conclusions on the combination effect of mixtures can often be drawn on surprisingly few data.

The method of isoboles does not include facilities, which are helpful in deciding whether deviations from the line of additivity are systematic or simply due to

chance or experimental error. One way of dealing with this problem is to calculate confidence intervals for the iso-effective doses of the single compounds and to add a confidence belt to the line of additivity (Kortenkamp and Altenburger, 1998).

Kortenkamp and Altenburger (1998) have evaluated a study by Gaido *et al.* (1997) applying the isobole method. The study focussed on binary mixtures of the hydroxylated polychlorinated biphenyls, 2,4,6-trichloro-4-biphenylol and 2,3,4,5-tetrachloro-4-biphenylol, which had been analysed by Arnold *et al.* (1996). The transcriptional activation of human estrogen receptors in human hepatoma cells (HepG2) was determined. The cells were transfected with a reporter plasmid containing an estrogen-responsive promoter linked to the luciferase gene. The estrogen receptor was expressed from a second plasmid, referred to as estrogen receptor expression plasmid. In order to study the influence of receptor concentration on response, the cells were transformed with varying amounts of this expression receptor plasmid (270, 27, and 2.7 ng plasmid per well).

Considering the experiments in which 27 ng plasmid was used, the evaluation resulted in a data point for the mixture of the two hydroxylated polychlorinated biphenyls which lied close to the additivity line. Thus, the hydroxy-PCBs act additively which support the conclusion made by the authors. In contrast, the results of experiments in which 270 ng expression plasmid per well were used for cell transformations indicate that the combination effect of the same binary mixture is synergistic. With the amount of expression plasmid further lowered to only 2.7 ng/well a strong antagonism between the two agents becomes apparent.

The work by Gaido *et al.* is interesting in that it shows how the type of combination effect can change as the concentration at the target site, the estrogen receptor, is varied. It is too early to suggest any explanations for this phenomenon, but it would be worthwhile to pursue studies of this kind by measuring the effects of combinations of the two hydroxy-PCBs at different mixture ratios.

7.5.5 Conclusion

Most of the work made within this field so far is based on *in vitro* experiments and only very few *in vivo* experiments on mixtures on EDCs have been performed so far. Such experiments will be one of the future challenges within the field of endocrine disruption.

The majority of the -especially older- studies were inconclusive. Most of them conclude that there appears to be additive effects, i.e. no interaction between compounds is found, although a detailed mechanistic analysis has not been applied in most cases. However, some studies, which showed absence of synergism, had in fact overlooked synergistic effects as determined by a detailed evaluation of the data. At least two studies, which involve the effect of hydroxy-PCBs on sex reversal in turtle eggs and estrogen receptor activation, respectively, have shown that synergism takes place.

However, the recent well-designed studies performed by Kortenkamp and co-workers clearly show that the effects of estrogenic compounds do not deviate from the expected additivity. In addition, additive effects of two antiandrogenic compounds given *in vivo* were found.

Overall, most studies conclude that compounds are acting additively, so at the present time there is no evidence pointing to the necessity of incorporating synergism in the hazard assessment of weakly estrogenic chemical mixtures.

7.6 Neurotoxicity

Prepared by Ole Ladefoged, Henrik Rye Lam and Grete Østergaard

7.6.1 Introduction

Exposure to chemicals and drugs has been linked to persistent changes in the nervous system. The nervous system has a long period of development during which it is particularly vulnerable to such exposures.

Because of the complexity and the hierarchical organisation of the functions of the nervous system there are a large number of possible target sites for neurotoxicants, and many possibilities for combined actions and interactions when various targets are affected simultaneously.

At present, there is no internationally accepted definition of neurotoxicity. In the scientific community, it is generally agreed that neurotoxicity is *any adverse effect on the chemistry, structure and/or function of the nervous system during development or at maturity induced by chemical or physical influences* (Ladefoged *et al.*, 1995).

In this context, it may sometimes be difficult to define what should be regarded as an 'adverse' effect for dose/response assessment purposes. Adversity may have one or more characteristics: The organism may be brought into a state of compensation in order to function normally, and/or have a diminished ability to function fully, or to survive, reproduce or adapt to the environment (Tilson, 1990).

A persistent (long-term, reversible or irreversible) effect should always be regarded as a serious indication of neurotoxicity. Acute reversible effects should also be included.

The complex structure and function of the nervous system is briefly described in the following text.

7.6.2 Complexity of the nervous system

7.6.2.1 Anatomy

The nervous system consists of the brain and spinal cord (Central Nervous System, CNS), peripheral nerves including nerve endings (Peripheral Nervous System, PNS), and the organs of special sense. Functionally, PNS is divided into the somatic (motor and sensory) and the autonomic nervous system.

7.6.2.2 Neurons

The neurons, with their long processes, provide a vast surface area for chemical attack and are therefore inherently susceptible to chemical interference. Intensive long-distance intracellular transport of essential compounds and intercellular communications are further special characteristics of nerve tissue. The brain requires a continuous and rich supply of oxygen and glucose, which is essential for its cellular energy turnover, the highest of all organs in the body. The brain has an extremely high rate of oxidative metabolism that may be accompanied by a high rate of generation of reactive oxygen species. Further, the brain has only low activity levels of the protective systems which function to minimise oxidative damage (Kehrer, 1993). Mature neurons are not capable of regeneration.

7.6.2.3 Receptors

A receptor is a binding site for endogenous and exogenous chemical substances. Receptor binding elicits a specific biological reaction. The nervous system is unique compared to other organs because of its large number of different types of receptors and receptor agonists (neurotransmitters). A single neuron can possess different receptors even for the same agonist, and these receptors may transduce receptor binding to different/opposing signals. It is the integrated sum of transduced signals that determines the overall effect on the neuron and its output. Furthermore, nervous system receptor interactions can exert their effect for a long time period in that receptors can ´remember´ binding and it is possible to make specific circuits among neurons more effective by potentiation and ´learning´. Many nervous system receptors are composed of more than one subunit and allosteric effects are common i.e. binding to one subunit affects (increase/decrease) binding characteristics of others.

7.6.2.4 CNS

In the CNS two main functional barriers protect the brain from entry of toxic chemicals present in the blood. The blood-brain barrier (BBB) is formed by close contact between the endothelial cells of brain capillaries and supported by astrocytes, which are found in close proximity to the endothelial cells. These cells cover more than 99% of the capillary surface in the brain. The brain-cerebrospinal fluid barrier is situated in defined small circumventricular regions, and covers approximately 0.5% of the capillary surface.

These barriers inhibit influx of hydrophilic substances along electrochemical gradients. They contain different transport systems each of selective affinity to physiological substances. The barriers also allow access of many unphysiological hydrophilic substances with carrier affinity (Pardridge, 1988). Lipophilic substances can penetrate the barriers by unspecific mechanisms due to their lipophilicity.

In spite of this protection, the brain is very susceptible to toxic compounds. This is mainly due to the structural, functional, and integral complexity, the specific metabolic characteristics, and the limited capacity for compensation and repair.

7.6.2.5 PNS

In the PNS, nerve axons are surrounded by myelin, high in lipid content, which protects and insulates the axon membrane. Further, axons are bundled together in fascicles that are protected by connective tissue, thus creating a blood nerve barrier. A special feature of the PNS is the long distance between cell body and axon terminal. Common modes of action for peripheral nerve toxicity are affection of bi-directional axonal transport mechanisms and of myelin integrity.

Substances and other factors (physical stimuli, infections) interfering with BBB and myelin sheet integrity may give access to chemicals normally not penetrating these protective barriers and thereby indirectly induce neurotoxicity.

7.6.3 Developmental neurotoxicity

It is generally agreed that the brain is highly susceptible to effects of exposure to xenobiotics during development. During development, there is extensive mutual interaction between the developing brain and other organs, especially the thyroid gland and sex organs. Disturbance of this intimate interconnection may affect structure and function of brain and peripheral organs. Therefore, chemicals that affect sex organs or thyroid may affect the developing nervous system, and interaction between such chemicals is possible.

Apoptosis, programmed cell death, plays a significant role during brain development. Interference by neurotoxicants on apoptotic processes during development may be important.

An example of toxic interaction in relation to developmental neurotoxicity is the interaction between dimethoate and lead. Predosing of rats with low doses of dimethoate (a pesticide) accentuates the developmental neurotoxicity of lead as measured by electrophysiological parameters. Predosing with lead has the same effect on dimethoate neurotoxicity, but not as pronounced (Nagymajtényi *et al.*, 1998).

7.6.4 Consequences of adverse effects on the nervous system

The nervous system is central in the physical and psychological performance of man. It is one of the most complex organ systems in terms of both structure and function.

The risk of permanent damage to the nervous system after toxic injury is greater than for many other target organs, as mature neurons are not capable of regeneration. The nervous system possesses great plasticity, which means that injuries do not necessarily become apparent immediately after the insult.

Proper function of the nervous system depends on a delicate electrochemical balance for uncompromized communication of information throughout the body. Large energy supply and unaffected neuron function maintain this. Chemicals affecting these elements may cause neurotoxicity. Theoretically, there are many possibilities for interaction between chemicals on the above-mentioned targets (membranes, intracellular transport, energy supply).

7.6.4.1 Mechanism of interaction

The mechanism of action is unknown for the majority of neurotoxicants, especially in the CNS. In the PNS, a well-known mode of action for peripheral neuropathy is the inhibition of the anterograde transport of cellular components. Interaction is possible between chemicals which both affect this transport system, even if the target is not identical.

7.6.4.2 Most important test systems

Animal studies performed according to present guideline methods give information about nervous system effects. Investigation of potential interaction with other chemicals is normally not a part of such studies. The complexity of research on mixtures and dose-response relations is obvious, considering the possible number of binary combinations for a single mixture.

It is difficult to study interactions in epidemiological investigations because of uncertainties regarding exposure levels and duration and because the effects of individual factors normally cannot be isolated.

7.6.5 Examples of interactions: Mechanisms

7.6.5.1 Reactive oxygen species

Generation of reactive oxygen species (ROS) is a mechanism whereby similar action by chemicals can occur. ROS generation may initiate cellular injury leading to neurotoxicity and/or neurodegeneration. Several chemicals may lead to increased ROS generation. Various ROS types may be generated, depending on the chemical. Although the neurotoxic effect of the various ROS types may vary, at least an additive effect is expected.

7.6.5.2 Kindling

Kindling is the phenomenon whereby repeated seizure activity in the brain is accompanied by increased synaptic strength. Experimental kindling is used to study changes in nervous system excitability. Repeated presentations of subthreshold seizure stimuli (electrical, chemical) decrease the threshold for induction of seizures.

Certain pesticides (lindane, dieldrin) may change seizure susceptibility. Chlorpyrifos administered to immature rats caused a more rapid occurrence of kindling. The effects were additive with xylene (Wurpel *et al.*, 1993). Exposure to lead, or to trimethyltin rendered rats more susceptible to effects of seizure-inducing stimuli (see Ladefoged *et al.*, 1995).

7.6.5.3 Multiple chemical sensitivity

Multiple Chemical Sensitivity (MCS) is often defined by reactivity to common environmental exposures at significantly lower exposure levels than those, which would cause noticeable illness in the general population, and at levels that the majority of individuals tolerate quite well. MCS symptoms include symptoms originating from CNS (concentration and memory difficulties, headache, fatigue, depression, and irritability). Suggested triggers of MCS include odorous and non-odorous volatile organic compounds, solvents, pesticides, industrial chemicals, and metals. An IPCS workshop (IPCS 1996b) on MCS suggested another term for this phenomenon i.e. "Idiopathic Environmental Intolerances" (IEI). A working definition was formulated as follows: Acquired disorder with multiple recurrent symptoms, associated with diverse environmental factors tolerated by the majority of people, cannot be explained by any known medical or psychiatric disorder.

Often mixed and multiple chemcial exposure is mentioned in connection with MCS, however it is not known whether the greatest risk for development of MCS is in connection with single chemcial exposure or mixed exposure.

A recent review suggests a physical explanation for MCS, that of limbic kindling (Graveling *et al.* 1999). However, there is not international agreement on the existence of this phenomenon.

7.6.5.4 Gulf war syndrome

During the Persian Gulf War, 1990-1991, military personnel were concurrently exposed to biological, chemical, and psychological environments. Some veterans have reported the development of headache, loss of memory, fatigue and different somatic symptoms.

Three chemicals used in combination by military Gulf War personnel have been studied in hens with emphasis on peripheral neuropathy. The chemicals were an anti-nerve gas agent (pyridostigmine bromide), an insect repellent (N,N-diethyl-m-toluamide), and an insecticide (permethrin). The response to individual or simultaneous administration was investigated. The single compounds were administered in doses causing minimal toxicity. Combinations of two agents produced greater neurotoxicity than that caused by individual agents. Neurotoxicity was further enhanced when all three agents were administered concurrently (Abou-Donia & Wilmarth, 1996).

A recent Danish survey of Danish Gulf War personnel concludes that the most likely explanation for the symptoms identified in this population is a post-traumatic stress disorder rather than chemical toxicity (Gyntelberg, 2000).

7.6.5.5 Conditioning

Odour conditioning describes the situation where an agent such as a volatile organic compound with a distinct odour, experienced at a high concentration, evokes illness. On subsequent occasions, lower concentrations of the agent evoke the feeling, but with shorter latencies (Weiss, 1998).

Other stimuli may be effective in causing conditioning. Possible stimuli include respiratory irritants, which may induce a neurogenic response at low sub-irritant concentrations after an initial conditioning episode with a higher irritation-producing concentration.

7.6.5.6 Potentiation

A chemical without neurotoxic effect may interact with a neurotoxicant and increase the neurotoxic response/effect of the neurotoxicant. Numerous examples of ketone-induced enhancement of n-hexane- or 2,5-hexanedione-induced neuropathy in animals or man have been reported. This means that hexane or hexanedione is more neurotoxic at the same dose level when a ketone is co-administered. Hexane was 5 times more toxic when methyl isobutyl ketone was co-administered, using a neurotoxicity index (Abou-Donia, 1992). Hexanedione was twice as toxic when acetone was co-administered, based on a behavioural test (Ladefoged *et al.*, 1994). Although the mechanism is not fully elucidated, it is generally believed that the interaction is related to changes in metabolism, distribution, or elimination of the neurotoxic agent.

A chemical without neurotoxic effect may open the BBB and thus give access to the brain for a number of neurotoxicants, which otherwise would have been excluded from this tissue. Mannitol is a non-neurotoxic substance, which opens the BBB. An example of a chemical, where enhanced neurotoxicity by mannitol-induced BBB-opening is important, is doxorubicin, a cytostatic agent.

7.6.5.7 Promotion

In the field of neurotoxicity, the term promotion is used to describe the phenomenon where administration of one chemical, which itself is not neurotoxic, enhances the toxicity of another neurotoxicant. The time interval between exposure to promoter and to neurotoxicant may be long.

The initial observation of promotion was made during the study of chlorpyrifos-induced polyneuropathy. Sulfonyl halide, which is not neurotoxic by itself, enhanced the neurotoxic effect of chlorpyrifos when administered after chlorpyrifos (Lotti, 1997).

Some organophosphates, which do not induce delayed neuropathy, cause a stronger effect of a subsequently administered true inducer of delayed neuropathy (Lotti, 1992). The mechanism is possibly an interaction by the promoter in the “ageing” process of neuropathy target esterase, NTE, which is believed to be related to the induction of delayed neuropathy.

Several NTE inhibitors, such as certain carbamates, phosphinates, and sulfonyl halides protect the animals from neuropathy when they are administered prior to dosing with neurotoxic organophosphorus esters.

7.6.6 Examples of interactions: Agents

7.6.6.1 Organophosphorus insecticides

The toxicity of combined exposure to organophosphorus insecticides is generally considered to be additive. The ILSI (International Life Science Institute) Risk

Science Institute (RSI) have convened a group to develop a comprehensive approach for grouping chemicals by a common mechanism of toxicity using the organophosphorus pesticides as a case study (Mileson *et al.*, 1998).

Three scenarios were considered:

- two compounds that cause the same effect and induce toxicity by the same molecular mechanism,
- two compounds that cause different toxic effects and induce toxicity via the same molecular mechanism
- two substances that cause the same toxic effect and induce toxicity by different molecular mechanisms

Overall, the expert panel concluded that the anticholinesterase organophosphorus pesticides are a group of structurally related compounds that share certain characteristic toxicological actions, specifically the inhibition of acetylcholinesterase by phosphorylation and the subsequent accumulation of acetylcholine in the nervous system of animals. However, although the anticholinesterase organophosphorus pesticides clearly share the mentioned characteristics, they also produce a variety of clinical signs of neurotoxicity that are not identical for all organophosphorus compounds.

Ortiz *et al.* (1994) examined the acute neurotoxicities of formulated products of methyl parathion and permethrin in male rats with that of a commercially formulated mixture of the compounds. Methyl parathion was found to increase the LD_{50} of permethrin because of an inhibition of the carboxylesterase involved in the main metabolic pathway of permethrin. The decrease in carboxylesterase activity probably caused an increase in the concentration of permethrin, which again caused a greater toxicity. They also found that permethrin decreased the methyl parathion-induced inhibition of brain cholinesterase activity. Methyl paraoxon is a metabolite of methyl parathion and also inhibits the activity of brain cholinesterase.

An inhibition of the brain cholinesterase activity of 90 % was found in rats treated with methyl parathion but the activity was only inhibited by 40 % in animals treated with a mixture of methyl parathion and permethrin. Ortiz *et al.* (1994) therefore assumed that in rats treated with the mixture there was only enough methyl paraoxon available for a partial inhibition of the cholinesterase activity.

Although they used commercially formulated products in their experiment, they argued that the decreased inhibition found for brain cholinesterase activity in rats was due to the action of the active ingredient in the permethrin formulation. This argument was based on the experimental data on cholinesterase activity in brain of rats treated with commercially formulated products of methyl parathion and permethrin or with the formulation alone. They concluded that when a mixture of commercially formulated products of permethrin and methyl parathion was administered to rats at high doses the toxicity differed from that of either pesticide given alone. More specifically, the two pesticides produced a synergistic effect at doses corresponding to LD_{50} and LD_{90}.

Marinovich *et al.* (1996) compared the effect of mixtures of dimethoate, diazinon and azinophos-methyl *in vitro* on acetylcholinesterase in nervous cells with the effect of the single pesticides. They examined the following three mixtures in concentrations ranging from 0.4 to 100 μg/ml of the single compound:

A. dimethoate, diazinon and azinophos-methyl
B. benomyl and pirimiphos methyl
C. Dimethoate, azinophos-methyl, diazinon, pirimiphos methyl and benomyl

They found that the effect of mixture A on acetylcholinesterase activity was equal to that of the most potent compound, diazinon. Dimethoate was not active at the concentrations used in the experiment (1, 10 and 100 μg/ml). Dimethoate, diazinon and azinophos-methyl all have the same mode of action (inhibition of acetylcholinesterase). One would therefore expect to observe a simple similar action of the compounds in the mixture, which in fact was reported. A significant inhibition of protein synthesis after 4 hours of treatment were only seen for azinophos-methyl at the highest concentration tested (60 μg/ml). Dimethoate and diazinon were not active at all. The inhibition of protein synthesis of mixture A at the highest concentrations (100+40+60 μg/ml) was nevertheless greater than that of azinophos at 4 hours thus showing a potentiation effect.

The authors claim that mixture B of benomyl and pirimiphos-methyl was ”more potent than benomyl” in inhibition of the protein synthesis. As pirimiphos-methyl did not inhibit the protein synthesis mixture B also showed a potentiation. However since the two compounds does not act by the same mode of action one would expect a simple dissimilar action so this result is somewhat surprising.

The acetylcholinesterase activity of mixture C containing all five pesticides was equal to that of the most potent agent in the mixture thus showing a simple similar action. Mixture C was found to potentiate the protein synthesis at 4 hours as the protein synthesis was higher for the mixture compared to that of the single compounds. In the experiment made in relation to this they found that only benomyl had a significant effect on protein synthesis at the concentrations used.

7.6.6.2 Organic solvents

Several studies indicate that exposure to mixtures of solvents may be more harmful than exposure to single solvents (WHO, 1985, Organic solvents and the nervous system, 1990). Among 30 publications investigating cytochrome P-450 isoenzyme activity caused by solvent combinations, effects greater than mere addition were reported for 11 of the 23 solvent combinations investigated. The interactions of the solvents styrene, n-hexane, xylene, dichloromethane and toluene were described. The authors concluded that the rule of additivity should be used with caution when dealing with combined exposure for organic solvents in industry. Certain solvents should not be used together (Noraberg, 1993). It is not known whether the combined effect is the result of each chemical acting via the same or via different mechanisms.

It is generally believed that the narcotic effect induced by a mixture of solvents, including ethanol, equals the sum of the effect of the individual solvents. If the effect observed is greater than additive, then this is probably related to kinetic interaction. A number of reports of ethanol-induced enhancement of solvent neurotoxicity can be explained by kinetic factors.

However, ethanol intake six days before and during p-xylene inhalation exposure reduced the severity of the neurotoxic effect in rats (Padilla *et al.*, 1992).

Methylethylketone-induced potentiation of n-hexane/methyl n-butylketone neurotoxicity, through enhanced formation of 2,5-hexanedione, appears to have been responsible for the outbreak of an occupational neuropathy among textile workers (*Allen et al.*, 1975).

7.6.6.3 Metals

Toxic metals cannot be degraded but may react chemically with important sites. Most metals affect multiple organ systems, and the targets for toxicity are specific enzymes and/or membranes of cells and organelles, and biochemical reactions (chelation,

reaction with sulfhydryl groups). Therefore, the possibility for interactions is potentially great (Cassarett & Doull, 1986).

Lead, mercury and manganese exhibit interaction with other neurotoxicants, possibly via kinetic mechanisms. Ethanol may increase the absorption of lead from the gastrointestinal tract (Barton & Conrad, 1978). Cadmium and lead absorption is increased in individuals with low iron status (Flanagan *et al.*, 1978, Berglund *et al.*, 1994, Watson *et al.*, 1980).

The neurotoxicity of toxic metals may be reduced by chelation with chelators as EDTA, n-acetyl-D-penicillamine, dimercaptosuccinic acid, and 2,3-dimercaptopropane (Goyer *et al.*, 1995).

7.6.6.4 Pesticides and manganese

Many epidemiological investigations demonstrate a relation between exposure to pesticides and increased incidence for the development of Parkinson's disease (Ho *et al.*, 1989; Koller *et al.*, 1990, Semchuk *et al.*, 1992, Gorell *et al.*, 1997, Golbe, 1998, Gorell *et al.*, 1998, Tüchsen & Jensen, 2000, Engel *et al.*, 2001). Inhalation exposure to manganese is known to induce symptoms of Parkinson's disease (Parkinsonism, manganism) (Zayed *et al.*, 1990, Calne *et al.*, 1994, Gorell *et al.*, 1997). The underlying mechanisms are not known in details (Veldman, 1998), but are proposed to include elements different from those supposed to be related to Parkinson's disease (Arlien-Søborg, 2001). Because of the similarity in effect, a potential for interaction between pesticides and manganese in the development of symptoms of Parkinson's disease exists. However, this potential has not been investigated.

7.6.6.5 PCB

Polychlorinated biphenyl (PCB) is a mixture of compounds, which may possess individual effects on the nervous system as well as exhibit interaction phenomena. Developmental neurotoxicity involving cognitive and behavioural disturbances has been implicated following perinatal exposure to environmental pollutants through fish. Mainly PCBs have been implicated (Jacobson & Jacobson, 1996). There are several studies, which show that PCBs produce a wide spectrum of neurochemical and neuroendocrine effects in animals. Ortho-substituted PCB congeners affect brain neurochemistry, while non-ortho-substituted PCBs having dioxin-like activity may have little or no activity in the nervous system (Tilson & Kodavanti, 1997). However, the fish contains a mixture of methylmercury, arsenic, lead, PCBs, DDT and DDE, and effects may be the end result of additive or synergistic interaction between these components (Mergler *et al.*, 1997).

7.6.6.6 γ-diketones

When γ-diketone precursors are co-administered, the neurotoxic effect observed equals the sum of effects caused by the individual substances.

7.6.6.7 Glutamate agonists

Injection of the glutamate agonist NMDA prior to injection of the causative agent for shellfish poisoning, domoic acid, which is also a glutamate agonist, potentiates the acute neurotoxicity of domoic acid in mice by a factor less than two (Tasker & Strain, 1998). This is an example of toxicodynamic interaction at the level of the glutamate receptor.

7.6.6.8 Physical stimuli

Physical stimuli may interact with the neurotoxicity of chemicals. One example is the increased hearing loss caused by interaction between organic solvents and noise (Morata *et al.*, 1993).

7.6.6.9 Stress

Stress may increase the brain's vulnerability to chemical substances by making the protective shield, the BBB, more permeable to neurotoxicants. This may be a possible mechanism for the entry of pyridostigmine into the brain of soldiers (Friedman *et al.*, 1996).

7.6.7 Conclusion

There are many possibilities for interaction between neurotoxicants because of the complex hierarchical structure of the nervous system. A number of examples have been described of which the most well-known are the additive effects of organic solvents with respect to narcotic effects, and organophosphorus insecticides and acetylcholinesterase inhibition. It is therefore evident that interaction is a real phenomenon and not just a theoretical possibility.

The strongest interaction found in the literature was a 5-fold increase in the neurotoxicity of hexane when methyl isobutyl ketone was co-administered. However, very few quantitative studies have been performed. Interaction has not been studied systematically, and the present state of knowledge does not allow general conclusions.

Potential neurotoxic interaction of chemicals by additive and synergistic effects and promotion should always be considered in the risk assessment procedure.

7.7 Immunotoxicity

Prepared by Charlotte Madsen

7.7.1 Direct toxic effect on the immune system

7.7.1.1 Introduction

The immune system is comprised of a number of organs and cells throughout the body. The cells are lymphoid cells of different lines and stromal cells in the lymphoid organs e.g. epithelial cells in thymus important for T-cell differentiation. A successful immune response is dependent on co-operation between different cell types. For example the production of antibodies against a foreign antigen requires macrophages presenting the antigen to T-cells, which as a consequence produces cytokines necessary for B-cells to differentiate into immunoglobulin producing plasma cells. The reactions of lymphoid cells are associated with gene amplification, transcription, and translation, and compounds that can affect these processes of cell proliferation and differentiation may exert immunotoxicity, in particular by affecting the rapidly dividing thymocytes and haematopoietic cells of the bone marrow (IPCS 1996a).

7.7.1.2 Measuring immunotoxic response

Toxic responses to the immune system can be measured experimentally in vivo using classical haematology and pathology, total and differential white blood cell counts, and weight and histology of lymphoid organs. Additional immunological methods include measurement of serum immunoglobulins, lymphocyte subsets, and unspecific lymphocyte reactivity to mitogens, specific lymphocyte reactivity to allogenic cells, Natural Killer cell activity and antibody production after immunisation with sheep red blood cells.

7.7.1.3 Mechanisms of immunotoxicity

A variety of mechanisms can be involved in immunotoxicity. Ultraviolet radiation and 7,12-dimethylbenz[*a*]anthracene can cause the disappearance of Langerhans cells from the skin (or the loss of their function), with consequent disturbance or dysregulation of the skins immune function. Cyclosporin A blocks the synthesis of lymphokines. The thymus toxicity of 2,3,7,8-TCDD is due to an effect on the epithelial cells of the thymus mediated by Ah-receptor. Bis(tri-N-butyltin)oxide is also toxic to the thymus but primarily because it induces cortical thymocyte cell death (De Wall *et al*. 1997). PCBs are mainly toxic to B-cells. Ozone modulates the non-specific defence mechanisms suppressing phagocytic activity in alveolar machropages. The immunotoxic effect of aflatoxin and maybe trichothecenes is probably linked to inhibition of protein synthesis (IPCS 1996a).

7.7.1.4 Combined action and interactions in immunotoxicity

Mixtures of chemicals such as tobacco smoke, engine exhaust and air pollutants may have an effect on the immune system (rewieved in Germolec and Luster 1994). There are few genuine immunotoxicology studies performed on chemical mixtures.

A mixture of 25 groundwater contaminants was studied in B6C3F1 mice in a 14 days and 90 days study. The mixture was found to be myelotoxic, suppressing the granulocyte-macrophage progenitor cells. In addition a decreased cellularity was observed in the bone marrow. Antibody formation to sheep red blood cells was decreased. An infection model showed an increased number of parasitised red blood cells in mice challenged with the malaria parasite P. yoelli. Several of the components of the mixture, including Aroclor, benzene, and heavy metals, had previously been shown to cause similar immmunological effects in laboratory animals. However, the authors conclude, that none of the individual contaminants were present at sufficient concentration in the chemical mixture to be solely responsible for the observed effects on the immune system (Germolec *et al.* 1989). Although all chemicals were administered in sub-immunotoxic doses the total dose in the highest dose groups was relatively high, i.e. 756 ppm in drinking water in the 14 days study and 378 ppm in the 90 days study, because of the many chemicals in the mixture. The result of this study indicates some kind of additive immunotoxic effect.

Hsieh *et al.* (1990) exposed mice to a mixture of benzene and toluene in drinking water. Benzene alone produced the well-characterised profile of anaemia and alterations in immune function. The mixture of benzene and toluene was completely devoid of these adverse effects probably because the two chemicals compete for the same metabolising enzymes and thus the bioavailability of toxic metabolites was decreased.

Omara *et al.* (1997, 1998) exposed rat splenocytes, thymocytes and peripheral blood lymphocytes *in vitro* for 24 or 72 h to methylmercury (MeHg), mixtures of PCDDs and PCDFs, and mixtures of PCBs alone and in combination. A variety of immmunological parameters were measured. The only immunotoxic effect found was a decreased response to T and B cell mitogens induced by MeHg. All combinations of MeHg/PCBs/PCDDs/PCDFs decreased the mitogenic response to levels similar to those of MeHg alone. In these studies no additive effect was found. The *in vitro* exposure of lymphocytes to these chemicals is not optimal for assessing immunotoxicity and may possibly explain the meagre results of the study.

The immunotoxic effects of TCDD are mediated by binding to a soluble cytosolic protein, the aryl hydrocarbon receptor, present in the thymus in the epithelial cells.

On the basis of a common receptor-mediated mechanism of toxic action, the relative immunotoxicity of individual PCDDs and PCDFs can be expressed relative to TCDD (Vos *et al.* 1997). When the immunotoxicity of a mixture of PCDDs and PCDFs are calculated and additive effect is assumed because of the common receptor mediated toxicity.

7.7.1.5 Conclusion

The many different cell types involved in the function of the immune system gives many theoretical possibilities of combined immunotoxic effects. As apparent from the above there are yet few experimental data to draw upon. Chemicals sharing a common toxic mechanism seem to have an additive effect. Competition for metabolising enzymes may antagonise the immunotoxic effect. How mixtures of chemicals that are toxic to different branches of the immune system will exert their combined immunotoxic effect remains to be established.

7.7.2 Allergy

7.7.2.1 Introduction

In the following the possible consequence of combined exposure to different chemical allergens on the skin will be discussed. Respiratory allergy to small molecular weight chemicals will not be covered because of lack of data.

7.7.2.2 Allergic contact dermatitis

Skin contact with chemical compounds may induce cellular mediated contact sensitisation. The consequence of this contact sensitisation can be allergic contact dermatitis.

Most contact allergens are small molecules with a molecular weight below 600 dalton. Contact sensitisation is not inborn but is always a consequence of earlier cutaneous contact. Contact sensitisation is considered to be life-long, but may become weaker if exposure is avoided. Contact sensitised individuals are at risk of developing the skin disease allergic contact dermatitis if re-exposed to the specific chemical (IPCS 1999).

7.7.2.3 Patch test

Contact sensitisation to environmental chemicals is diagnosed by the use of patch testing. The test is a biological test where contact allergy is proven by re-exposing the skin to the specific chemical under occlusion on a 0.5 cm^2 large skin area on the upper back for 2 days. A positive test is a reproduction of the clinical disease showing redness, infiltration and eventual vesicles. All patients are primarily tested with the Standard series including the most frequent sensitising chemicals such as metals, preservatives, fragrances, rubber additives, and topically used medicaments. Sensitisation can be quantified according to the degree of positive patch test reaction (+ to +++), patch test concentration threshold defined by dilution series, and finally by the “Use test”. In the latter test the individual is exposed to the chemical simulating normal use (IPCS 1999).

7.7.3 Patch test results with mixtures of chemicals

It is common clinical experience that testing with a mixture of substances, for instance a cosmetic product may cause a positive patch test reaction and subsequent testing of the single ingredients a negative response (Johansen et al 1998). In accordance with this Johansen and Menné (1995) found that only half of the patients reacting to fragrance mix from the European Standard patch test series also gave a positive response to at least one of the eight fragrance mix constituents

tested separately. This is a phenomenon also known from testing with other mixtures such as rubber allergens (Menné et al 1992) and preservatives (Menné and Hjort 1988).

These results have often been interpreted as a false-positive patch test to the mix. "False-positive" patch tests may have several explanations: cumulative irritancy of allergens in combination, false-negative reactions to the constituents, unequal absorption of the allergens, generation of a new hapten as a reaction product, etc. (Johansen and Menné 1995). However, despite a number of changes carried out in the composition of the fragrance mix to overcome problems related to irritancy and unequal absorption of allergens, the frequency of false-positive patch tests has remained stable over the years (Krasteva et al 1996).

7.7.4 Experimental studies on the elicitation of allergic response to mixtures of chemicals.

Fourteen patients with positive patch tests to two unrelated allergens were studied and the response to those two allergens was measured when tested singly or in combination, using 10 different paired combinations from 15 common allergens. Two-fold serial dilutions of the allergens were used for patch tests and change in skin thickness used as the objective measure of response.

Subthreshold doses: The threshold concentration at which a response occurred was established. Two further subthreshold dilutions were made for each of the allergens and when a subthreshold dose of each allergen was combined on one patch, a measurable response was produced. The threshold at which a response occurred to the combination of allergens was significantly lower than the threshold for a response to the individual allergens. It was stated that at subthreshold doses a purely additive effect would be indistinguishable from synergy.

Linear part of dose-response curve: 2-6 antigen concentrations were used, which produced a patch-test response to the individual allergen on the linear part of the dose-response curve. A greater response was elicited by the dilution of allergens in combination than to either of the individual allergens. The response to the mixture of the allergens was the same as the arithmetical sum of the responses to the individual allergens as measured by change in skin fold thickness. Thus the authors conclude that the increase in response to the mixture of allergens is additive (McLelland and Shuster 1990).

Female Balb/C mice were experimentally sensitised to the two contact sensitisers 2,4-dinitroflurobenzene (DNFB) and 4-ethoxy-methylene-2-phenyloxasol-5-one (oxazolone). The sensitisation was measured by applying the chemicals to the ear and measuring increase in ear thickness. When optimal challenge concentration of the single chemical and the chemicals in combination were used there was no significant difference in the ear swelling between the single chemicals and the mixture. As the two single chemicals were used in a concentration giving a maximum response reaching the plateau of the dose response curve it is not possible to induce a further reaction by mixing the two chemicals. When the mice were challenged with suboptimal or subthreshold concentrations of the two chemicals in combination strong positive responses were induced. The authors conclude that the results suggest that a "false-positive" reaction to a mixture of allergens may reveal a genuine sensitisation to the constituents (Krasteva et al 1996).

Two groups of patients with allergic contact dermatitis to perfume ingredients were included in the study, 18 subjects with a contact allergy to two fragrance

substances and 15 allergic to only one of the same two fragrances. The test and matched control subjects were patch tested with two allergens applied in serial dilutions in separate chambers on one side and combined in one chamber on the other side of the upper back. The assessment of reaction was carried out on day 3 by clinical grading and by measuring the blood flow using laser Doppler flowmetry. The extent of the reaction was measured in millimetres. The 1:1 mixture of the two allergens elicited responses as if the doses were three to four times higher than those actually used. This result was obtained both for the clinical grading, the blood flow and the size of reaction in mm. The data were analysed by logistic dose-response models comparing the horizontal displacement of the dose-response curves. The conclusion of the study is that the combination of two allergens had a synergistic effect on the elicitation response (Johansen et al 1998).

In all three studies elicitation of an allergic response is enhanced if two unrelated allergens are mixed. It is possible to elicit a response by using a subthreshold dose for both allergens and it is possible to enhance the response at levels above the threshold dose. McLelland and Shuster conclude that the effect is additive and Johansen that it is synergistic. Johansen uses a statistical method that is used in pharmacology and toxicology to assess relative potency and possible interaction between two drugs. McLelland and Shuster add the results from the linear part of the dose-response curve only. Even though they show that there is synergy below the threshold level they do not include this in the conclusion. The data presented by Johansen and by McLelland and Shuster are very similar. The discrepancy in conclusions stems from the different use of statistics.

7.7.5 Sensitisation

Inhibition of sensitisation, also called quenching, has been described .for chemicals in essential oils thought to be able to inhibit sensitisation to contact allergens in the same oil. The phenomenon has been studied extensively by Basketter and Allenby (1991). However, these investigators were not able to confirm the existence of inhibition of sensitisation of one chemical by the simultaneous application of another.

In a study of the influence of the skin irritant sodium lauryl sulphate (SLS) on sensitisation to 2,4,-dinitrochlorobenzene Cumberbatch et al (1993) found that SLS did not enhance sensitisation by increased skin penetration but by an increase in the number of immunostimulatory dendritic cells from the skin which reach the draining lymph node. van't Erve et al (1998) found that the cellular and humoral response to the contact allergen oxazolone was dissimilary affected by the vehicles used. A wet work environment will also enhance the possibility of sensitisation as water helps to break down the skin barrier. Occlusion e.g. gloves, armpits, will also facilitate sensitisation. Skin disease will do the same. The increased sensitisation may be induced by increased penetration of the skin, or other mechanisms as in the SLS study.

7.7.6 Conclusion

If a person allergic to two unrelated allergens is challenged with these two allergens in combination the subsequent elicitation response will be the sum or greater than the expected sum of response to the two allergens alone. The challenge with two allergens combined at subthreshold doses can elicit a response that none of the allergens alone at that dose may elicit.

The consequence of this is that a person may be negative in a diagnostic patch test with single chemicals although a mixture of chemicals would produce a reaction.

In practical life an allergen may be tolerated in one situation but not in another where it is in combination with another allergen. The threshold dose for elicitation may be very low for allergens in mixture e.g. fragrances in perfume.

A chemical or a physical condition (occlusion, disease) that facilitates penetration of the skin by a contact allergen or have an effect on the skin immune system may enhance the possibility of sensitisation.

8 Books, articles and reports

Several reference books, review articles and reports have been published on the issue of toxicological effects from combined exposure to chemicals. The more important are:

8.1 Books

Multiple Chemical Interactions (Calabrese 1991) by Edward J Calabrese is an exhaustive textbook. It consists of six parts:

- General principles, which contain chapters on terminology, analysis of combined effects, and pharmacokinetic and pharmacodynamic foundations of interactions.
- Carcinogenesis and teratogenesis.
- Interactions by chemical class.
- Interactions between medical drugs.
- The drug-pollutant interface.
- Regulatory perspectives.

The main emphasis is related to drug therapy and drug-pollutant interactions. However, other authors often make references to the first chapters of the book.

Toxicology of Chemical Mixtures – Case studies, Mechanisms, and Novel Approaches (Yang 1994) edited by Raymond SH Yang is an important work in the field of combination toxicology. The book includes basic concepts and mechanisms of interactions in target organ toxicities and it contains a number of informative case studies on different types of industrial and environmental chemicals, such as pesticides and hazardous waste. In addition, it offers novel approaches in the risk assessment of chemical mixtures. Special attention is paied to low-level, long-term exposures.

8.2 Review articles

Recent review articles have been published by Dutch researchers from the TNO Nutrition and Research Institute and The National Institute for Public Health and the Environment, Holland. *Toxicological Evaluation and Risk Assessment of Chemical Mixtures* was published in Critical Review of Toxicology by Cassee *et al.* (1998). *Toxicology of simple and complex mixtures* was published in Trends in Pharmacological Sciences by Groten *et al.* (2001) and *Toxicological evaluation of chemical mixtures* was published by Feron and Groten (2002) in Food and Chemical Toxicology. Concepts and perspectives in the area are comprehensively described and illustrated by results of toxicological studies on well-defined chemical mixtures performed by the same research group. A pragmatic approach for the study, hazard identification, and risk assessment of chemical mixtures is offered based on the hypothesis of this group of researchers (around Victor Feron) that exposure to mixtures of chemicals at non-toxic doses of the individual constituents is of no health concern.

8.3 Conference proceedings

Proceedings of a symposium sponsored by the Health Effect Research Laboratory of the U.S. Environmental Protection Agency, November 7-10, 1994 entitled *Chemical mixtures and quantitative risk assessment* has been published as a special issue of Toxicology (vol. 105 (2-3), 1995). It contains 8 session summaries and a total of 29 scientific papers presenting updated knowledge on combination toxicology.

Proceedings of *The European Conference on Combination Toxicology*, held in Veldhoven, The Netherlands, October 11-13, 1995 was published in Food and Chemical Toxicology (34 (11/12), 1996). Nineteen fairly brief articles present the European state of the art in combination toxicology.

8.4 Reports

Combination Effects. A survey of biological responses to mixtures of industrial chemicals and pesticides was prepared by Steen Clemmensen in 1986 for the Nordic Council of Ministers. This report was the first Danish effort to highlight the importance of evaluating the possible combined effects of chemical mixtures. The report deals with the fundamental concepts, biological mechanisms of interaction, combined actions after acute or prolonged exposure to chemical mixtures, including reproductive, mutagenic and carcinogenic efects, as well as how to extrapolate from high dose exposure to low dose exposure.

The report: Sundhedsmæssig vurdering af luftforurening fra vejtrafik (*"Health Evaluation of Air-Pollution from Road Traffic"*) by Larsen PB, Larsen JC, Fenger J and Jensen SS (1997) published in Danish by the Danish Environment Protection Agency contains an extensive Summary and Conclusions in English. The report contains evaluations of health effects from combined exposures to traffic related air pollutants especially in the areas of airway irritation, allergy and hypersensitivity, and cancer.

Combined actions of pesticides in food was prepared by Trine Klein Reffstrup in 2002, Institute of Food Safety and Nutrition at the Danish Veterinary and Food Administration (FødevareRapport 2002:19). The report summarises and evaluates the current knowledge on combined toxic effects of mixtures of pesticides. The most important models for risk assessment of pesticide residues in food are discussed and recommendations for future approaches in risk assessments in Denmark are presented.

Guidance manual for the assessment of joint toxic action of chemical mixtures was prepared by the Agency of Toxic Substances and Disease Registry (ATSDR) under the U.S. Department of Health and Human Services (ATSDR 2002). This report summarises terminology and basic concepts of toxicological interactions among chemicals and gives an overview of the approaches used for risk assessments by various North American agencies. It also provides its own strategies for exposure-based assessments for noncarcinogenic and carcinogenic effects, respectively.

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