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COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COC)

INITIAL DISCUSSION PAPER ON THE ASSESSMENT OF CHEMICAL MIXTURES FOR CARCINOGENICITY

Introduction

1. The COC expressed an interest in current developments in the assessment of chemical mixtures with regards to carcinogens and their modes of action. This paper aims to provide a simple overview of mixtures toxicology and a representative selection of published investigations, using receptor mediated responses (dioxins and estrogenic chemicals) and assessments of drinking water by-product mixtures as examples of the available literature.

2. Chemical mixtures are considered to be simple or complex. A simple mixture is one which is defined as a mixture consisting of a relatively small number of different chemicals (arbitrarily less than 10) whilst a complex mixture comprises a much larger number of chemicals, potentially hundreds or thousands and it is likely the composition, both qualitatively and quantitatively, will be poorly defined.

Basic models and concepts of mixtures assessments are based upon the classification of three possible combined (joint) toxic actions of the compounds in a mixture as follows:

1. Simple similar action (non-interaction, dose addition)
2. Simple dissimilar action (non-interaction, response addition)
3. Interaction (synergism/potentiation or antagonism/inhibition)

When there is evidence that the members of a group of chemicals elicit their effects by the same mechanism or mode of action, their combined effects can be determined by using Toxic Equivalency factors (TEF). TEFs are relative potencies, expressed relative to an 'index compound' and are used to normalize exposures of chemicals within such a common mechanism group to the 'index compound'. The TEF system was first developed to facilitate risk assessment for dioxins and related chemical classes (Safe 1990). The TEF for each chemical is derived from its point of departure (e.g. a Bench Mark Dose such as BMD10) relative to that of the index chemical, which is generally the one for which toxicity and ADME profiles are best characterised.

3. A variety of designs to study the toxicity of simple mixtures as well as methods to analyze the data obtained are available. These designs and methods include traditional empirical approaches (for simple mixtures for which toxicological information is available on the constituents), models for competitive agonism, or statistical designs such

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as full or fractional factorial designs, ray designs, and central composite designs. Factorial designs, where n chemicals are tested at x dose levels (i.e. x^n dose level combinations) and compared to the mixture, provide the most statistically valid assessment of a chemical mixture. However this approach is generally very expensive and once statistically useful group sizes are used, are also costly regards animal usage. As such this approach is more widely applied to high throughput type *in vitro* screening.

4. Members will be aware of the COT WiGRAMP report on the risk assessment of mixtures of pesticides and similar substances (WiGRAMP 2002) which provides comprehensive information specifically on the mixtures arising from dietary exposure to residues of pesticides or veterinary medicines. Regulatory approaches, evidence of exposure, and biomonitoring strategies were examined as well as the methodological approaches that can be adopted. A summary of the literature available at the time and which pertains to carcinogenicity is provided (APPENDIX 1). Overall conclusions were that chemicals with the same mode of action could be considered to be dose additive at levels occurring as residues in food.

5. The COM have recently considered in some depth the mutagenicity testing of chemical mixtures; the papers provide a comprehensive evaluation of current thoughts on mixtures of genotoxic substances. The papers can be found on the COM website (MUT/07/03 and MUT/07/15). The papers focused on three main areas: testing of whole mixtures, approaches to fractionation of a mixture (with view to monitoring of occupational and environmental sources and aiding risk reduction strategies), evaluating potential interactions using mutagenicity tests. The principle outcomes of the COM discussions were:

a) an outline proposal for a strategy for fractionation and monitoring of the mutagenicity of chemical mixtures and

b) that a number of studies provided examples of and hypotheses regarding the potential interactions between DNA reactive chemicals.

6. There are a number of international collaborative efforts to establish strategies for the assessment of chemical mixtures, for example ILSI-HESI are addressing the potential from synergy at low dose environmental exposures and International Programme on Chemical Safety (IPCS) are examining cumulative exposures in a tiered approach to combined exposure.

<http://www.who.int/ipcs/methods/harmonization/areas/aggregate/en/index.html>

7. With regards to the concept of assessing potential interactions and synergistic behaviour of chemicals in the context of carcinogenicity, the multi-stage nature of the process means it is less straightforward than assessing simple genotoxic events or a target organ toxic effect. Initiation – promotion models of cancer, for example mouse skin and rat liver, are examples of a synergistic response. In the skin model a promoter is applied, usually sub-chronically, to shaved skin treated with a single dose of a chemical which is anticipated to be a genotoxin (initiator) and then tumour formation is examined after about 10 weeks. Similarly in rodent liver, a number of initiator-promoter dosing regimens are routinely used to investigate stages of carcinogenesis. For example, in the

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Solt-Farber model comprising DEN initiation, followed by sub-chronic 2-AAF administration and partial hepatectomy, providing cell proliferative promotion, enzyme foci are produced within 7 days. With regards to human carcinogenesis, an example of a 'mixture' effect is the observation that there is a significantly much higher incidence of liver tumours in people exposed to aflatoxin B₁ who have hepatitis B (the proliferative response) then in those who do not. Similarly, lung cancer rates in those who smoke cigarettes and who are also exposed to asbestos are much more than additive. In this manner it could be postulated, and there is good supporting experimental evidence, that the combination of any chemical that causes DNA damage (genotoxicity) coupled with any chemical (or factor) that enhances cell proliferation could would act synergistically, the response being more than additive of the two chemicals (insults) independently.

Is it possible that this concept can be introduced into risk assessment strategies for environmental or occupational exposure to potential carcinogens?

Receptor mediated responses and equivalency factors

8. The term common mechanism group (CMG) is frequently used in mixture risk assessment and applies to a class of chemical with the same mode of action. Most simply, this applies to chemicals which act through the same molecular target, e.g. a receptor, such as the AhR receptor or the estrogen receptor.

Dioxins:

9. In terms of mixtures toxicology, the most comprehensively examined class of compounds are the dioxins and thus these have the most extensively defined TEFs. A recent WHO-IPCS meeting re-evaluated TEFs for dioxins (Van de Berg et al 2006). However the end-points examined were primarily effects mediated by the AhR receptor other than carcinogenicity. However, it seems likely that similar considerations and perhaps even values for the TEFs (which represent ranges and not point determinations) would apply.

The US National Toxicology Program (NTP) evaluated the carcinogenicity of a number of mixtures of dioxin like compounds (DLC's). In 2 year bioassays, varying the ratio of a binary mixture of PCB 126 (TEF 0.1) and PCB153 (ratio of 99:1) demonstrated some different effects across the groups, notably that an increase in PCB153 was associated with nonneoplastic changes in the liver (NTP 2006a). However there was some indication that the TEF values may not be wholly predictive although no attempts were made to address the possibility of interactions.

More recently, Walker et al (2005; appended) examined the dose additivity of the carcinogenicity of a group of dioxins, based on their respective TEFs. Doses were selected to test the hypothesis that the tumour response caused by a mixture can be predicted from potency and the dose additivity model. Using WHO TEQ of 1 for TCDD, 0.1 for PCB-126 and 0.5 for PeCDF specific target doses were constructed and administered to Sprague-Dawley rats in a classical 2-year bioassay. The TCDD

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equivalent doses used were 10, 22, 46 and 100 ng/kg/day (e.g 10 ng/kg comprised of 3.3 ng/kg TCDD, 6.6 ng/kg PeCDF and 33.3ng/kg PCB-126) and a range of doses of the individual dioxins was also examined. Analysis used statistical modelling (Hill function) to assess the accuracy of predictions based on the dose additivity model. Broadly it was found that for chloangiosarcoma, hepatocellular adenoma, cystic keratonizing epithelioma (CKE) and gingival SCC the results were generally consistent with dose additivity on the basis of the WHO TEF values. This is a robust study which has thoroughly examined the appropriateness of the assumption of dose additivity and the use of TEFs for chemicals acting via the AhR receptor

10. Maruyama and Aoki (2006; appended) re-examined the relative equivalence potencies (REP) for TCDD, PeCDD and PeCDF by focusing on tumour promoting activity with the aim of quantifying the health risk at current (Japanese) levels of contamination. A liver foci formation assay in female SD rats was used; initiation with diethylnitrosamine was followed on day 13 with dioxin orally, 70% partial hepatectomy on day 21, and then a further 4 dioxin administrations at weekly intervals. A PBPK model was then used for interspecies extrapolation to predict REP in man. It was concluded that the relative risk of excess liver cancer fro Japanese people at the current level of contamination was $1.7-6.5 \times 10^{-7}$ for TCDD and $2.9-11 \times 10^{-7}$ for the three in combination.

Estrogens :

11. The concept of a 'cocktail' effect of chemicals has persisted following a report describing a synergistic effect of combinations of pesticides in an *in vitro* assay for estrogen receptor activation (Arnold et al 1996), despite the fact that the results were never reproduced and the original findings were later withdrawn (McLachlan et al 1997). However, this is still an area of interest which has merited further attention, although from a review of the literature it appears that combinations of estrogens are being more widely investigated in relation to their environmental impact particularly in fish, and that the human health concerns are those relating to reproductive rather than carcinogenicity. From the studies that were retrieved it appears that this is an area where wide use is made of *in vitro* high-throughput screens which can therefore be used with factorial designs to fully evaluate mixture effects. However, this is obviously a complex area, and interaction at the estrogen receptor is just one way in which estrogenic effects can be induced. For example, it also possible to change endogenous estrogen production, through effects at a number of sites.

12. A representative, comprehensive study conducted as part of the ATSDR mixtures programme looked at 6 organochlorine pesticides (4'4'-DDT, 4'4'-DDD, 4'4'-DDE, aldrin, dieldrin and endrin). Herein HeLa cells were used to examine the ability of individual and pairs of chemicals to transcriptionally activate cotransfected estrogen receptors containing the CAT reporter plasmid (Mumtaz et al 2002). Little induction was observed following dosing with any of the pesticides compared to the estradiol positive control and certainly no additive or greater responses were reported. This paper also presents a study which investigated the use of a commercially available assay system,

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CAT-Tox, a battery of HepG2 cell lines designed to test the transcriptional activation capacity of xenobiotics in a number of different signal transduction pathways (such as NF κ BRE, CYP1A1, XRE, hMTIIA). The metals As(V), Cd(II), Cr (III,IV) and Pb(III) induced individual and complex induction profiles and the authors comment on the insight that these data provide to understanding the modes of action by which they elicit their effects. With regards to the combinations of metals, it was demonstrated that there were no results which provide evidence of possible synergistic responses of co-administration of these metals. It does appear that these types of *in vitro* based systems will be extremely useful for examining potential interactions.

13. Another *in vitro* approach, the MCF-7 cell ER- α reporter gene system, was investigated with a view to examining its usefulness to identify potential interactions of estrogenic mixtures of chemicals (Charles et al 2002; appended). A mixture of 17 β estradiol (E2), ethinyl estradiol, and diethylstilboestrol, assumed to be dose additive, and a mixture of 17 β -estradiol, epidermal growth factor and insulin like growth factor, selected to model greater than additive response were tested using complex full factorial study designs (4 concentrations of each chemical, 64 treatment groups). The observed responses were as predicted, notably the known interaction and therefore greater than additive effect of E2 and EGF. The authors comment that it was possible to observe that the nature of the interaction is dependent on dose and conclude that the system employed would be useful to investigate potential environmental chemical mixtures at relevant concentrations.

14. Payne et al (2001) used the MCF-7 proliferation assay to produce extensive concentration-response analyses from the effects of the organochlorines o,p'-DDT, p,p'-DDE, β -hexachlorocyclohexane, p,p'-DDT and their mixtures. The predicted concentration addition and dose addition responses for combinations of chemicals from individual responses were achieved. The thresholds for the effect of the individual chemicals and the mixtures were also examined. As discussed previously, it was noted that effects may differ at high and low doses thus indicating a need to consider relevant exposures when investigating mixture responses.

15. Van Meeuwen et al (2007) looked at mixture effects of estrogenic compounds, both food-borne phytochemicals and synthetic estrogens, on proliferation in MCF-7 cell line. Again, dose additivity was demonstrated.

***In vivo* approaches – drinking water by-products**

16. There are a few examples of studies which have aimed to investigate *in vivo* responses to mixtures of carcinogens; these include either classical oncogenicity studies, responses in models of carcinogenesis or biomarkers of relevant endpoints. Here a number of reports of investigations of drinking water disinfection by-products are summarised which indicate how investigators are attempting to understand the nature of potential interactions in realistic scenarios.

17. Pereira et al (1997; appended) addressed the hepatic tumour promoting ability of dichloroacetic acid (DCA) and trichloroacetic acid (TCA) and a combination of the two

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in female B6C3F1 mice. Following initiation with a single dose of N-methyl-N-nitrosourea (MNU) at 25mg/kg at 15 days of age, DCA (7.8, 15.6 or 25mmol/L in drinking water) or TCA (6.0 or 25.0 mmol/L) or combinations of DCA and TCA (25+6, 15.6+6, 7.8+6, 15.6+25mmol/L) were given from 6-50 weeks of age. Promotion activity was assessed as the number of GST positive foci in the liver. The increase in DCA induced foci (principally eosinophilic, GST+ve), was not linear, with a greater than dose proportional increase at 25 mmol/L and the same shape dose response curve was generated when the two chemicals were administered together and the response induced by 25mmol/L DCA was not enhanced when 6.0mmol/L TCA was co-administered. For TCA the number of proliferative lesions, both altered foci and adenomas, increased linearly with dose and the lesions were principally basophilic and GST⁻ve. When 25mmol/L TCA and DCA 15.6mmol/L were given a greater than additive number of lesions were found and interestingly these were predominantly eosinophilic. Thus it is concluded that DCA predominates over TCA with regards to mechanism of promotion and that there is a potential synergistic response.

18. Hooth et al (2002) assessed the carcinogenicity of a mixture of water disinfection by-products in the Eker rat (Tsc2 Mutant Long Evans), a model of hereditary renal cancer. A number of known renal carcinogens or nephrotoxicants, namely potassium bromate (KBrO₃), 3-chloro-4-(dichloro-methyl)-5-hydroxy-2-(5H)-furanone (MX), chloroform (CHCl₃) and bromodichloromethane (BDM) were tested. Animals were exposed to the chemicals in drinking water for 4 or 10 months as high or low doses and a mixture of all the high doses or all the low doses based on previous carcinogenicity data as follows:

KBrO₃ at 0.02 or 0.40g/L, MX 0.005 and 0.07 g/L; CHCl₃ 0.40 g/L and 1.8 g/L; BDCM 0.07 and 0.7 g/L. Groups of 8 male and 8 females were used for the single chemicals and 10 or 14 per group for the mixtures. In this model the generation of early, easily identified proliferative lesions enables an assessment of carcinogenic potential and potency to be made. A number of non-neoplastic findings were reported, including proliferative lesions in the spleen and uterus, as well as renal adenomas and carcinomas after 4 and 10 months treatment in both males and females. This model results in tumours in untreated rats at these time-points and therefore the number of tumours/rat is considered to be indicative of carcinogen potency. The greatest incidence of tumours was observed following administration of MX at 0.7 g/L for 10 months, maximum 35.4 tumours/animal in females. In the high dose mixture group there were 18.6 tumours/animal. After 4 months the total tumours/animal were 4.1 and 5.3 for MX and high-dose mixture groups respectively. The authors concluded that the default assumption of additivity may overestimate the carcinogenic effect of a mixture of disinfectant by products in water.

19. A mechanistic study assessed oxidative damage induced by these same drinking water contaminants and the mixture (McDorman et al 2005). Briefly, KBrO₃ at 0.40g/L, MX 0.07 g/L; CHCl₃ 1.8 g/L; BDCM 0.7 g/L and a mixture of these were administered in the drinking water for 3 weeks to Long Evans rats and the Tsc mutant strain, after which 8-oxoguanine (8-oxoG) levels were assessed in kidney DNA. 8-oxoG was increased only by KBrO₃, and the levels were less in the kidneys of animals given the mixture.

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Analyses

20. The relationships between thresholds for single chemicals and interaction thresholds of mixtures were the key focus of the analyses of a number of different types of mixtures studies undertaken by Yang and Dennison (2007). A number of *in vitro* studies were selected for the analysis, three assessing metal-induced responses in keratinocytes, an estrogen receptor induction study in MCF-7 cells and a PBPK modelling of gasoline components study. Data from these papers was analysed using Benchmark Dose Software to calculate BMD₀₁, BMD₀₅ and BMD₁₀ values from which dose response curves were plotted and single chemicals vs mixtures were compared. Linear relationships were apparent for all BMDs in all studies and the plot for the mixture was consistently with the plots determined for the individual components of the mixture. This analysis represents a method by which synergistic results can be readily identified.

Summary and questions for the Committee:

21. This preliminary review of the literature indicates that most mixture toxicology assessments are still confined to specific single event toxicities rather than multi stage carcinogenesis. However, there are some studies of carcinogenicity being conducted on combinations, although these are generally assessing chemicals from the same CMG's. *In vitro* studies evaluating receptor mediated responses do provide robust end-point assessments as it is possible to produce detailed dose responses and thus more closely assess the potential for any interactions. However, it is recognised that the endpoint investigated in these studies usually reflects a simple response to the interaction with a defined molecular target. As such it does not model the multiple stages of a carcinogenic response *in vivo*.

Questions;

- Do Committee members have any general comments on the studies carried out to investigate carcinogenic effects of mixtures of chemicals?
- Does the Committee agree that for common mechanism groups, the evidence supports a default assumption of dose additivity
- Does the Committee believe that there are instances where synergy can be anticipated, e.g. initiation – promotion, and if so is it likely that such an interaction would be so significant as to alter the risk assessment of a carcinogen?
- Does the Committee have suggestions for further work that it wishes undertaken in this area or ideas for approaches that might be used? If so how do they suggest we take this forward.

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DH Toxicology Unit

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Appendix 1 to CC/08/6

Extract from WIGRAMP report, September 2002

Carcinogenicity

8.52 Because pesticides that are believed to be genotoxic *in vivo* are not normally approved for use (except for spindle inhibitors, where there is a clear biological basis for the expectation of a threshold), a few carcinogenicity studies have been performed to test combination treatments. Where data exist, relatively few studies include concurrent data regarding the effects of the individual constituents alone. In many cases, short-term models of carcinogenicity have been used, particularly when studying large numbers of dose groups. How predictive these models are of tumorigenic potential for different classes of chemical is open to question.

8.53 Ito *et al* investigated the carcinogenic potential of pesticide combinations at low doses. The test protocol adopted a short-term initiation/promotion model of carcinogenesis with glutathione S-transferase (GST) (placental or pi form) positive (GSTp+ve) hepatocyte foci as a preneoplastic endpoint marker. After tumour initiation with diethylnitrosamine (DEN) and a two-thirds partial hepatectomy, young adult rats received the test chemicals in their diets for 6 weeks. Frequency and size of GSTp+ve foci were examined in the livers of animals following termination. A combination of twenty pesticides was tested (19 OPs and one organochlorine compound), administered at 1 x the acceptable daily intake (ADI) and 100 x ADI for each individual chemical. At 1 x the ADI level, there was no enhancement of the development of preneoplastic lesions initiated by DEN. At 100 x the ADI, both the number and the area of the lesions was increased. According to Ito *et al*⁴⁶ the combination effects observed at the higher (100 x ADI) dose suggested several of the pesticides were acting as tumour promoters in the liver. It is also worth noting that several constituents were Ames test positive. Also, similar enhancing effects had been demonstrated previously for methidathion and malathion, both of which were included in the present study.

8.54 In a second study from the same group,^{46,47} using a medium-term multiorgan protocol of 28 weeks (in which tumours were initiated by five known potent carcinogens in combination), the carcinogenicity of a mixture of 40 pesticides (high volume compounds) and another of 20 pesticides (suspected carcinogens) was investigated at the constituents' respective ADIs. There was no enhancement of carcinogenesis.

8.55 While these studies provide reassurance in the fact that no enhancement of observed effects is observed at low levels of exposure, positive effects were seen at multiples of the ADI dose. However, these studies provide little or no information regarding the nature of any interactions that may have occurred. The following paragraphs describe some other studies that have investigated the nature of interactions within mixtures that contain chemicals other than pesticides.

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8.56 To determine whether combinations of two carcinogens with the same target can act synergistically, the long-term dietary effects of hepatocarcinogens, cycad flour, lasiocarpine, aflatoxin and dipentylnitrosamine (DPN) were studied in pair-wise combinations in male and female F344 rats.⁴⁸ Each of the six possible pairs was studied in a 4 x 4 factorial study design (including a zero and 3 non-zero dose groups for each agent). The maximum dose level used for each individual chemical was high enough to cause tumours in a large proportion of animals without producing toxicity sufficient to reduce survival. Other doses were equally spaced on a log scale. Data were analysed by traditional methods and methods specifically designed to determine the additivity index and test for deviation from simple additivity (for parameters of time to death, tumorigenicity, intercurrent mortality/occult tumours). No chemical was found to antagonise the effects of any other. Some chemicals were reported to act synergistically, for example cycad flour and lasiocarpine, when the low dose or the mid dose of cycad flour was combined with the mid-dose or the high dose of lasiocarpine either where the endpoint was taken as time of death or time to death with malignant liver tumour. Lasiocarpine and DPN were reported to act synergistically when the endpoint was taken as time to death or time to death with a liver tumour. Cycad flour and DPN were reported to act synergistically when the endpoint was taken as time to death. Findings in male and female animals were generally in agreement. Although clear excess toxicity was seen with some of the mixtures, the study design precludes clear definition of the type of combined toxicity seen.

8.57 A further study reported by the same group investigated the outcome of exposure to combinations of carcinogens that independently act on different organ systems.⁴⁹ Four carcinogens, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-butanol-butyl nitrosamine (NBBN), nitrilotriacetic acid and DPN were studied in pair-wise combinations in F344 rats, again using a 4 x 4 factorial design. Data were analysed by methods specifically designed to determine the additivity index and test for deviation from simple additivity. Antagonism was reported for some mixtures containing nitriloacetic acid. Other combinations were found not to interact. Findings in male and female animals were generally in agreement.

8.58 Potential synergism among five heterocyclic amines was investigated in a short-term initiation/promotion model of carcinogenesis with glutathione S-transferase (placental form) positive (GSTp+ve) hepatocyte foci as a preneoplastic endpoint marker.⁵⁰ Separate groups (n = 15 – 18 per group) were treated with a combination of the five chemicals or each chemical individually at the following dose levels incorporated in the diet: 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2, 500 ppm); 2-amino-6-methyldipyridol[1,2-a:3',2'-d]-imidazole (Glu-P-1, 500 ppm); 2-amino-3-methyl-9Hpyrido[2,3b]indole (MeA_C, 800 ppm); 2-amino-9H-pyrido[2,3-b]indole (A_C, 800 ppm); 2-amino-1-methyl-6-phenylimadazol[4,5-b]pyridine (PhIP, 400 ppm), and at 1/5 and 1/25 these levels. With the exception of PhIP, all chemicals individually at the highest dose increased the numbers and areas of GSTp+ve foci. Data were analysed for additive or synergistic effects using a test for linear statistical inference, assuming dose-linearity in response. Combined treatment at the 1/5 dose level, but not at 1/25 level, resulted in what the authors described as a synergistic enhancement of foci parameters in that the numbers and areas of foci were significantly increased above the sums of the individual data. However, the individual dose-response relationships were not characterised in sufficient detail to support this conclusion.

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8.59 Using a similar experimental protocol, separate groups of animals were treated with carcinogenic doses of 3-amino-1,4-dimethyl 5H-pyrido[4,3-b]indole (Trp-P-1, 150 ppm), 2-aminodipyrido[1,2-a:3',2'-d]imidazole (Glu-P-2, 500 ppm), 2-amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQ, 300 ppm), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ, 300 ppm) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx, 400 ppm) and 1/5 and 1/25 these dose levels. Further groups received the chemicals in combination at 1/5 and 1/25 the individual carcinogenic dose levels.^{51,52} All chemicals significantly increased GSTp+ve foci numbers at the highest dose levels. Trp-P-1, MeIQ and IQ also exerted a positive influence at the 1/5 dose level. With the exception of Glu-P-2, similar results were obtained at the highest dose level regarding foci area. An increase was also observed with MeIQ at the 1/5 dose level. The authors suggested that the data were consistent with the occurrence of both additive and synergistic effects (as determined by modified Yoshimura's *t*-test) in animals treated with the combination at both the 1/5 and 1/25 dose levels. However, the results may have simply represented dose-addition.

8.60 Similarly, combination effects of 10 heterocyclic amines were investigated at the following doses: Trp-P-2 (500 ppm); Glu-P-1(500 ppm); MeA_C (800 ppm); A_C (800 ppm); PhIP (400 ppm); Trp-P-1 (150 ppm); Glu-P-2 (500 ppm); MeIQ (300 ppm); IQ (300 ppm) and MeIQx (400 ppm), and at 1/10th and at 1/100 of these dose levels.⁵³ Chemicals were tested individually at the same dose levels. The authors claimed that synergism was observed at the 1/10 dose level but not at the 1/100th. However, the results may have simply represented dose-addition.

8.61 PhIP, Glu-P-1, Glu-P-2, IQ and MeIQ were also tested individually and in combination in a medium term (28 week) multi-organ model of carcinogenesis (involving initiation with 5 potent carcinogens) in male F344 rats at levels of 300, 300, 600, 300 and 200 ppm, respectively and at 1/5th and 1/25th these levels in the diet.^{52,54} The combination was reported to act synergistically in relation to the multiplicity of adenocarcinomas in the small intestine and multiplicity of Zymbal gland tumours at the 1/25 but not at the 1/5 dose level, thus failing to demonstrate a dose response. Furthermore, none of these findings were statistically significant. The results may have simply represented dose-addition.

8.62 The carcinogenic potential of low dietary levels of antioxidants known to target the rodent forestomach or glandular stomach, either alone or in combination, was investigated in a long term feeding study and in a medium (28 week) term multi-organ model, the latter involving pre-initiation with several potent carcinogens.⁵⁵ In the long-term test, butylated hydroxyanisole (BHA, 0.4%), caffeic acid (0.4%), sesamol (0.4%), 4-methoxyphenol (4-MP) (0.4%) and catechol (0.16%), alone or in combination, were fed to male F344 rats (n = 30-31/group) for up to 104 weeks. Slight increases in forestomach papillomas (3-16% incidence compared to 0 % incidence with the basal diet) were observed in all groups except the BHA group. Incidence in the group receiving the combination was 43%. Enhanced multiplicity of papillomas in the combination group was interpreted as evidence for a synergistic interaction. Furthermore, this was the only group in which carcinoma of the stomach was observed albeit only in a single animal. The incidence of papillary or nodular hyperplasia was reported as less than additive, with

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the response in the combination group being similar to the response observed with caffeic acid alone. However, the nature of the combined effect, particularly whether any potentiation was present cannot be assessed from these data as only a single dose level was tested.

8.63 In the medium-term test, BHA, caffeic acid, sesamol and 4-MP were administered at doses of 0.4% or 0.08% and catechol at doses of 0.16% or 0.032%, individually or as a high or a low dose combination (n = 15/group). Incidences of forestomach papillomas and papillary or nodular hyperplasia were increased in each of the high dose groups, significantly so in the caffeic acid and 4-MP groups. The incidence of forestomach papillomas was also significantly increased in the catechol high dose group. The effects of the high dose combination were less than would be predicted by dose addition. Compared to the spontaneous incidence, there was a tendency for a reduction in numbers of carcinomas and adenocarcinomas of the large intestine with each individual antioxidant treatment. However, this reduction became significant with the combination treatment. In the low dose groups, the incidence of forestomach papillomas was increased only in those animals receiving the combination.

8.64 Nesnow *et al*⁵⁶ studied the binary, ternary, quaternary and quintuple interactive effects of a five component mixture of environmental polyaromatic hydrocarbons (PAHs) on the development of lung tumours in male A/J mice. Individual dose-response data (lung adenoma score following a single i.p. administration 8 months prior to termination) were obtained for benzo[a]pyrene, benzo[b]fluoranthene, dibenz[a,h]anthracene, 5-methylchrysene and cyclopenta[c,d]pyrene. From these data, quintuple mixture doses were selected, based on survival, range of dose response and predicted tumour yield. The ratios of chemicals within the mixture were design to be representative of the ratios found in the air or from combustion samples. A 2⁵ factorial study design (32 groups, n = 20 per group) was employed, incorporating high and low dose level groups. This scheme allowed the calculation of five PAH dose parameters, 10 binary interaction parameters, ten ternary interaction parameters, five quaternary interaction parameters and one quintuple interaction parameter. Comparison of observed lung adenoma score with that expected from additivity identified a greater than additive response at the low dose and a less than additive response at the high dose. Less than additive interactions were observed under most mixture conditions and binary interactions were dominated by the inhibitory effect of dibenz[a,h]anthracene. Surface response analysis (using response addition) predicted the observed lung tumorigenic responses of quintuple mixtures. Data suggested that interactions between PAHs do occur but to a limited extent.

8.65 Further to this, an analysis of the binary carcinogen interaction literature that encompasses multiple species, organs and routes of administration has identified both greater than and less than additive effects for PAHs, depending on target tissue, species and route of administration.⁵⁷ That is to say that the occurrence of interactions may be dependent on the mixture, dose, individual target, experimental model employed and/or the route of administration.

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Appendix 2 to CC/08/6

Key papers

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