

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT**Chemical Mixtures: the potential for chemical interactions in carcinogenesis.****Introduction:**

1) This paper has been prepared as part of the ongoing evaluation of chemical mixtures by the COC which aims to explore the potential for chemicals to interact in the carcinogenic process. An initial paper looked at the concepts of dose additively within a receptor mediated, common mechanism group using dioxins and estrogens as examples. At the COC meeting in July 2008, a strategy was proposed in which it was agreed to consider the multi-stage nature of the carcinogenic process and to identify publications which attempt to address how chemicals may interact at different points in this process.

2) The potential for interactions to occur during the initiation stage of carcinogenesis is considered to have been effectively covered by the COM review, where classical genotoxicity endpoints, such as bacterial mutagenicity, DNA strand breaks and micronucleus induction were addressed. Some individual papers described synergy; one example suggested that 4-nitroquinoline-1-oxide (4NQO) enhanced methylmethane sulphonate (MMS) induced mutation via DNA unwinding thus enabling increased levels of methylation and mutation. Overall conclusions of the COM were that potential for such interactions needed to be studied on a case-by case basis.

3) Assessment of papers according to the quality criteria laid out in Borgert (2001) was undertaken in the COM review and it was suggested that this also be used for the current review. A summary of these criteria is provided in Appendix 1. It is noteworthy that, of the five criteria, two pertain to close characterisation of the dose responses of the individual chemicals in questions. Although this was possible for many of the approaches used when assessing genotoxicity endpoints, as full dose response data could be more easily generated using *in vitro* methods, it was not considered appropriate for the review of carcinogenesis due to the complexity and size of studies that would be needed to provide the requisite dose response characterisation. Nevertheless, there are many good examples of studies where laboratories have included a number of different dose levels in an attempt to provide dose-response information. Furthermore, many have carefully considered the various processes involved and often appraise a number of end-points with the aim of exploring mechanisms which may explain synergistic and inhibitory responses.

4) Pharmacokinetic interactions, a consequence of metabolising enzyme induction or inhibition, is an area identified as pivotal in some of the synergistic or antagonistic responses observed. This has been addressed by evaluating effects on cytochromes P450 (CYP) which have been assessed variously by monooxygenase activity, e.g. ethoxyresorufin-O-deethylase (EROD) activity, Western blotting and gene expression analysis (microarray). Impact on detoxification pathways is also considered.

5) The most widely investigated chemicals are polyaromatic hydrocarbons (PAH's), where both individual chemicals in this class and complex environmental mixtures such as urban dust particulate matter and diesel exhaust have been evaluated. Using established initiation-promotion models in liver and skin, rapid assessment of initiation potential is possible. Investigators have often concurrently evaluated DNA adduction and strand breaks. Lung tumourigenic responses to PAH mixtures have also been reported.

6) Food heterocyclic amines (HCA) are also a group where potential interactions have been considered, although there is only a limited attempt has been made to provide hypotheses to explain the effects observed. The studies presented have generally investigated their potential tumour or foci inducing ability of HCA using initiation-promotion models.

7) The 'chemo-protective' effects of dietary constituents, such as vitamins and phytochemicals, on HCA mutagenicity and carcinogenicity, and the implication that this may impact on human cancer incidence, represents an area where interactions in the carcinogenic process can occur. However this was considered to be outside of the scope of the current review. This area of research has been succinctly reviewed by Dashwood (2002).

8) The literature was searched also for studies in which the epidemiology of co-exposure to chemicals was studied. The only significant examples are of alcohol and tobacco, presented at the COC in July 2008, and asbestos and smoking, presented in the accompanying paper.

Polyaromatic hydrocarbons: DNA binding and tumour initiation

***In vitro* approaches**

9) The effects of a complex mixture of PAH's, extracted from coal tar (Standard Reference Material SRM₁₅₉₇; previously shown to act as an initiator in an initiation-promotion model), on the activation of benzo[a]pyrene (B[a]P) and dibenzo[a,l] pyrene (DB[a,l]P) was investigated in an *in vitro* system by measuring adduct formation (Mahadevan et al 2005). The role of CYP induction in potential interactions was also considered. MCF-7 cells (a human mammary carcinoma derived cell line) were incubated with SRM₁₅₉₇ (400 µg), B[a]P (20.2 µg) or DB[a,l]P (0.2 µg) and combinations of SRM₁₅₉₇ with one or both of the PAH's for 6-192 hours. ³²P-postlabelling was used to detect adducts and CYP1A1 and 1B1 levels were assessed by

Western blotting after 24 and 120 hours in culture. It was demonstrated that the presence of SRM₁₅₉₇ significantly decreased PAH-DNA adduct formation in both B[a]P and DB[a,l]P exposed cells, the effect being more pronounced and of longer duration in those co-treated with DB[a,l]P. SRM₁₅₉₇ and B[a]P, but not DB[a,l]P, were shown to induce CYP1A1 and 1B1, although this had diminished by 120 hours in the B[a]P only treated cells.

10) In a follow up study, Mahedevan and colleagues (2007) aimed to further investigate the role of metabolising enzymes in possible antagonistic, additive or synergistic effects of the same coal tar mixture. Again the effects of SRM₁₅₉₇ on the metabolic activation of B[a]P and DB[a,l]P were examined by assessing DNA adducts using ³²P-postlabelling, this time in Chinese hamster V79 cell lines which expressed human CYP1A1 or 1B1. Cells were treated for 6, 12, 24, 48 and 72 hours (with SRM 20 µg/mL, B[a]P 80 nmol, DB[a,l]P 0.8 nmol and combinations of these doses based upon relative carcinogenic potencies in mouse skin assays). In addition, EROD activity was measured in CYP1A1 or 1B1-containing microsomes following incubation with 0-10 µg SRM 1597/mL. A significant reduction of DNA binding was observed when SRM was co-administered with the two PAH's in cells expressing CYP1B1, the effect was not apparent to the same extent in CYP1A1 expressing cells. EROD activity indicated that SRM competitively inhibited the activity of both isoforms, more strongly on 1B1. From an overall assessment of the two studies, the authors conclude that the individual components of a complex environmental mixture may have the potential to impact on the bioactivation of components within the mixture or other carcinogens by induction or inhibition of CYP enzymes and therefore impact on carcinogenic activity.

11) Similarly, in a series of *in vitro* experiments, the effects of SRM₁₉₇₅ on B[a]P and DB[a,l]P adduct formation, metabolic activation, gene expression and 8-oxodG adduct levels in a human breast epithelial cell line (MCF-10A) were examined (Courter et al 2007a). Cells were exposed to SRM₁₉₇₅ (400 µg), B[a]P (20.2 µg), SRM+B[a]P, DB[a,l]P (0.2 µg) or SRM+DB[a,l]P for 24 or 48 hours. A significant reduction in B[a]P adducts was observed following co-administration with SRM. Only low levels of DB[a,l]P adducts were detected and this was not affected by SRM. Conversely SRM had no impact on B[a]P induced EROD activity but significantly increased DB[a,l]P induced activity. Gene expression confirmed the induction was a consequence of CYP1B1 activity. 8-oxodG adducts were increased when cells were treated with D[a]P and SRM, but combined treatment was less than additive.

12) A recent, well designed study assessed, *in vitro*, the effects of binary PAH mixtures on DNA adduct formation, gene expression, apoptosis and cell cycle in human hepatoma cells (HepG2) (Staal et al 2007, Appendix 2). Cells were incubated with 1 nM - 30 µM B[a]P, DB[a,l]P, dibenzo[a,h]anthracene (DB[a,h]A), benzo[b]fluoranthene (B[b]F), fluoranthene (FA) or 1-methylphenanthrene (1-MPA) or equimolar or equitoxic binary combinations of these for 24 hours. Flow cytometry was used to assess movement through the cell cycle and apoptosis and microarray analysis was used to assess gene expression changes. PAH DNA adducts were also assessed.

13) DB[a,l]P, B[a]P, DB[a,h]A and B[b]F dose-dependently increased apoptosis and blocked cell cycle in S-phase and the PAH mixtures showed a concentration additive effect on apoptosis and on cell cycle blockage. DNA adduct formation in mixtures was higher than expected based on the individual compounds, indicating a synergistic effect of PAH mixtures. Gene expression data showed varying modulation of expression. The combinations of B[a]P with B[b]F, DB[a,h]A or FA showed concentration additivity, whereas B[a]P with DB[a,l]P or 1-MPA showed antagonism. It was noted that weak or non-carcinogenic PAH's such as FA and 1-MCA can also have the potential to affect the carcinogenic potency. Many individual genes showed additivity in mixtures, but some genes showed mostly antagonism or synergism. For example, CYP1A2 expression was more than additive when B[a]P was given with DB[a,h]A or DB[a,l]P. However, the results are not conclusive as the effects of binary mixtures of PAHs on DNA adduct formation generally show synergism, indicating increased carcinogenic potency, whereas effects on gene expression are generally concentration additive or slightly antagonistic, suggesting no effect or decreased carcinogenic potency.

In vivo approaches

14) In an early investigation also addressing the impact of B[a]P on the tumour initiating potential of a number of coal derived complex organic mixtures (com) utilised a mouse skin model and correlated tumour initiation with DNA binding and adducts (Springer et al 1989). The com's were fractionated by distillation (300-700, 700-750, 750-800, 800-850 and >850 °F). These test materials (5 mg in 50 µl acetone) were applied to the shaved skins of female CD-1 mice with or without 25 µg of B[a]P. Two weeks after initiation, the sites were promoted using twice weekly applications of 5 µg TPA for 24 weeks. At the end of this period the numbers of tumours/mouse were assessed and adducts were measured. The following results were obtained:

Initiator	No. mice/group	Number of tumours/animal SEM	In vivo binding pmol/mg DNA
Solvent	30	0.17 ± 0.07	
BaP	30	7.07 ± 0.67	6.30 ± 0.63
300-700 °F + BaP	30	6.63 ± 0.50	2.94 ± 0.59
700-750 °F + BaP	29	4.14 ± 0.49	1.28 ± 0.10
750-800 °F + BaP	29	2.93 ± 0.33	1.31 ± 0.13
800-850 °F + BaP	30	3.00 ± 0.36	0.87 ± 0.06
>850 °F + BaP	30	6.33 ± 0.75	0.90 ± 0.12
300-700 °F	30	0.37 ± 0.13	ND
700-750 °F	30	0.57 ± 0.14	ND
750-800 °F	30	0.60 ± 0.18	ND
800-850 °F	30	1.23 ± 0.43	ND
>850 °F	29	4.52 ± 0.43	ND

ND: not determined

It was demonstrated that B[a]P initiating activity was significantly reduced by the presence of the com's, some of which had inducing potential themselves. The most significant reduction seen was in the presence of the 750-800 °F fraction.

15) A number of papers by Courter and colleagues were retrieved, which had a similar aim to that of Mahedevan presented above, to test the hypothesis that complex mixtures of pollutants may impact on the carcinogenic potency of individual PAH's.

16) In the first, it was proposed that urban dust particulate matter (UDPM) alters PAH bioactivation via inhibition of CYP1A1 and 1B1 (Courter et al 2007b). A mouse skin initiation model was used to investigate a number of relevant end-points. The shaved skin of 6 Groups of SENCAR mice (n=6) was initiated with vehicle control (toluene), UDPM (1 mg), benzo[a]pyrene (B[a]P; 200 nmol), UDPM+B[a]P, dibenzo[a,l]pyrene (DB[a,l]P, 2 nmol) or UDPM+DB[a,l]P. Two weeks after initiation, 12-O-tetradecanoylphorbol-13 acetate (TPA) was administered weekly for 25 weeks and then the skin was examined for the presence of skin papillomas. UDPM alone exhibited only weak tumour initiating activity (5.7% of treated animals with tumours) and these developed later than those seen with the PAH treatment. Interestingly, UDPM decreased the tumour incidence induced by both B[a]P and DB[a,l]P alone (51.4% compared to 71.4% and 88.6% compared to 100% respectively) and also delayed the onset of time to tumours induced by B[a]P.

17) To evaluate the potential stages at which these interactions may occur, skin DNA was isolated and examined using ³²-P-postlabelling to assess PAH adducts and the COMET assay was used to assess DNA strand breakage. Western blotting for CYP1A1 and 1B1 was conducted on skin microsomes and ethoxyresorufin O-deethylase (EROD) activity was measured in cells expressing CYP1A1 and 1B1 in the presence of UDPM. A good correlation between tumour incidence and DNA adducts was apparent for all groups. Conversely, B[a]P and DB[a,l]P induced DNA strand breaks were elevated in groups in which they were co-treated with UDPM. Induction of CYP as examined by Western blotting was less clear; approximately additive effects were noted for CYP1A1 expression when both PAH's were co-administered with UDPM. However, it appeared that UDPM significantly decreased B[a]P-induced CYP1B1 expression, whilst significantly increasing that induced by DB[a,l]P. Alone UDPM was a weak inducer. Assessment of EROD activity revealed some decreased CYP activity and it was concluded that UDPM inhibits CYP metabolic capacity in a noncompetitive manner. Overall it was demonstrated that UDPM modified the tumour initiating potential of two PAH's by altering bioactivation pathways

18) In a comparable study, the effects of the diesel exhaust particulate matter SRM₁₉₇₅ on the effects of B[a]P and DB[a,l]P were investigated in SENCAR mice using a similar dosing protocol to the previous study (Courter et al 2008 Appendix 2) . The SRM₁₉₇₅ dose, used instead of the UDPM, was 50 mg applied with and without B[a]P and DP[a,l]P, followed by TPA promotion for 25 weeks. Papillomas and DNA adducts were measured and EROD activity

assessed in skin microsomes prepared from the treated mice. SRM₁₉₇₅ had little tumour promoting activity itself, which correlated with very low levels of DNA adducts, but enhanced the tumour promoting activity of B[a]P (71.4% compared to 84.6% with B[a]P alone) and significantly reduced the number of carcinomas induced by DB[a,l]P (3 compared to 9), although papillomas were increased in the co-administered group (37 compared to 14). However, SRM₁₉₇₅ had a slight enhancing effect on DNA adduct levels for both combined treatments, although this was not statistically significant. An assessment of EROD activity in skin microsomes showed that SRM, whilst not significantly inducing activity itself, reduced EROD activity induced by B[a]P. Gene expression changes indicated that B[a]P significantly increased the expression of CYP1A1 but not 1B1, whilst DB[a,l] had greatest impact on CYP1B1. It was concluded that the SRM acted in an antagonistic manner on carcinogenic potency when metabolic activation by 1B1 is required, as with DB[a,l]P.

19) Marston et al (2001) also investigated the interactions of SRM₁₅₉₇ with B[a]P and DB[a,l]P in SENCAR mouse skin. SRM (1mg), B[a]P (50.4 µg, 200 nmol), DB[a,l]P (0.6 µg), SRM+B[a]P or SRM+DB[a,l]P were applied to shaved skin followed by twice weekly promotion with TPA for 25 weeks (controls n=8-9, treated n=26-35). Again ³²P-postlabelling was used to measure adducts and CYP1A1 and 1B1 levels were assessed Western blotting.

The following tumour data were reported:

Compound	No tumours/TBA	TBA %	N° tumours	N° tumours/mouse
Control	0	0	0	0.12
SRM	5.3	92.3	128	4.92
B[a]P	8.9	90	241	8.03
SRM+B[a]P	8.8	100	256	8.72
Control	1	22.2	2	0.25
SRM	4.1	96.3	107	3.41
DB[a,l]P	8.1	100	242	7.87
SRM+DB[a,l]P	5.2	96.7	150	4.67

TBA: tumour bearing animals

20) The latency period to tumour appearance did not differ significantly across groups. In contrast to Courter et al (2008), SRM was shown to have tumour inducing activity despite being applied at a considerably lower dose. An effect on B[a]P activity was not observed but a significant reduction in the total number of tumours and tumours/TBA were noted when SRM was applied with DB[a,l]P.

21) Interestingly in this study SRM was shown to significantly reduce both B[a]P and DB[a,l]P DNA adducts. SRM induced both CYP1A1 and 1B1, B[a]P was a weak inducer of both whilst DB[a,l]P did not appear to increase either.

22) Inhibition of detoxification pathways, rather than activation pathways, is also a potential point where interactions can occur in the carcinogenic process. This has been elegantly investigated in a study which has assessed the impact of pentachlorophenol (PCP), known to interfere with a number of detoxification pathways, on B[a]P DNA adducts and 8-OHdG formation in the liver and lung of adult and infant mice (Bordelon et al 2001). Groups of 15 day old and 6 weeks old B6C3F1 mice were given i.p. doses of B[a]P (55 mg/kg) alone or with PCP (10, 25 and 50 mg/kg). Twenty four hours later lung and liver tissue were taken for 8-OHdG and DNA adducts assessment. In adult mice adducts were significantly increased when B[a]P was administered with PCP (52.5 nmol adduct/mol nucleotide compared to 6.7 in the liver, and 88.7 compared to 19.4 in the lung), but 8-OHdG was not detected at any dose. However, in infant mice, where a number of time points had also been looked at, a significant antagonistic effect was observed and adducts were significantly lower when PCP had also been given (12.8 compared to 50.7 at 12 hours in the liver and 10.6 compared to 30.0 at 12 hours in the lung). These data clearly show that PCP is able to impact upon the availability of DNA reactive metabolites of B[a]P although by different mechanisms in infant and adult mice. The inhibition of microsomal epoxide hydrolase and glutathione S- and sulfo-transferase are the suggested pathways affected.

23) These studies provide good examples of how chemicals, including complex environmental mixtures, can impact on the carcinogenic potential of other PAH's. In testing the hypothesis of competitive inhibition of enzymes responsible for the metabolic activation of PAH's it was broadly demonstrated that tumour promotion and DNA adduction were affected by the mixtures and that some CYP expression was altered which could explain this. For example, it is proposed that B[a]P is more readily activated by CYP1A1 than by 1B1, such that the competitive inhibition of this isoform would result in reduced activity. Furthermore, it was suggested that the effects of environmental mixtures on the metabolism of DB[a,l]P differs from B[a]P although this is not supported by all the available data. This likely indicates the complexity of the interactions, both metabolic and genotoxic, involved in the processes and the dose dependency of these interactions.

Evaluation of mixtures of HCA's using initiation-promotion models

24) An investigation of the potential synergistic effects of the tumour promoting activity of a series of heterocyclic amines was undertaken in the 1990's by a group from Japan using initiation promotion models.

In the first study, a medium term bioassay was utilised in which groups of male F334 rats were given diethyl nitrosoamine (DEN -200mg/kg), followed two weeks later by administration of the HCAs in the diet for 6 weeks and partial hepatectomy at week 3. (Ito et al 1991, Appendix 2). The HCA's studied were Trp-P-1 (150 ppm), Glu-P-2 (500 ppm), IQ (300 ppm), MeIQ (300

ppm) and MeIQx (400 ppm) and these were administered as 1/1, 1/5 and 1/25 of the given dose (the known carcinogenic dose) and as a combination of all 5 at 1/5 and 1/25 of the dose. GST-P-positive foci >0.1mm were the selected endpoint. The results obtained are presented below:

Treatment		Number foci/cm ²	Area foci mm ² /cm ²
1/1	Trp-P-1	69.55 ± 14.79 ^a	3.43 ± 0.94 ^a
	Glu-P-1	29.78 ± 5.90 ^a	0.95 ± 0.19
	IQ	85.70 ± 14.39 ^a	4.08 ± 1.30 ^a
	MeIQ	37.82 ± 7.15 ^a	1.66 ± 0.83 ^b
	MeIQX	48.74 ± 11.1 ^a	1.86 ± 0.42 ^a
1/5	Trp-P-1	28.38 ± 5.65 ^a	1.02 ± 0.32
	Glu-P-1	22.24 ± 3.80	0.85 ± 0.28
	IQ	28.27 ± 5.00 ^a	1.10 ± 0.30
	MeIQ	34.68 ± 6.77 ^a	1.25 ± 0.30 ^b
	MeIQX	21.55 ± 3.88	0.79 ± 0.37
	Mixture	56.85 ± 9.69 ^a	32.02 ± 0.45 ^a
1/25	Trp-P-1	21.20 ± 3.88	0.72 ± 0.16
	Glu-P-1	20.18 ± 4.48	0.66 ± 0.25
	IQ	21.07 ± 3.94	0.67 ± 0.15
	MeIQ	19.49 ± 4.05	0.78 ± 0.35
	MeIQX	20.02 ± 2.45	0.78 ± 0.24
	Mixture	26.80 ± 4.86 ^b	1.39 ± 0.92 ^c
	Control	20.38 ± 4.58	0.80 ± 0.45

a: significantly different from control p<0.001

b: significantly different from control p<0.01

c: significantly different from control p<0.05

25) From the foci data derived, the authors calculated the net values by subtracting the background control tumour incidence. Assessing the data in this manner it was demonstrated that the combined treatments at 1/5 doses was greater than the sum of the individual treatments, an apparent synergistic effect. Furthermore, the combined mixture foci incidence of the 1/5 group is slightly higher than the mean incidence of the individual HCA's given at 1/1. The combined 1/25 group induced foci (both number and area) above the background level, whilst none of the individual 1/25 dose increased the number of foci compared to the controls. However, it is noted that the mean number of foci of the five 1/1 in treatments is 54.3, which compared to 56.9 achieved in the 1/5 mixture group, which does not convincingly corroborate the claimed synergy. Similarly, the mean value of the five 1/5 treatments is 21.3 compared to 26.8 in the 1/25 mixture group. Notably comparing the values for area of foci, there does appear to be clear synergism in the 1/5 mixture group compared to the 1/1 individual treatments (32.0 compared to 2.4).

26) Data are also provided from groups given the HCA's and combinations in the absence of DEN initiation. The maximum effect, 4.82 foci/cm², was induced by IQ 1/1, whilst MeIQ and Glu-P-2 did not increase the incidence over controls (0.16). The combined treatment at 1/5

induced 1.44 foci/cm², and 0.16 at 1/25, and the authors claim that these represent additive effects of the individual treatments, although these appear marginal. The null hypothesis in this study appears to have been response additivity rather than dose additivity. In addition, the background incidence of foci was not insignificant, so that subtraction from a modest response due to the HCA's would be subject to significant error.

27) This study was followed up with another short term investigation, which examined combinations of the HCA's used in the previous study and 5 additional ones, but at lower (1/10) doses (Hasegawa et al 1994a). The same dosing protocol as in the previous study (Ito et al 1991) was used. At the end of the dosing period, livers were examined for GST-P-positive foci. The HCA dose groups were Trp-P-1, Glu-P-2, IQ, MeIQ, MeIQx (used in the first study at the same doses) and Trp-P-2 (500 ppm), Glu-P-1 (500 ppm), MeAαC (800 ppm), AαC (800ppm), PhIP (400 ppm) and combined doses of these at 1/10 and 1/100 of the selected dose. The additive dose model, where the background incidence was subtracted from those in the treated groups was used. It was demonstrated that the combination of chemicals at 1/10 significantly increased the number of foci but the individual chemicals at this dose level and the 1/100 combination did not induce increases above the untreated control group. The authors compared these data to that from the previous study, including an evaluation of the average values of the individual HCA's as follows :

Treatment		Relative dose levels				
		1/1	1/5	1/10	1/25	1/100
Combination 1	average	33.95	6.64	-	-0.01	-
	mixture	-	34.67	-	6.42	-
Combination 2	average	17.30	5.78	-	3.16	-
	mixture	-	42.39	-	7.56	-
Combination 3	average	25.62	6.23	-0.99	1.58	-
	mixture		-	42.32	-	-1.82

Combination 1 the 5 HCA's used in both studies

Combination 2 ; the additional 5 HCA's used in the second study

Combination 3 : all 10 HCA's

28) These studies, of apparently robust design, show clear synergistic effects when the effects are assessed in this manner, notably the 1/10 mixture when compared to the average of the individuals at 1/1. The authors note that PhIP and Trp-P-2 are not liver carcinogens but may exert metabolic effects. A detailed consideration of mechanisms of the effect was not made.

29) In a third paper, Ito et al (1998) presented some of the data from the previous paper (Hasegawa et al 1994) in more detail. The following results, are provided again as relative number of preneoplastic foci in the liver compared to the 1/1 control group.

Chemical	Numbers of GST positive foci		
	Relative dose levels		
	1/1	1/10	1/100
Trp-P-1	49.17	-0.82	-
Glu-P-2	9.40	-4.73	-
IQ	65.32	0.87	-
MeIQ	17.44	-1.56	-
MeIQX	28.36	-1.77	-
Trp-P-2	5.97	-2.37	-
Glu-P-1	55.48	5.27	-
MeAαC	17.93	0.38	-
AαC	9.42	-4.05	-
PhIP	-2.31	-0.10	-
Combination	-	42.32	-1.82

It was concluded that there was clear synergism following administration of all 10 at the 1/10 dose level.

30) It is clear from these data that the potency of the different HCA's to induce preneoplastic foci in this model differs quite substantially and it can also be seen that the dose responses are different. Therefore it is not clear that subtracting the incidence from background provides a good estimate of the magnitude of the synergism. However, there does appear to be evidence of an effect greater than additive at the low doses used.

31) A similar approach by the same group utilised a rat intestine initiation promotion model to examine the combined effects of some of the 10 HCA's used in the liver studies (Hasegawa et al 1994b Appendix 2). The initiation regime used 5 known carcinogens (dimethylnitrosamine, N-methyl-N-nitrosourea, N-butly-N-(4-hydroxybutyl-N-nitrosamine), 1,2 dimethylhydrazine and 2,2'-dihydroxy-di-n-propylnitrosamine) to induce preneoplastic lesions (4 weeks), and this was followed by treatment with single or combinations of 5 HCA's (PhIP, Glu-P-1, Glu-P-2, IQ, MeIQ) in the diet for 24 weeks . Doses of the individual chemicals were based on previous rat liver assays and were again given at 1/1, 1/5 and 1/25 of this dose. When given in combination, the doses were 1/5 and 1/25. The intestinal tumour data obtained are presented below:

32) It is noted that the averages of the numbers of small intestine tumours/rat of the individual chemicals at 1/1 gave a similar values to that achieved when the mixture was given as the 1/5 mixture (4.91 vs 5.75) and a similar comparison was made for the 1/5 doses and the 1/25 mixture (2.43 vs 2.95). This is indicative of dose additivity. In the large intestine the average for number of tumours/rat for the individual 1/1 chemicals was 3.3 whereas the value for the mixture at 1/5 was 6.05 Although dose dependent increases in tumours were observed following administration of the individual chemicals, it is clear from these data that the dose response for tumours is not linear with dose. The percentage of animals affected was high, even in the 1/25 dose groups and

this may mask any potential synergistic or antagonistic activity, although it is clear that additivity occurred as would be expected.

Treatment	Small intestine - tumours		Large intestine -tumours	
	%	No/rat	%	No/rat
1/1 PhIP	95 ^c	6.60 ± 4.82 ^a	90 ^b	3.85 ± 3.63 ^b
Glu-P-1	80	3.00 ± 2.58 ^b	75	2.05 ± 2.11 ^c
Glu-P-2	84	4.84 ± 4.21 ^b	79	2.84 ± 2.75 ^b
IQ	90 ^c	4.30 ± 4.33 ^b	90 ^b	4.25 ± 4.15 ^b
MeIQ	85	5.80 ± 4.86 ^a	75	3.45 ± 3.50 ^b
1/5 Mixture	90 ^c	5.75 ± 5.11 ^a	90 ^b	6.05 ± 5.11 ^a
PhIP	61	1.39 ± 1.65	83	1.89 ± 1.45 ^c
Glu-P-1	85	3.30 ± 2.08 ^a	70	1.51 ± 1.51
Glu-P-2	35	0.85 ± 1.46	50	1.05 ± 1.33
IQ	80	1.85 ± 1.87	55	2.13 ± 2.18 ^b
MeIQ	95 ^c	4.75 ± 3.57 ^a	85 ^c	1.85 ± 1.31 ^b
1/25 Mixture	90 ^c	2.95 ± 2.06 ^b	85 ^c	1.05 ± 1.03
PhIP	37	0.89 ± 1.59	63	0.70 ± 1.03
Glu-P-1	65	1.20 ± 1.40	40	0.63 ± 1.21
Glu-P-2	53	0.89 ± 1.05	37	1.63 ± 1.61
IQ	47	1.0 1.37	68	0.95 ± 1.32
MeIQ	75	1.30 ± 1.03	50	0.95 ± 1.32
Control	55	1.0 ± 1.49	45	0.80 ± 1.06

a: significantly different from control p<0.001

b: significantly different from control p<0.01

c: significantly different from control p<0.05

33) This study also looked at the incidence of a number of other tumours including kidney, and bladder. A synergistic effect of the 1/25 mixture was demonstrated in the Zymbal gland compared to the individual HCA's at 1/5.

34) In another study investigating HCA's, Hirata et al (2008, Appendix 2) utilised a different initiation-promotion model and used microarray analysis of metabolic enzyme genes with a view to elucidating potential mechanisms of interactions. Groups of male F344 rats (n= 15) were subjected to partial hepatectomy (PH) and then 12 hours later given single oral doses of PhIP (200 mg/kg) or MeIQx (25 mg/kg), neither, both together or staggered at 12 and 30 hours post-PH (where each was given first in different groups) followed by basal diet for 2 weeks and then 2-AAF as a proliferative stimulus for a further 2 weeks. Livers were then scored for GST-P positive foci. Appropriate control groups of single chemicals at each time-point were also included. When MeIQx was given prior to PhIP significantly greater number of foci were induced than when either was given alone; however when the two were given in the reverse order or at the same time the number of foci were less than when each was given individually. Expression of CYP1A1 was significantly increased in the combined and PhIP groups but not in the MeIQx group, whereas CYP1A2 expression was broadly similar across groups. It was speculated that the apparent inhibitory effect of pre-administration of PhIP on MeIQx induced

foci is as a consequence of metabolising enzyme induction. This is broadly supported by the results of the CYP induction arm of the study in which PhIP but not MeIQx induced CYP1A1 and the inference that this leads ultimately to MeIQx detoxification.

35) Tsuda et al (1999) investigated combinations of HCA's on tumour incidence and also the impact of caffeine on the induced carcinogenic response. IQ, MeIQx and PhIP, all at 50 ppm in the diet, were administered alone to groups of Fischer 344 rats, in pairs or all three together for 16 weeks after which GST positive foci and aberrant crypt foci (ACF) in the liver were scored. In a second experiment, PhIP was given at 400 ppm with or without caffeine at 500 or 1000 ppm in the drinking water for 2 weeks. A third experiment used reduced doses of caffeine (10, 100 and 1000 ppm), given alone and together with PhIP. HCA-adducts and CYP levels in livers (in microsomes and by Western blotting for CYP1A2) were determined.

36) At the doses administered, IQ and MeIQx induced foci individually and were more than additive when given together. PhIP alone did not induce foci and reduced the number of foci when given with the other HCA's. Adducts in the liver were detected following IQ and MeIQx administration but not PhIP, correlating with the foci response. Conversely, PhIP apparently enhanced MeIQx adduct formation. The adduct levels for the 3 HCA combination was similar to that seen for the IQ+MeIQx and IQ+PhIP combinations. Caffeine at 500 and 1000 ppm induced CYP1A2 itself but did not enhance PhIP responses. No ACF was seen in this experiment. However in the third experiment, ACF were induced by PhIP and it was shown that the co-administration of caffeine significantly increased the number of foci. It was concluded that HCA's themselves do not act synergistically but caffeine may act as a potentiating agent for HCA carcinogenesis.

37) The studies presented provide some evidence that the combined administration of different HCA's may enhance tumour initiating potential and this may be relevant at relatively low doses. The role of CYP induction is implicated in having a role in the synergism observed although the investigations are preliminary.

Pharmacokinetic modelling:

38) A study to evaluate the potential pharmacokinetic interactions of a number of carcinogenic trihalomethanes (THM) in the rat was undertaken in an attempt to understand the role of each on previously reported kinetic differences of a quaternary mixture (da Silva et al 2008). Sprague-Dawley rats (n=5) were given oral doses of chloroform, bromodichloromethane (BDCM), dibromochloromethane (DBCM) and bromoform (TBM) at 0.5 mmol/kg and binary combinations of these. Blood concentrations were measured at 20, 40, 60, 120, 180, 270, 360 and 480 mins after dosing. Modifications of the blood kinetics of each THM were assessed using a physiologically based toxicokinetic model. The blood concentrations of each THM were significantly enhanced when administered in a binary mixture, the effects were initially more

pronounced with chloroform, but with TBM and DBCM during the elimination phase. It is inferred that these data are consistent with metabolic inhibition as all are metabolised by the same CYP isoform, CYP2E1. It is noted that the dose levels used are high, and no attempts were made to speculate on the relevance of these findings to environmentally relevant doses.

Tumourigenesis:

39) A number of studies were retrieved which utilised longer term assays, rather than initiation promotion models.

An early, comprehensive study evaluating the carcinogenicity of mixtures used a study design with low doses of nitrosamines (N-nitrosodiethylamine, NDEA; N-nitrosopyrrolidine, NPYR; N-nitrosodiethanolamine, NDEIA) (Berger et al 1987). Groups of rats were administered the chemicals in the drinking water according to the table below for a maximum period of 170 weeks, and killed when moribund. Livers were then inspected for preneoplastic foci and tumours.

The following results were obtained:

Group	No	Chemical +dose mg/kg/day	% animals with tumours		% with tumours of				
			malignant	benign	liver	GI*	neurogenic	haem	urinary
1	500	Control	29	72	0.6	5.2	10.8	4.6	0.2
2a	80	NDEA - 0.1	65	66	45	31.3	5	5	1.3
2b	80	0.032	34	68	3.8	8.8	13.8	5	1.3
2c	80	0.01	29	73	2.5	11.3	12.5	2.5	2.5
3a	80	NPYR - 0.4	44	69	21.3	7.5	12.5	5	0
3b	80	0.13	35	74	5	8.8	11.3	6.3	1.3
3c	80	0.04	29	79	1.3	7.5	6.3	7.5	2.5
4a	80	NDEIA 2.0	35	80	7.5	7.5	13.8	8.8	1.3
4b	80	0.63	36	75	1.3	3.8	20	6.3	1.3
4c	80	0.20	36	76	2.5	8.8	10	10	0
5a	100	NDEA 0.032 NPYR 0.13 NDEIA 0.63	48	76	16	9	17	3	3
5b	240	NDEA 0.01 NPYR 0.04 NDEIA 0.20	33	75	4.2	7.9	10	4.2	0.8
5c	240	NDEA 0.0032 NPYR 0.013 NDEIA 0.063	31	74	1.7	5.8	12.1	7.9	0.4

*: included oral cavity, oesophagus, stomach and intestine.

40) The use of three dose levels of the individual chemical and high, mid and low dose mixtures enabled dose responses to be established. It is noted that the dose response curves are generally

less steep at the low-dose range used although this varies considerably between tissues, the clearest dose response being observed in the liver. It was concluded that the carcinogenic effects on the combined treatment regimes were generally additive and that the dose response of the mixture was linear.

41) The potential for enhancement or inhibition of the carcinogenic potential of PAH's by other PAH's and also the effects of a complex mixture, coal tar was investigated by Warshawsky et al (1993). The aim was to evaluate the impact of non-carcinogenic PAH's on the effects of a low dose of B[a]P in a mouse skin painting model. Briefly, 50 µL of 0.1% solutions of anthracene, chrysene, fluoroanthrene, phenanthrene or pyrene, individually, or as a mixture with and without 0.001% BaP were painted on the clipped skin of groups of C3H/HeJ mice (n=20) twice weekly for 6 months and then assessed for tumours (number and latency). Applied individually, none of the PAH's induced tumours under the conditions of the assay; when applied all together, 2 malignant and 1 benign tumours were reported with mean latency period of 73 weeks. Chrysene and B[a]P (which alone did not induce tumours) induced 2 malignant and 1 benign tumour and when all the PAH's were applied together with B[a]P, the incidence increased to 5 malignant and 3 benign with a slightly reduced mean latency of 66 weeks. In a second study it was demonstrated that the use of n-dodecane as the solvent instead of toluene consistently increased tumour numbers and decreased latency of tumours induced by a series of methyl benz[a]anthracenes. In a third arm of the study, coal tar, determined to contain 0.0006% B[a]P, was applied to skin and compared to 0.0006% B[a]P. The effect of solvent (toluene and n-dodecane) was also examined. Here, B[a]P alone did not cause tumours in either solvent; coal tar in toluene induced 20 malignant and 19 benign tumours and coal tar in n-dodecane induced 7 malignant and 10 benign. It is surmised that the solvent has capacity to alter the absorption, distribution and metabolism of the PAH's and that the low non-carcinogenic doses contribute to the overall carcinogenicity of a mixture.

42) A comprehensive investigation examined the effects of 5 PAH's on lung tumourigenesis in a 2⁵ factorial designed study, using a number of models to assess interactions (Nesnow et al 1998, Appendix 2). The PAH's, B[a]P, benzo[b]fluoroanthene (B[b]F), dibenz[a,h]anthracene (DBA), 5-methylchrysene (5MC) and cyclopenta[cd]pyrene (CPP), were selected to represent structural and metabolic diversity and a range of tumourigenic potentials. In the first part of the study, groups of male A/J mice (20/group) were administered the individual PAH's i.p. at a variety of doses and lung adenomas scored 8 months later. From these preliminary dose response data, intermediate doses were selected for the quintary experiment in which a dose of each PAH was administered concurrently; B[a]P and B[b]F at 30 and 75 mg/kg, DBA at 2.5 and 10 mg/kg, 5MC at 10 and 30 mg/kg and CPP at 30 and 100 mg/kg, resulting in 32 dose groups.

Additive responses were modelled and specific interactions were assessed using a response surface analysis model which utilised dose response relationships for each individual chemical to predict responses which deviated from additivity. Statistically significant responses were noted for 16 combinations. These analyses indicated both greater than additive and less than additive

responses, generally apparent at the lower and higher doses respectively. However it was concluded that the magnitude of these interactions was relatively small and thus occur only to a limited extent.

Discussion:

43) Reports have been reviewed which have explored various ways in which carcinogens can interact with one another at different stages of the carcinogenic process. Many of the studies have examined low doses with the aim of making the findings more relevant to human exposure scenarios.

44) The majority of the papers retrieved investigated PAH's, and complex mixtures containing PAH's, or food HCA's although a few others have been considered. There is evidence that some PAH's may have the potential to decrease the potency of others, although this appears to be very chemical specific. The roles of CYP1A1 and 1B1 in metabolically activating this class of chemical have been singled out as of significance in these interactions and the principle that CYP inhibition and induction can impact on relevant endpoints such as adduct or foci formation has broadly been demonstrated. However it can be speculated that other CYP isoforms could also be involved and this may explain the lack of consistency between some studies. There is often a lack of concordance between the end-points measured, for example degree of enzyme induction, DNA adducts and/or tumour/foci induction, and between studies. A single study assessing the role of detoxifying enzymes shows that there is also potential for interaction during this process. It would be of interest to see if the elevated level of DNA adducts observed translated to an increase in tumours in a longer term study but no follow up studies are available.

45) A number of studies have suggested that HCA's may act synergistically in promoting tumours. Again CYP induction and or inhibition are implicated in the mechanism of action, although there is little mechanistic information on which to base firm conclusions. It is likely that a general mechanism could not be applied and that the different HCA's would impact differently on the various isoforms. Interpretation of these studies is also complicated by the way in which the data were analysed.

46) Overall, there are some studies which have effectively evaluated how mixtures can impact on the different stages of the carcinogenic process, although the numbers retrieved of good design were limited. In addition, many of the more mechanistic studies focused on earlier stages of the cancer process. Indeed, the roles of metabolic interactions are the prevalent hypotheses tested. Investigators also appear to restricted studies to looking at combinations of chemicals from within the same class. However, there are examples where investigators have looked across chemical classes when an *a priori* hypothesis on potential mechanism of interactions has been formulated.

Questions for the Committee:

Members are asked to consider the following questions:

- 1) Do members have any opinions on the types of studies reviewed and the end points used to examine chemical interactions in the process of carcinogenesis?
- 2) What are the members opinions of the postulated mechanism of interaction of complex environmental mixtures and PAH's?
- 3) What are the member's opinions on the proposed synergistic effects of HCA's?
- 4) Can members comment on the relevance of the studies to human exposure scenarios and the implications for carcinogenicity testing ?
- 5) Do members have suggestions of other experimental work which may contribute to establishing testing strategies or evaluating the significance of chemical interactions in carcinogenesis?

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