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Genotoxicity under extreme culture conditions

A Report from ICPEMC Task Group 9

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1. Introduction

Chemicals that are genotoxic *in vitro* in mammalian cells often fail to give positive results when tested *in vivo* in experimental animals. For example, approximately 50% of chemicals that are clastogenic *in vitro* do not induce chromosome aberrations or micronuclei in the bone marrow of rodents (Thompson, 1986; Ishidate et al., 1988). There are many possible reasons for such discrepancies (see Waters et al., 1988) including: (a) the absence of detoxification or excretion processes *in vitro*; (b) metabolic processes, unique to the cells used in *in vitro* bioassays, that create active genotoxins; (c) the insensitivity of *in vivo* assays; (d) the use of conditions for *in vitro* tests that are so extreme and artificial as to be irrelevant to the situation *in vivo*.

This Report addresses the latter possibility. Four *in vitro* conditions have been considered as possibly generating such 'false positive' results: excessively high concentrations, high levels of cytotoxicity, the use of metabolic activation systems which in themselves may be genotoxic, and extremes of pH. Most of the data available relate to clastogenesis but other genotoxic endpoints have been considered when appropriate.

2. Genotoxicity at high concentrations *in vitro*

Genotoxicity assays *in vitro* are often conducted at concentrations up to the maximum solubility of the test compound. Indeed such a procedure is recommended in several Guidelines (Table 1, for clastogenesis) to optimise detection. For highly soluble, relatively non-toxic substances this can mean testing chemicals at tens of milligrams

per millilitre or up to almost molar concentrations in the test medium. Concern has been expressed that at such high concentrations of test agent the assay system will itself become subverted, i.e., disturbances to chromatin structure may result from the test chemical perturbing cellular homeostasis, rather than itself directly modifying chromatin structure. Further, such effects may have no relevance to the situation *in vivo*, particularly to human exposure, because of the high cellular concentrations required.

2.1. Genotoxicity associated with high osmolality

Ishidate et al. (1984) were the first to suggest that the clastogenicity of certain chemicals (e.g. sucrose, propylene glycol) at high doses might be a consequence of the elevated osmolality of the culture medium rather than to the test compounds themselves. More recently, several chemicals, which are probably not DNA-reactive, have been found to induce a variety of genotoxic effects (e.g. clastogenesis, mutations at the TK locus in mouse lymphoma cells, DNA-strand breakage and morphological transformation) at high osmolality (Table 2). None of these agents induce gene mutations in Ames tests, but sodium and potassium chloride induce base substitutions and frameshift mutations in yeast at the very high concentration of 2000 mM (Parker and von Borstel, 1987).

Few of these studies have been sufficiently extensive to establish accurate dose/response relationships but a threshold response is suggested by some investigations (e.g. Ishidate et al., 1984; Ashby and Ishidate, 1986). The lowest concentration at which putative osmolality-related genotoxicity has so far been observed is 19.5 mM (4.0 mg/ml) of sodium saccharin which induces chro-

TABLE 1
GUIDELINES ON EXPOSURE CONCENTRATIONS IN CLASTOGENICITY TESTS IN VITRO

Recommendation	Source
'...the highest dose suppressing the mitotic activity by approximately 50%'	Health and Safety Commission (1982)
'The highest test substance concentration...should suppress mitotic activity by approximately 50 percent. Relatively insoluble substances should be tested <i>up to the limit of solubility</i> . For freely-soluble non-toxic substances the <i>upper test substance concentration should be determined on a case-by-case basis</i> .'	Organisation for Economic Cooperation and Development (1983)
'The highest dose chosen for testing should be one which causes a significant reduction in mitotic index... Agents that are non-cytotoxic should be tested <i>up to their maximum solubility</i> .'	United Kingdom Environmental Mutagen Society (1983)
'...the highest dose suppressing the mitotic activity by approximately 50%.'	European Communities (1984)
'Generally the highest test substance concentration...should show evidence of cytotoxicity or reduced mitotic activity. Relatively insoluble substances should be tested <i>up to the limit of solubility</i> . For freely soluble nontoxic chemicals, <i>the upper test chemical concentration should be determined on a case by case basis</i> '	USA Environmental Protection Agency (1985, 1987)
'Perform the test with the concentration of the test substance at which it produced a 50% or greater inhibition of cell growth or mitosis at the maximum dose level... In case of a test substance devoid of cytotoxic activity, <i>a concentration of 5 mg/ml (or equivalent of 10 mM)</i> should be employed at the maximum dose level...'	Japanese Guidelines (1987)
'For agents where no cytotoxicity can be demonstrated a...maximum of... <i>5 mg/ml</i> is frequently used. Use as a maximum concentration one that reduces MI and PI (proliferation index) by about 50%'	American Society for Testing and Materials (see Preston et al., 1987)

mosome aberrations in CHL cells (Ashby and Ishidate, 1986). Typically such genotoxic effects are observed when the osmolality of the culture medium increases by > 100 milliosmoles/kg. However, there is no simple relationship between osmolality and clastogenesis when all chemicals are considered and Marzin et al. (1986) detected a significant increase in aberrations in human lymphocytes treated with urea with an increase in osmolality of less than 50 mOsm/kg (from 275 to 320 mOsm/kg).

Genotoxic effects that are found only at high levels of osmolality are unlikely to occur in humans. Although sodium saccharin (see above) is weakly mutagenic in rodents (Ashby, 1985) this is at doses of about 10 g/kg, and an effect is demonstrable only because the chemical is tolerated at high levels in experimental animals ($LD_{50} = 17$ g/kg in mice). Brusick (1987) speculated that results from some cancer studies in rodents where dietary levels of the materials would lead to high consumption of sodium or potassium ions could

be interpreted solely on the basis of ion levels in the target organ (typically the urinary bladder or kidney). Further research will be required to determine if hyper-osmolality can induce chromosome aberrations in vivo which might lead to tumour induction in experimental animals or whether other alterations in target organs are responsible. However, the relevance of such observations to human exposure and consequent risk has been seriously questioned (Ashby, 1985).

Osmotic effects are induced by diffusible molecules, and these can be ionic (e.g. NaCl) or neutral (e.g. glycerol). It is, at this stage, not possible to separate the several possible mechanisms by which high-dose genotoxicity may be produced. For example, the effects produced by high dose-levels of sodium saccharin may represent the result of non-specific osmotic effects, or the intracellular presence of high concentrations of ionic species, or the presence of high concentrations of sodium ions — the latter explanation being able to also accommodate the clastogenicity of sodium chlo-

TABLE 2
GENOTOXIC EFFECTS IN VITRO ASSOCIATED WITH HIGH OSMOLALITY OF THE CULTURE MEDIUM

Chemical	Endpoint	Cell type	Result	LEC		Treatment time (h)	Toxicity at LEC	Comment	Reference
				mg/ml	mOsm/kg				
Calcium saccharin	Chromosome aberrations	CHL	+	8.0	19.8 327	24, 48			Ashby and Ishidate (1986)
Dimethyl sulphoxide	Mutation (TK)	Mouse lymphoma	+	108	1 390 2 383	4	Total growth 49% of control		Wangenheim and Bolesfoldi (1988)
Ethylene glycol	Chromosome aberrations	CHL	+	60	968	48			Ishidate (1988)
	Gene mutation	CHL (+ S9) Salmonella	+	20	322	6			Ishidate (1988) Ishidate (1988)
Glucose	Mutation (TK)	Mouse lymphoma	+	37	204 350	4	Total growth 43% of control		Wangenheim and Bolesfoldi (1988)
Magnesium chloride	Chromosome aberrations	CHL	+	8.0	59.0	24, 48			Ashby and Ishidate (1986)
Magnesium saccharin	Chromosome aberrations	CHL	+	8.0	20.6	24, 48			Ashby and Ishidate (1986)
D-Mannitol	Chromosome aberrations	Human lymphocytes CHL	+	13.6	75 370	24	M.I. = 70% of control		Marzin et al. (1986)
Potassium chloride	Gene mutation	Salmonella	-			24, 48		No significant increase up to 11 mM	Ishidate (1988)
	Chromosome aberrations	CHO	+	6.0	80 445	22	CFE = 45%		Ishidate (1988)
	SCE	CHO	+	10.4	140 527	4	CFE = 71%		Galloway et al., in Brusick (1986)
		CHO	±	5.6	75 425	24	CFE = 44% MI = 46% of control		Galloway et al. (1987a) Seeberg et al. (1988)
	Mutation (TK)	CHO (+ S9)	+	3.7	49 536	4	CFE = 28% MI = 112% of control		Seeberg et al. (1988)
		CHO	-	4.0	53 405	24, 48			Ishidate (1988)
	Mutation (HPRT)	CHO	±	13.4	180 626	4		Cell density effect? (see footnote)	Galloway et al. (1987a)
		Mouse lymphoma (+ S9)	+	4.0	54		Total growth 26-69% of control		Myhr, in Brusick (1986)
	Mutation (HPRT)	V79	+	5.6	75 425	3	None	Effective over narrow dose range	Seeberg et al. (1988)
		V79 (+ S9)	+	2.8	37.5 357	3		Poorly reproducible. Effective over narrow dose range	Seeberg et al. (1988)

Gene mutation	Salmonella (5 strains \pm S9)	-						No mutants at 1.9-30 mg per plate	Seeberg et al. (1988)
Gene mutation	Saccharomyces	+	150	2000	1.0	CFE = 67%	No mutation in stationary phase cells	Parker and Von Borstel (1987)	
Transformation	Balb/c-3T3	+	4.0	54	400			Rundell and Matthews, in Brusick (1986)	
UDS	HeLa (\pm S9)	-					No UDS up to 200 mM	Seeberg et al. (1988)	
DNA ssb	CHO	+	15	200	689	4	No direct measurement but 160 mM gave CFA = 3% in same series of experiments	Galloway et al. (1987a)	
DNA dsb	CHO	-				4	No dsb detected up to 260 mM (756 mOsm/kg)	Galloway et al. (1987a)	
Potassium saccharin	Chromosome aberrations	CHL	+	8.0	347	24, 48		Ashby and Ishidate (1986)	
Polyethylene glycol	Mutation (TK)	Mouse lymphoma	+	150	> 620	4	Total growth 84% of control	Wangenheim and Bolcsfoldi (1988)	
Propylene glycol	Chromosome aberrations	CHL (+ S9)	+	32	421	534	48	Ishidate (1988)	
	Gene mutation	Salmonella	-	64	842	1000	3	Ishidate (1988)	
Sodium chloride	Chromosome aberrations	CHL	+	7.0	120	512	24	Ishidate (1988)	
		CHO	+	8.2	140	560	22	CFE = 40%	
			+	8.8	150	559	4	CFE = 89%	
			+	8.8	150	559	24	CFE = 12% MI = 3% of control	
		CHO (+ S9)	+	11.7	200	643	4	CFE = 36% MI = 97% of control	
		Human Lymphocytes	+	2.7	50	372	24	MI = 40% of control	
								Seeberg et al. (1988)	
								Marzin et al. (1986)	

TABLE 2 (continued)

Chemical	Endpoint	Cell type	Result	LEC		Treatment time (h)	Toxicity at LEC	Comment	Reference
				mg/ml	mM				
	Micronuclei	Human fibroblasts	+	2.5	43	48	MI = 90% of control	Scott and Roberts (1987)	
	SCE	CHO	-			4		25% increase above control at 250 mM (754 mOsm/kg) but not significant (see footnote)	Galloway et al. (1987a)
	Mutation (TK)	Mouse lymphoma (\pm S9)	+	4.0	68		Total growth 20-40% of control	Myhr, in Brusick (1986)	
	Mutation (HPRT)	V79 (\pm S9)	\pm	5.6	94.3	4	Total growth 25% of control	Wangenheim and Bolcsfoldi (1988)	
	Gene mutation	Salmonella (5 strains \pm S9)	-			3		Sporadic increases at 100-150 mM (550-600 mOsm/kg)	Seeberg et al. (1988)
	Gene Mutation	Saccharomyces	+	117	2000	1.0	CFE = 65%	No mutants at 1.5-23 mg per plate	Seeberg et al. (1988)
	Transformation	Balb/c-3T3	+	3.3	57	400		No mutations in stationary phase cells	Parker and von Borstel (1987)
	UDS	HeLa (\pm S9)	-			3		No UDS up to 200 mM	Rundell and Matthews, in Brusick (1986)
	DNA ssb	CHO	+	21.9	375	937			Seeberg et al. (1988)
	DNA dbp	CHO	+	21.9	375	937		No direct measurement but 200 mM gave CFE = 71% in same series of expts ditto	Galloway et al. (1987a)
	Chromosome aberrations	DON	+	10.3	50	322		'Considerable mitotic inhibition'	Abe and Sasaki (1977)
	SCE	CHL DON	+	4.0	19.5	24, 48		\times 2 spontaneous frequency. No dose response.	Ashby and Ishidate (1986)
		DON	\pm	1.0	4.8	26		'Considerable mitotic inhibition' see footnote	Abe and Sasaki (1977)
		CHO	\pm	1.0	4.8	24		4.8 mM induced <10% increase required to give increase up to 50%. See footnote	Wolff and Rodin (1978)

	Human lymphocytes	±	1.0	4.8	72	4.8 mM induced < 20% increase. 24 mM required to give increase > 50%. See footnote 70% increase with 48 mM See footnote	Wolff and Rodin (1978)
	Human lymphocytes	±	10	48	24		Brogger et al. (1979)
	Mutation (TK) lymphoma (+ S9)	±	17	83	4	Survival 7%	Clive et al. (1979)
	Gene mutation Salmonella	-					Reviewed by Ashby (1985)
Sorbitol	Chromosome aberrations	+	55	300	4	CFE = 100%	Galloway et al. (1987a)
	CHL	-			24, 48		Ishidate (1988)
	Gene mutation Salmonella	-					Ishidate (1988)
Sucrose	Chromosome aberrations	+	70	204	24	CFE = 60%	Ishidate et al. (1984)
	CHO	+	94	275	4	CFE = 60%	Galloway et al. (1985)
	CHO	-			4		Galloway et al. (1987a)
	DNA ssb	-			4	No direct measurement but 325 mM gave CFE = 6% in same series of expts.	Galloway et al. (1987a)
	DNA dsb	-			4	no dsb detected up to 400 mM (779 mOsm/kg)	Galloway et al. (1987a)
Urea	Chromosome aberrations	+	3.0	50	72	14% pycnotic cells MI = 70% of control	Oppenheim and Fishbein (1965)
	CHL	+	12.0	200	24		Marzin et al. (1986)
	Mutation (TK) lymphoma	+	31.8	530	> 816	Total growth 24% of control	Ishidate (1988)
	Gene mutation Salmonella	-					Wangenheim and Bolcsfoldi (1988)
							Ishidate (1988)

Chemicals have been tested without S9 mix unless specified. Galloway et al. (1987) suggest that the apparent induction of SCEs by hyperosmotic conditions may be a consequence of reduced cell density and increased BrdUrd uptake by remaining cells.

CFE, colony-forming efficiency. MI, mitotic index.

ride itself. Detailed studies will be required to define the mechanisms of action; all we have at present are rather diffuse empirical observations. Galloway et al. (1987a) have suggested that the intracellular disturbances produced by high dose treatments may lead to changes in chromatin structure and/or enzyme activity.

Not all chemicals are genotoxic at high osmolality (Ishidate et al., 1984; Galloway et al., 1987a). For example, glycerol is non-clastogenic in CHO cells even with an increase in osmolality of more than 1100 mOsm/kg, at a concentration of 1000 mM, probably because rapid equilibration across the cell membrane precludes excessive osmotic stress (Galloway et al., 1987a).

In an attempt to avoid the problems associated with high osmolality, the Japanese Guidelines (1987) for clastogenicity testing *in vitro* recommend the use of a maximum concentration level of 10 mM. The validity of this recommendation can be investigated by examining dose-response data *in vitro* for those chemicals which are clastogenic *in vivo*. The possibility of undertaking such an analysis has been facilitated by two recent literature reviews. Ishidate et al. (1988) have listed the lowest effective concentrations (LEC) for clastogenicity of 466 chemicals tested in a variety of mammalian cell types *in vitro*. (Designation of a lowest effective concentration does not necessarily imply the existence of a threshold. Extensive dose-response data are required for this purpose.) About 20% of these chemicals have also been tested for micronucleus induction *in vivo* in rodent bone marrow. Thompson published a similar paper in 1986 which was restricted to chemicals which had been tested both *in vivo* and *in vitro* and found to be positive in one or both tests. In Thompson's review the *in vivo* clastogenicity data included tests for both micronucleus and metaphase aberration induction in rodent marrow. However, LEC values for *in vitro* testing were not given. We have therefore listed from these reviews (Tables 3 and 4) those chemicals which have been tested both *in vivo* and *in vitro*, which are positive in one or both tests, where the *in vivo* endpoint is either micronucleus or metaphase aberration induction, and for which *in vitro* LEC data are available from the review of Ishidate et al. (1988).

2.2. Lowest effective concentrations for clastogenicity *in vitro*

Before considering those chemicals which have been tested both *in vivo* and *in vitro* (Tables 3 and 4) it is worthwhile examining the range of LEC values for all the *in vitro* clastogens reviewed by Ishidate et al. The LEC values of the 466 clastogens varied over more than 10 orders of magnitude, from 4.3×10^{-8} mM (Trenimon at 10^{-5} $\mu\text{g}/\text{ml}$) to 6.9×10^2 mM (acetone at 4×10^4 $\mu\text{g}/\text{ml}$); 125 (27%) had LEC values > 1.0 mM in all cell types tested and 37 (8%) had values > 10 mM. The latter group is listed in Table 6 and comprises a wide range of chemical species that are sufficiently soluble and non-toxic to enable cytogenetic data to be obtained at these high concentrations. Clastogenesis is not, however, an inevitable consequence of *in vitro* exposure to high concentrations of chemicals. Of 377 chemicals reported as non-clastogenic in the review of Ishidate et al., 91 were tested at > 10 mM.

A number of potentially DNA reactive agents are included in Table 6 because they have been tested without metabolic activation (e.g. diethanolnitrosamine), but others, even with activation (e.g. diethylnitrosamine, LEC 29 mM or 3 mg/ml), require these high doses for detection. Some chemicals (e.g. polyethylene glycol, urea) are probably clastogenic through osmotic effects. It should be noted that the majority of these chemicals have only been tested in Chinese hamster cells and have not been tested for activity *in vivo* (see right-hand column in Table 6).

2.2.1. LEC values *in vitro* for agents which are clastogenic *in vivo*

Table 3 lists 66 chemicals that induce metaphase aberrations and/or micronuclei in rodent bone marrow and have been tested for clastogenicity *in vitro* in a number of mammalian test systems whose descriptions and abbreviations are given in Table 5. All but 4 of the chemicals were clastogenic *in vitro*. Again, LEC values range over almost 10 orders of magnitude from 4.3×10^{-8} mM to 100 mM (Fig. 1). Thus, even for chemicals that are clastogenic *in vivo*, some require very high exposure concentrations to be detected *in vitro* in

some cell systems. In part, this probably reflects the inadequacies of metabolic activation systems.

From the data in Table 3 and Fig. 1 we have listed (Table 7) those chemicals whose clastogenicity would have been missed if upper concentration limits had been applied to in vitro tests. Upper limits of 1, 5, 10, 20 and 50 mM are considered. Reading vertically, one can see which chemicals would be missed if tested in particular cell types if a given upper limit was used for testing.

Of the 66 in vivo clastogens, 7 had LEC values in vitro of > 10 mM in at least one cell type or were non-clastogenic at > 10 mM. Since most testing laboratories use only one cell type for their in vitro clastogenicity assays, the potential of these 7 chemicals for in vivo clastogenesis might have been missed if an upper limit of 10 mM had been adopted for in vitro testing. However, the testing of these 7 chemicals may not in all cases have utilised suitable protocols; indeed many of the studies were not intended to establish LEC values. The data on these chemicals is evaluated below:

(a) *Barbital*. Tested only in Chinese hamster cells (CHL and DON) without metabolic activation. Positive in CHL at 11 mM after treatment for 48 h (negative at 24 h), inconclusive at the next lowest concentration (5.5 mM) for 48 h after an analysis of 100 cells (Ishidate and Odashima, 1977). Negative in DON after 26 h treatment at up to 8 mM (Abe and Sasaki, 1977).

Verdict: Probably detectable at < 10 mM in CHL if more cells were analysed. Possibly detectable at < 10 mM in DON cells with a longer treatment time. May require metabolic activation.

(b) *Benzene*. Detectable at < 10 mM in Chinese hamster cells only with metabolic activation [CHO (Palitti et al., 1985) and CHL (Ishidate and Sofuni, 1985)] and human lymphocytes with or without activation (Howard et al., 1985). Negative in RL4 (Priston and Dean, 1985) up to 12.8 mM (24 h). RL4 cells may not have the necessary metabolic capacity to activate benzene. Dean et al. (1985) caution that benzene floats on top of the medium so agitation of cultures is required and Proctor et al. (1986) have pointed out the high volatility of the chemical such that most is 'lost to the head space' in a closed treatment vessel.

Verdict: Probably detectable at < 10 mM in RL4 cells with activation.

(c) *Dimethylaminobenzene*. Positive in CH1-L at 0.11 mM (Lafi, 1985). Negative in CHO up to 55 mM even with activation, but the treatment time was only 1 h and sampling was at 12 h (Natarajan and Van Kesteren-van Leeuwen, 1981). Negative in RL4, but because of cytotoxicity it was possible only to test up to 0.36 mM (Malallah et al., 1982). Difficult to detect in bacterial mutation assays because of problems with metabolic activation (Parry and Arlett, 1985).

Verdict: May be detectable in CHO at < 10 mM with optimal conditions for metabolic activation and a later sampling time to allow for mitotic delay.

(d) *Dimethylnitrosamine*. Requires > 10 mM, with activation, for a positive result in CHO (Natarajan et al., 1976), human lymphocytes (HL) (Bimboes and Greim, 1976) and Syrian hamster fibroblasts (SHF) (Nishi et al., 1980). In CHL, LEC = 6.7 mM (Ishidate, 1988).

CHO: Doses used 8, 27 and 135 mM for 1 h. High yields of aberrations at 27 mM (152 per 100 cells); no significant increase at 8 mM but 6 exchanges per 100 cells observed.

Verdict: Probably detectable at 10 mM with a longer duration of treatment and analysis of 200 cells per sample.

HL: Only one dose used (50 mM) for 45 min. Low aberration yield (5 per 100 cells).

Verdict: May be detectable at < 10 mM with longer treatment time to ensure adequate activation.

SHF: Only one concentration used (100 mM for 3 h), which gave a high aberration yield (76 per 100 cells).

Verdict: May be detectable at < 10 mM.

Overall verdict: Probably detectable in various cell types, in addition to CHL, at < 10 mM with adequate protocols.

(e) *Hexamethylphosphoramide (HMPA)*. Positive in HL (Ashby et al., 1985) and CH1-L (Danford, 1985) at < 10 mM. Positive in CHL at 33.5 mM without activation but inconclusive at 22.3 and 11.2 mM even after 200 cells analysed (Ishi-

TABLE 3

CHEMICALS WHICH INDUCE MICRONUCLEI OR METAPHASE ABERRATIONS IN RODENT BONE MARROW IN VIVO WHICH HAVE ALSO BEEN TESTED FOR CLASTOGENICITY IN VITRO

Data from Ishidate et al. (1988) and Thompson (1986).

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration		TRT (h)	REC (h)	Ref.
							(μ g/ml)	(mM)			
4	Acetaldehyde	75-07-0	[- M]	+	[6] Rat fibro	-	4.4	0.1	24	0	32
12 a	2-Acetylaminofluorene	53-96-3	[+ m +]	+	[4] CHL	+	500	2.2	3	21	174
b				[4] CHL	-	2000	9	3	21	174	
c				[6] RL1	+	30	0.13	24	0	61	
d				[4] V79-4	-	5	0.022	24	0	227	
25 a	Actinomycin D	50-76-0	[- m +]	+	[1] Hum lymph	-	1.8	0.0014	1	0	113
b				[1] Hum lymph	+	3.5	0.0028	1.5	0	47	
c				[4] CHL	+	0.0125	24	0	121		
d				[5] A(T)CL-3	+	0.05	0.00004	24	0	28	
27 a	Adriamycin	23214-92-8	[+ m ?]	+	[4] CHO	+	0.1	0.00018	5	0	19
b				[1] Hum lymph	+	5.4	0.01	1	0	113	
c				[1] Hum lymph	+	0.06	0.00011	48	0	198	
d				[1] Hum lymph	+	0.02	0.000037	24	0	200	
e				[1] Hum lymph	+	0.1	0.00018	4	48	318	
f				[1] Hum lymph	+	0.05	0.00009	4	24	317	
g				[2] Hum fibro	+	0.01	0.000018	1	5	200	
h				[4] CHO	+	0.1	0.00018	5	0	19	
i				[4] CHO	+	0.1	0.00018	0.5	7	103	
28	Aflatoxin B1	1162-65-8	[+ m +]	+	[4] V79	+	0.5	0.0016	1	26	23
30	Alachlor	15972-60-8	[M]	+	[1] Hum lymph	-	2	0.0074	24	0	88
88 a	Azathioprine	446-86-6	[+ m +]	+	[1] Hum lymph	-	50	0.2	24	0	210
b				[1] Hum lymph	+	23	0.083	24	0	313	
c				[1] Hum lymph	-	40	0.14	24	0	7	
92 a	Barbital	57-44-3	[- M]	+	[4] CHL	-	2000	11	48	0	122
b				[4] DON	-	1470	8	26	0	1	
98 a	Benzene	71-43-2	[- M +]	+	[1] Hum lymph	+	9	0.11	3	25	110
b				[4] CHL	+	550	7.6	6	18	123	
c				[4] CHO	+	200	2.6	3	15	218	
d				[1] Hum lymph	+	16	0.2	53	0	183	
e				[1] Hum lymph	+	9	0.11	3	25	110	
f				[1] Hum lymph	+	86	1.1	72	0	148	
g				[4] CHO	-	5000	64	2	10	92	

TABLE 3 (continued)

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration		TRT (h)	REC (h)	Ref.
							($\mu\text{g}/\text{ml}$)	(mM)			
295	Diglycidylaniline	2095-06-9	[m]	+	[4] CHO-K1	-	8	0.039	24	0	258
305 a	4-Dimethylaminoazo-benzene	60-11-7	[+ m +]	+	[4] CH1-L	-	25	0.11	36	0	152
b				+	[4] CHO	-	12500	55	1	12	191
c				-	[4] CHO	-	12500	55	1	12	191
d				-	[6] RL4	-	50	0.22	24	0	230
e				-	[6] RL4	-	80	0.36	24	0	170
308 a	7,12-Dimethylbenz[a]-anthracene	57-97-6	[+ m +]	+	[4] V79-4	+	1	0.0039	24	0	227
b				+	[4] CHL	+	50	0.2	6	18	121
c				+	[6] RL1	-	12.5	0.049	24	0	61
d				-	[4] CHL	-	200	0.78	6	18	121
309 a	Dimethylcarbamoylechloride	79-44-7	[+ m +]	+	[4] CHO	+	0.0167 $\mu\text{l}/\text{ml}$	1	12	191	
b				+	[4] CHO	-	0.1 $\mu\text{l}/\text{ml}$	1	12	191	
312 a	Dimethylnitrosamine	62-75-9	[+ m +]	+	[1] Hum lymph	+	3750	50	0.75	24	31
b				+	[4] CHO	+	2000	27	1	24	194
c				+	[4] CHL	+	500	6.7	6	18	121
d				+	[5] S H Fibro	+	7400	100	3	24	203
e				-	[4] CHL	-	2000	27	48	0	122
f				-	[5] S H Fibro	-	7400	100	3	24	203
g				-	[6] Rat lymph	-	6.7	0.09	24	0	162
331 a	Epichlorohydrin	106-89-8	[+ M +]	+	[4] CHO	+	40	0.42	1	12	191
b				+	[1] Hum lymph	-	18.5	0.2	48	0	207
c				+	[4] CHO	-	120	1.3	1	12	191
d				+	[4] CHL	-	62.5	0.68	24	0	121
e				+	[6] RL1	-	5	0.054	24	0	61
355	Ethylene oxide	75-21-8	[+ m ?]	+	[3] FL	-	220	5	1	48	226
360 a	Ethyl methanesulfonate	62-50-0	[+ m +]	+	[1] SN1029	-	0.003	0.000024	48	0	263
b				+	[1] HLCL	-	0.03	0.00024	48	0	263
c				+	[4] CHL	-	500	4	24	0	121
364 a	N-Ethyl-N-nitrosourea	759-73-9	[+ M +]	+	[2] Hum fibro	-	11.7	0.1	1	5	249
b				+	[4] CHL	-	62.5	0.53	48	0	122
378 a	Fenitrothion	122-14-5	[+ M -]	+	[4] CHL	+	50	0.18	3	21	121
b				+	[4] CHL	-	100	0.36	24	0	121
388 a	5-Fluorouracil	51-21-8	[+ m ?]	+	[4] CHO	-	100	0.77	24	0	168
b				+	[4] CHL	-	1	0.008	24	22	333
c				+	[4] CHL	-	3.125	0.024	48	0	121

393 a	Furylfuramide (AF-2)	3 688-53-7	[+ m +]	+	[4] CHL	+	80	0.32	3	21	121
b				+	[1] Hum lymph	-	7.4	0.03	24	0	296
c				+	[4] CHL	-	5	0.02	48	0	121
d				+	[7] FM3A	-	7.4	0.03	48	0	310
433 a	Hexamethylphosphor- amide (HMPA)	680-31-9	[- m +]	+	[1] Hum lymph	+	100	0.56	3	25	9
b				+	[1] Hum lymph	+	500	2.8	3	25	110
c				+	[1] Hum lymph	-	100	0.56	3	25	9
d				+	[1] Hum lymph	-	500	2.8	3	25	110
e				+	[4] CH1-L	-	2	0.01	36	0	54
f				+	[4] CHL	-	6000	33.5	48	0	123
g				-	[4] CHO	+	340	1.9	1	12	191
h				-	[4] CHO	+	5000	28	2	10	92
i				-	[4] CHO	+	10000	56	3	15	218
j				-	[4] CHO	-	340	1.9	1	12	191
k				-	[4] CHO	-	5000	28	10	3	92
l				-	[4] CHO	-	10000	56	3	15	218
m				-	[6] RL4	-	2000	11.2	24	24	229
440	Hycanthon	3105-97-3	[+ m ?]	+	[5] A(T1)CL-3	-	1	0.0028	24	0	28
441	Hycanthon methane- sulfonate	23 255-93-8	[+ m ?]	+	[1] Hum lymph	-	5	0.011	24	0	236
443 a	Hydralazine	304-20-1	[M]	+	[4] CHL	-	3.75	0.019	48	0	121
b	hydrochlorine			+	[7] FM3A	-	3.9	0.02	48	0	147
460	N-Hydroxyurethane	589-41-3	[+ m +]	+	[1] Hum lymph	-	105	1	48	0	214
486 a	Kaempferol	520-18-3	[+ m]	+	[4] CHO-AT3-2	+	35	0.12	15	0	45
b				+	[4] CHO-AT3-2	-	25	0.09	15	0	45
506 a	Malathion	121-75-5	[- M -]	+	[4] CHL	+	100	0.3	3	21	121
b				+	[1] Hum lymph	-	10	0.03	24	0	322
c				+	[4] CHL	-	60	0.18	48	0	121
d				-	[1] B411-4	-	100	0.3	50	0	115
510	Mancozeb	8018-01-7	[M]	+	[1] Hum lymph	-	4		24	0	88
522 a	6-Mercaptopurine	50-44-2	[+ m ?]	+	[1] Hum lymph	-	0.01	0.00007	52	0	190
b				+	[4] CHO	-	10	0.066	24	0	168
c				+	[4] CHL	-	2.5	0.016	24	22	333
d				+	[4] CHL	-	0.75	0.0049	48	0	121
e				-	[1] Hum lymph	-	5	0.03	24	0	210
f				-	[5] A(T1)CL-3	-	100	0.66	24	0	28
530 a	Methotrexate	59-05-2	[- m ?]	+	[4] CHO	+	5	0.011	5	0	19
b				+	[4] CHO	-	1	0.0022	24	0	168
c				+	[4] CHO	-	5	0.011	5	0	19
d				+	[5] A(T1)CL-3	-	1	0.0022	24	0	28

TABLE 3 (continued)

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration		TRT (h)	REC (h)	Ref.			
							($\mu\text{g}/\text{ml}$)	(mM)						
572 a	Methyl methanesulfonate	66-27-3	[+M+]	+	[1] Hum lymph	-	22	0.2	24	0	42			
b				+	[1] SN1029	-	0.003	0.000027	48	0	263			
c				+	[1] HLCL	-	0.003	0.000027	48	0	263			
d				+	[4] V79	-	27.5	0.25	24	24	24	141		
e				+	[4] CHL	-	10	0.09	24	0	24	121		
575 a	<i>N</i> -Methyl- <i>N</i> '-nitro- <i>N</i> -nitrosoguanidine	70-25-7	[+m+]	+	[1] Hum lymph	-	2.9	0.02	1	0	113			
b				+	[1] SN1029	-	0.003	0.00002	48	0	263			
c				+	[1] HLCL	-	0.003	0.00002	48	0	263			
d				+	[2] Hum fibro	-	7.4	0.05	3	20	20	323		
e				+	[2] W1-38	-	1	0.0068	24	0	24	328		
f				+	[4] CHO	-	7.4	0.05	3	20	20	323		
g				+	[4] CHO	-	3	0.02	2	24	24	112		
h				+	[4] CHL	-	5	0.034	48	0	48	122		
i				+	[4] DON, D-6	-	1.5	0.01	1	24	24	139		
j				+	[4] V79-4	-	0.5	0.0034	24	0	24	227		
k				+	[5] S H Fibro	-	0.3	0.002	3	24	24	120		
l				+	[6] RL1	-	1	0.0068	24	0	24	61		
584 a	<i>N</i> -Methyl- <i>N</i> -nitrosoarea	684-93-5	[+M+]	+	[2] Hum Fibro	-	10	0.1	1	23	249			
b				+	[4] DON	-	10	0.1	26	0	1			
c				+	[4] CHL	-	25	0.24	48	0	122			
d				+	[6] Rat lymph	-	93	0.9	24	0	162			
604 a	Mitomycin C	50-07-7	[-M+]	+	[4] CHO	+	1	0.003	5	0	19			
b				+	[1] Hum lymph	-	0.1	0.0003	24	0	0	50		
c				+	[1] Hum lymph	-	0.1	0.0003	24	0	0	39		
d				+	[1] SN1029	-	0.03	0.00009	48	0	263			
e				+	[1] HLCL	-	0.03	0.00009	48	0	263			
f				+	[4] CHO	-	1	0.003	5	0	0	19		
g				+	[4] DON, D-6	-	0.33	0.001	1	24	24	139		
h				+	[4] DON, D-6	-	0.017	0.00005	8	0	0	140		
i				+	[4] CHL	-	0.04	0.00011	48	0	0	121		
j				+	[8] MA61	-	0.6	0.0018	3	15	15	196		
k				-	[1] Hum lymph	-	30	0.1	1	0	0	113		
612 a				1-Naphthylamine	134-32-7	[+m?]	+	[4] CHO	+	17	0.12	1	12	191
b							-	[4] CHO	-	333	2.3	1	12	191
c	-	[4] CHL	-				60	0.42	48	0	122			
613 a	2-Naphthylamine	91-59-8	[+m+]	+	[4] CHO	+	3.33	0.023	1	12	191			
b				+	[4] CHO	-	5	0.035	1	12	191			
632	Nitrofurantoin	67-20-9	[+m?]	+	[4] CHL	-	60	0.25	24	0	121			

635 a	Nitrogen mustard	51-75-2	[+ m +]	+	-	0.02	0.0001	52	0	190
b				+	-	15.6	0.1	2	0	104
644 a	4-Nitroquinoline	56-57-5	[+ m +]	+	+	0.05	0.00025	1	24	328
b	1-oxide			+	+	3	0.016	1	12	191
c				+	-	0.03	0.00016	48	0	263
d				+	-	0.03	0.00016	48	0	263
e				+	-	0.057	0.0003	3	20	323
f				+	-	0.03	0.00015	24	0	328
g				+	-	2.3	0.012	1	12	191
h				+	-	0.19	0.001	3	20	323
i				+	-	0.2	0.001	24	0	122
j				+	-	0.19	0.001	1	24	139
k				+	-	0.5	0.0026	4	21	279
l				+	-	0.02	0.00011	24	0	61
m				+	-	0.025	0.00013	9	0	280
n				+	-	0.025	0.00013	12	0	280
o				+	-	0.025	0.00013	12	0	280
718	Potassium bromate	7758-01-2	[+ m +]	+	-	62.5	0.37	24	0	121
721 a	Potassium chromate	7789-00-6	[+ m +]	+	-	0.78	0.004	28	0	187
b				+	-	0.06	0.0012	48	0	149
c				+	-	0.25	0.0048	30	0	158
d				+	-	0.3	0.002	48	0	308
725 a	Potassium dichromate	7778-50-9	[+ M]	+	-	0.073	0.00025	70	0	271
b				+	-	0.15	0.0005	28	0	187
c				+	-	0.3	0.001	32	0	157
d				+	-	0.1	0.002	32	0	316
e				+	-	0.1	0.0019	30	0	158
f				+	-	0.35	0.001	24	24	199
g				+	-	0.1	0.0003	24	0	298
h				+	-	0.1	0.00034	24	24	299
i				+	-	0.02	0.00064	24	0	308
735 a	Propanesultone	1120-71-4	[+ M +]	+	-	12	0.1	26	0	1
b				+	-	62.5	0.5	24	0	122
771 a	Quercetin	117-39-5	[+ m ?]	+	+	200	0.66	3	21	121
b				+	-	6	0.02	15	0	45
c				+	-	100	0.33	3	20	277
d				+	-	60	0.2	48	0	121
773	Quinacrine mustard	64046-79-3	[+ m]	+	-	9.4	0.02	2	24	112
780 a	Rhodamine B	81-88-9	[+ M +]	+	-	10	0.02	5	0	15
b				+	-	60	0.13	48	0	121
c				+	-	4	0.0083	24	0	160
818	Sodium chlorite	7758-19-2	[+ m]	+	-	20	0.22	24	0	121

TABLE 3 (continued)

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration		TRT (h)	REC (h)	Ref.
							(μ E/ml)	(mM)			
858	Streptonigrin	3930-19-6	[M]	+	[1] Hum lymph	-	0.001	0.000002	6	0	52
878 a	3,3',5,5'-Tetra-methylbenzidine	54827-17-7	[- m]	-	[4] CHO	+	5000	21	1	12	191
b					[4] CHO	-	5000	21	1	12	191
890 a	Thio-TEPA	52-24-4	[+ m +]	+	[1] Hum lymph	-	1	0.0053	32	0	36
b					[4] CHO	-	10	0.053	24	0	168
c					[4] CHL	-	0.94	0.0049	48	0	121
d					[5] A(T1)CL-3	-	1	0.0053	24	0	28
898	Tolbutamide	64-77-7	[- m -]	-	[4] CHL	-	500	1.8	48	0	121
903 a	Trenimon	68-76-8	[+ m +]	+	[1] Hum lymph	-	0.002	0.0000086	24	0	8
b					[2] Hum fibro	-	0.00002	0.00000086	24	0	8
c					[4] C H Fibro	-	0.00001	0.000000043	24	0	8
d					[4] CHO	-	0.23	0.001	2	0	104
917 a	Triethylenemelamine	51-18-3	[+ m +]	+	[3] HeLa	-	0.03	0.00015	24	0	334
b					[4] C H Fibro	-	0.1	0.00049	24	0	334
934 a	Urethane	51-79-6	[- m +]	+	[4] CHL	-	8000	90	48	0	122
b					[4] DON	-	7130	80	26	0	1
c					[4] V79-4	-	25	0.28	24	0	227
940	Vinblastine	865-21-4	[- m ?]	+	[4] DON	-	0.0039	0.000005	10	0	257
941 a	Vincristine	57-22-7	[- m ?]	-	[4] CHO	+	10	0.012	5	0	19
b					[4] CHO	-	1	0.0012	24	0	168
c					[4] CHO	-	10	0.012	5	0	19
d					[5] A(T1)CL-3	-	0.05	0.000061	24	0	28

No. The number of the test substance is that used by Ishidate et al. (1988) in their listing of all substances tested in vitro.

[ABC] Results from other tests. A, Ames test (data from Ishidate et al., 1988); B, bone marrow test in rodents [m, micronuclei (Ishidate et al., 1988); M, metaphase aberrations (Thompson, 1986)]; C, carcinogenicity tests in rodents (Ishidate et al., 1988). +, Positive; -, negative; ?, unresolved; blank, untested.

Results in vitro +, clearly positive; -, negative or not clearly positive.

Cell type See Table 5.

MET ACT -, without metabolic activation; +, with metabolic activation (usually S9 from rat liver).

Concentration The lowest effective concentration (LEC) for positive (+) results or the maximum tested dose for negative (-) results.

TRT Treatment time in h (H).

REC Recovery time in h (H).

Ref. Reference number as in Ishidate et al. (1988), in vitro clastogenicity data.

date and Sofuni, 1985). May require metabolic activation; Ashby et al. (1985) suggest that the genotoxic effects of HMPA may be *via* enzyme-mediated formation of formaldehyde which is clastogenic (Natarajan et al., 1983; Levy et al., 1983). Negative in RL4 (Priston and Dean, 1985) and CHO (Palitti et al., 1985) with or without activation, up to 11.2 and 56 mM respectively.

Verdict: Possibly detectable at < 10 mM in CHL with activation. RL4 and CHO appear unresponsive.

(f) *Tetramethylbenzidine.* Tested only in CHO (Natarajan and Van Kesteren-van Leeuwen, 1981); negative up to 21 mM with or without activation. One hour treatment only, sampled at 12 h.

Verdict: May be detectable at < 10 mM with a longer duration of treatment and later sampling to allow for mitotic delay. The *in vivo* data require confirmation.

(g) *Urethane.* Positive in CHL (Ishidate and Odashima, 1977) at 90 mM for 48 h without activation; inconclusive at 45 mM. With activation (6 h treatment) LEC = 225 mM (Ishidate, personal communication). Negative in DON up to 80 mM (Abe and Sasaki, 1977).

Verdict: Not detectable at 10 mM (or even 50 mM).

Conclusions

(a) Those assays that required doses in excess of 10 mM to detect clastogenicity would probably have given positive results at < 10 mM if current testing guidelines had been adopted. The exception is urethane which, even with rigorous testing, had an LEC value of 90 mM in CHL cells. There is an urgent need for LEC estimates for urethane in cell systems other than Chinese hamster. It should be noted also that urethane is non-mutagenic in Ames tests. [*Note added in proof:* Frölich and Würzler (1990) have recently shown in the somatic mutation and recombination test (SMART) in *Drosophila* that urethane requires to be metabolically activated probably *via* cytochrome P450-dependent enzyme activities.]

(b) In a few instances certain cell types appear to be totally unresponsive to particular chemicals even under optimal conditions. Reasons other than

an insufficiency of test chemical must be sought to explain these observations.

(c) If this database can be taken as representative of clastogenic chemicals, we conclude that the great majority of chemicals which are clastogenic *in vivo* will be detected *in vitro* even if an upper dose limit of 10 mM is used, provided that a rigorous protocol is adopted. Of particular importance are the following:

(i) The use of optimal conditions for metabolic activation. A positive control which requires activation (e.g. cyclophosphamide) should always be included and used at a relatively low concentration (Preston et al., 1987) to test the efficiency of activation.

(ii) An adequate duration of treatment. When activation is used, the duration of treatment with the test chemical is limited by the toxicity of the S9 mix. The longest possible treatment time consistent with the non-toxicity and non-mutagenicity (Section 4) of the S9 mix alone should be used; e.g., at least 3 h in human lymphocytes and CHO cells. 1-h treatments are insufficient.

(iii) An appropriate sampling time to allow for cell-cycle delay.

(iv) The analysis of at least 200 cells per dose. With a typical background frequency of 2% aberrant cells, when 100 cells are scored there is less than a 40% chance of detecting even a quadrupling in aberration frequency. Increasing the sample size to 200 cells increases this power to about 70% (Margolin et al., 1986).

(d) The advantage of adopting an upper concentration limit of 10 mM for testing is that it will, rightly, exclude those chemicals which are only clastogenic at very high doses which are irrelevant to human exposure. This assumes that such chemicals show threshold responses, which appears to be the case for non-DNA reactive chemicals which are clastogenic at concentrations producing significant changes in the osmolality of the culture medium (section 2.1). The disadvantage is that a few chemicals which are potentially clastogenic *in vivo* will be 'missed'; on the basis of the Ishidate/Thompson database we conclude that the frequency will be low ($1/66 = 1.5\%$). Lowering the cut-off concentration would certainly increase this frequency (Table 7) whereas raising the level to 20 mM would begin to pick up effects associated

TABLE 4

CHEMICALS WHICH ARE CLASTOGENIC IN VITRO BUT DO NOT INDUCE MICRONUCLEI OR METAPHASE ABERRATIONS IN RODENT BONE MARROW IN VIVO

Data from Ishidate et al. (1988) and Thompson (1986).

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration		TRT (h)	REC (h)	Ref.
							($\mu\text{g}/\text{ml}$)	(mM)			
5 a	Acetaminophen	103-90-2	[-M]	+	[1] Hum lymph	-	200	1.3	72	0	325
b				+	[4] CHO-K1	-	70	0.46	24	0	250
c				+	[4] CHL	-	30	0.2	48	0	121
17	Acid red (C I acid red 52)	3520-42-1	[-M]	+	[4] CHL	-	9000	15	48	0	121
18 a	Acridine	260-94-6	[-m-]	+	[4] CHL	+	200	1.1	3	21	173
b				+	[4] CHL	-	30	0.17	24	0	173
43	2-Amino-4-nitrophenol	99-57-0	[+m]	+	[4] CHL	-	15	0.1	48	0	121
48	4-Aminoquinoline-1-oxide	2508-86-3	[+M-]	+	[4] CHL	-	200	1.3	24	0	122
52	Ammonium chloride	12125-02-9	[-m]	+	[4] CHL	-	300	5.6	48	0	121
86	Auramine O	2465-27-2	[-m?]	+	[4] CHO	-	6.4	0.02	5	0	15
159 a	Cadmium chloride	10108-64-2	[+m+]	+	[1] Hum lymph	-	0.3	0.0016	24	0	46
b				+	[4] CHO	-	5.5	0.001	36	0	63
c				+	[4] V79	-	1.8	0.01	2	22	211
d				+	[4] V79	-	1.8	0.01	2	22	213
e				-	[7] FM3A	-	5.9	0.032	48	0	308
162 a	Caffeine	58-08-2	[-m-]	+	[2] W1-38	+	2000	10	1	24	328
b				+	[1] Hum lymph	-	250	1.3	24	0	327
176 a	Captan	2425-06-1	[+M]	+	[4] V79-CL-15	-	3.5	0.01	27	0	287
b				+	[4] CHL	-	4	0.011	24	0	121
c				-	[4] CHL	+	16	0.046	3	21	121
177 a	Captan	133-06-2	[+ _M +]	+	[4] V79-CL-15	-	14	0.045	27	0	287
b				+	[4] CHL	-	7	0.023	24	0	121
c				-	[2] Hum fibro	-	4	0.013	24	0	286
227 a	Cinnamic aldehyde	104-55-2	[+m?]	+	[4] B241	-	0.001	0.00001	24	24	138
b				+	[4] CHL	-	10	0.076	48	0	121
238	Cochineal	1260-17-9	[+M]	+	[4] CHL	-	12000	20	48	0	121
252 a	DDT	50-29-3	[-m?]	+	[4] B14F28	-	8.1	0.023	4	0	167
b				-	[4] V79	-	45	0.13	24	24	141
258 a	2,4-Diaminoanisole	615-05-4	[+m+]	+	[4] CHO	+	50	0.36	1	16	57
b				+	[4] CHO	-	50	0.36	1	16	57

272 a	Dibromochloromethane	124-48-1	[+ m]	+	[4] CHL	+	98	0.47	3	21	121
b				-	[4] CHL	-	610	2.9	48	0	121
285 a	Dichlorvos	62-73-7	[+ M ?]	+	[1] Hum lymph	-	10	0.045	24	0	59
b				+	[4] V79-CL-15	-	110	0.5	27	0	287
c				+	[4] CHL	-	125	0.57	48	0	121
291 a	Diethylnitrosamine	55-18-5	[+ m +]	+	[4] CHL	+	3000	29	3	21	174
b				+	[4] CHO	+	10200	100	3	24	194
c				-	[4] CHL	-	3000	29	3	21	121
302	Dimethoate	60-51-5	[+ M ?]	+	[4] CHL	-	500	2.2	48	0	121
325	Disodium glycyrrhizinate	71 277-789-7	[- m]	+	[4] CHL	-	2000	2.3	48	0	121
339 a	Ethenzamide	938-73-8	[M]	+	[4] CHL	+	600	3.6	3	21	174
b				+	[4] CHL	-	500	3	48	0	121
341	Ethionamide	536-33-4	[- m ?]	+	[4] CHL	-	400	2.4	48	0	121
344	Ethylacetate	141-78-6	[- m ?]	+	[4] CHL	-	9000	100	48	0	121
373 a	Eugenol	97-53-0	[- m ?]	+	[4] CHL	-	125	0.76	48	0	121
b				-	[4] CHO	-	200	1.2	3	20	277
376	Fast green FCF (crude)	2353-45-9	[+ m +]	+	[4] CHL	-	2000	2.5	48	0	121
379	Ferrous sulphate	7720-78-7	[m]	+	[4] CHL	-	1250	8.2	24	0	121
389 a	Formaldehyde	50-00-0	[+ m +]	+	[4] CHO	+	6	0.2	2	20	193
b				+	[2] Hum fibro	-	60	2	24	0	159
c				+	[4] CHO	-	6	0.2	2	20	193
420 a	Griseofulvin	126-07-8	[- m +]	+	[1] Hum lymph	-	40	0.11	60	0	155
b				+	[2] Hum eue	-	40	0.11	60	0	155
444 a	Hydrazine sulphate	10034-93-2	[+ m +]	+	[4] CHO	+	158	1.2	1	12	191
b				+	[4] CHO	-	158	1.2	1	12	191
449 a	Hydrogen peroxide	7722-84-1	[+ M]	+	[4] CHO-K1	-	3.4	0.1	3	24	297
b				+	[4] V79	-	3.4	0.1	3	24	297
c				+	[4] CHL	-	125	3.7	24	0	121
d				+	[5] SH Fibro	-	3.4	0.1	3	24	297
e				+	[7] Mus fibro	-	0.34	0.01	3	24	297
457	N-Hydroxymethyl dimethyl phosphonopropionamide	20120-33-6	[- M]	+	[4] CHL	-	1000	4.4	24	0	121
459 a	Hydroxyurea	127-07-1	[- m ?]	+	[1] Hum lymph	-	25.3	0.333	48	0	214
b				-	[1] Hum lymph	-	37	0.1	1	0	113

TABLE 4 (continued)

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration		TRT (h)	REC (h)	Ref.
							($\mu\text{g}/\text{ml}$)	(mM)			
467	Iron and sodium succinate citrate		[- m]	+	[4] CHL	-	2000		24	0	121
482	Isoniazid	54-85-3	[+ M]	+	[4] CHL	-	2000	15	24	0	121
				+	[7] FM3A	-	440	3.2	48	0	147
				-	[4] CHL	+	8000	58	3	21	174
491	Lacchaic acid A	60687-93-6	[- M]	+	[4] CHL	-	1500		48	0	121
				-	[4] CHL	+	500		3	21	121
494	Lead acetate	301-04-2	[- m +]	+	[1] Hum lymph	-	3.3	0.01	24	0	26
				-	[1] Hum lymph	-	330	1	3	48	86
				-	[1] Hum lymph	-	330	1	72	0	254
501	D-Lysergic acid diethylamide (LSD)	50-37-3	[- m]	+	[4] CHL	+	1600	4.9	6	18	121
				-	[4] CHL	-	62.5	0.19	48	0	121
507	Maleic anhydride	108-31-6	[- ^M m ?]	+	[4] CHL	-	125	1.3	48	0	121
538	Methyl acrylate	96-33-3	[- m]	+	[4] CHL	-	7.5	0.087	24	0	121
559	3-Methylcholanthrene	56-49-5	[+ m +]	+	[4] CHL	+	20	0.075	6	18	121
				+	[6] RL1	-	2	0.0075	24	0	61
				-	[4] V79-4	+	1	0.0037	24	0	227
				-	[2] W1-38	-	10	0.037	24	0	328
				-	[4] CHL	-	80	0.3	6	18	121
560	2-Methyl-4-dimethylaminoazobenzene	54-88-6	[+ M]	+	[4] CHL	+	120	0.5	3	21	121
				-	[4] DON	-	24	0.1	26	0	1
				-	[4] CHL	-	30	0.13	48	0	122
633	Nitrofurazone	59-87-0	[+ ^M m ?]	+	[4] CHL	+	500	0.5	3	21	174
				-	[4] CHL	-	10	0.05	48	0	122
642	4-Nitro-O-phenylenediamine	99-56-9	[+ m -]	+	[4] CHL	-	30	0.2	48	0	122
				+	[4] CHMP/E	-	25	0.16	24	0	144
651	N-Nitroso-ethylenethiourea	3715-92-2	[m]	+	[4] V79-CL-15	-	66	0.5	16	0	287
680	Perillaaldehyde	2111-75-3	[- m]	+	[4] CHL	-	40	0.27	24	0	121
687	Phenobarbital	50-06-6	[+ m ?]	+	[4] CHO	+	500	2.2	2	10	92
				+	[4] CHO	+	3500	15	1	19	195
				+	[4] CH1-L	-	100	0.43	36	0	54
				+	[4] CHO	-	1000	4.3	10	3	92
				-	[4] CHO	+	400	1.7	1	25	240
				-	[4] CHO	-	400	1.7	1	25	240
				-	[4] CHL	-	2000	8.6	48	0	123
				-	[4] CHO	-	3500	15	1	19	195
				-	[6] RL4	-	1000	4.3	24	24	229

691 a	Phenylbutazone	50-33-9	[- m ?]	+	-		98.6	0.32	24	0	331
b				+	-	[1] Hum lymph [4] CHL	800	2.6	48	0	121
696 a	P-Phenylenediamine	106-50-3	[+ m]	+	+	[4] CHL	500	4.6	3	21	121
b				-	-	[4] CHL	500	4.6	3	21	121
730 a	Potassium sorbate	24634-61-5	[- M]	+	-	[4] DON	3000	20	26	0	1
b				+	-	[4] CHL	4000	27	48	0	122
737 a	β -Propiolactone	57-57-8	[+ m +]	+	-	[4] DON	72	1	26	0	1
b				+	-	[4] CHL	30	0.42	24	0	122
738 a	Propylene glycol	57-55-6	[- m]	+	-	[4] CHL	32000	420	48	0	121
b				-	+	[4] CHL	64000	840	3	21	121
742	Propyl gallate	121-79-9	[- M]	+	-	[4] CHL	20	0.094	24	0	121
772	Quinaacrine dihydrochloride	69-05-6	[m]	+	-	[4] CHO	9.5	0.02	2	24	112
775 a	Quinoline	91-22-5	[+ m +]	+	+	[4] CHL	300	2.3	3	21	174
b				-	-	[4] DON	129	1	26	0	1
c				-	-	[4] CHL	250	1.9	48	0	122
777 a	Resorcinol	108-46-3	[- m ?]	+	-	[1] Hum lymph	20	0.18	24	0	56
b				+	-	[1] Hum lymph	80	0.73	24	0	255
c				+	-	[3] Hum amm	40	0.36	24	0	255
d				-	+	[4] CHO	1600	14	1	16	56
e				-	-	[2] Hum fibro	50	0.45	24	0	56
f				-	-	[4] CHO	400	3.6	16	0	56
778	Resorcinol diglycidyl ether	101-90-6	[m]	+	-	[4] CHO-K1	8	0.036	24	0	258
792 a	Safrole	94-59-7	[+ m +]	+	+	[4] CHL	175	1.1	6	18	123
b				+	+	[4] CHO	83.3	0.5	3	15	218
c				-	-	[4] CHO	365	2.3	1	12	191
d				-	-	[4] CHO	50	0.31	2	10	92
e				-	-	[4] CHO	365	2.3	1	12	191
f				-	-	[4] CH1-L	60	0.37	36	0	54
g				-	-	[4] CHO	100	0.62	10	3	92
h				-	-	[4] CHL	150	0.92	6	18	123
i				-	-	[4] CHO	250	1.5	3	15	218
j				-	-	[6] RL4	100	0.62	24	24	229
824 a	Sodium dehydroacetate	4418-26-2	[- m]	+	-	[4] CHL	2000	5.3	48	0	122
b				-	-	[4] DON	19	1	26	0	1
829 a	Sodium fluoride	7681-49-4	[M ?]	+	-	[2] JHU-1	20	0.48	24	0	302
b				+	-	[5] SH Fibro	100	2.4	28	0	305
c				-	-	[1] Hum lymph	130	3	48	0	290
831 a	Sodium hypochlorite	7681-52-9	[+ m]	+	-	[4] CHL	500	6.7	48	0	121
b				-	+	[4] CHL	125	1.7	3	21	121

TABLE 4 (continued)

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration ($\mu\text{g}/\text{ml}$)	Concentration (mM)	TRT (h)	REC (h)	Ref.
839	Sodium nitrite	7632-00-0	[+ m ?]	+	[4] CHL	-	500	7.2	48	0	122
860 a	Styrene oxide	96-09-3	[+ m ?]	+	[1] Hum lymph	-	80	0.67	8	0	165
b				+	[1] Hum lymph	-	12	0.1	48	0	75
c				+	[1] Hum lymph	-	24	0.2	48	0	207
d				+	[1] Hum lymph	-	80	0.67	8	0	164
e				+	[4] CHL	-	3.75	0.031	24	0	121
880 a	Theophylline	58-55-9	[- M]	+	[1] Hum lymph	-	500	2.8	24	0	327
b				+	[4] CHL	-	500	2.8	48	0	121
c				+	[7] FM3A	-	580	3.2	48	0	147
d				-	[4] CHL	+	2000	11	3	21	121
e				-	[1] Hum lymph	-	1800	10	72	0	295
904 a	Triamterene	396-01-0	[- M]	+	[4] CHL	-	3.75	0.015	48	0	121
b				-	[4] CHL	+	30	0.12	3	21	121
906 a	Tribromomethane	75-25-2	[+ m ?]	+	[4] CHL	+	116	0.46	3	21	121
b	(bromoform)			-	[4] CHL	-	440	1.7	48	0	121
907	Trichlorfon	52-68-6	[+ m]	+	[4] CHL	-	125	0.49	48	0	121
923 a	Trisodium glycyrrhizinate	71277-78-6	[- m]	+	[4] CHL	+	4000	4.5	3	21	121
b				+	[4] CHL	-	2000	2.2	48	0	121

See Footnote to Table 2 for explanations of headings and symbols except for M and m in the column headed [ABC]. In this Table M (metaphase aberrations) and m (micronuclei) refer to the tests performed, not to positive results obtained.

with high levels of osmolality. On balance, we recommend an upper concentration limit of 10 mM.

(e) If clastogenicity tests *are* performed at concentrations above 10 mM the osmolality of the culture medium should be measured. If there is a substantial increase (> 50 mOsm/kg) and the chemical nature of the test agent does not suggest DNA reactivity, clastogenesis as a consequence of the high osmolality of the culture medium should be suspected. For endpoints other than clastoge-

nicity (Table 2) there are insufficient data to specify the osmolality levels at which these effects are likely to be seen.

2.2.2. LEC values in vitro for agents which are non-clastogenic in vivo

Table 4 lists, with LEC values, 68 chemicals which are clastogenic in vitro but not in vivo. These are also depicted in Fig. 2. Fig. 3 compares the LEC values of chemicals which are clastogenic in vivo and those which are not. There is a distinct

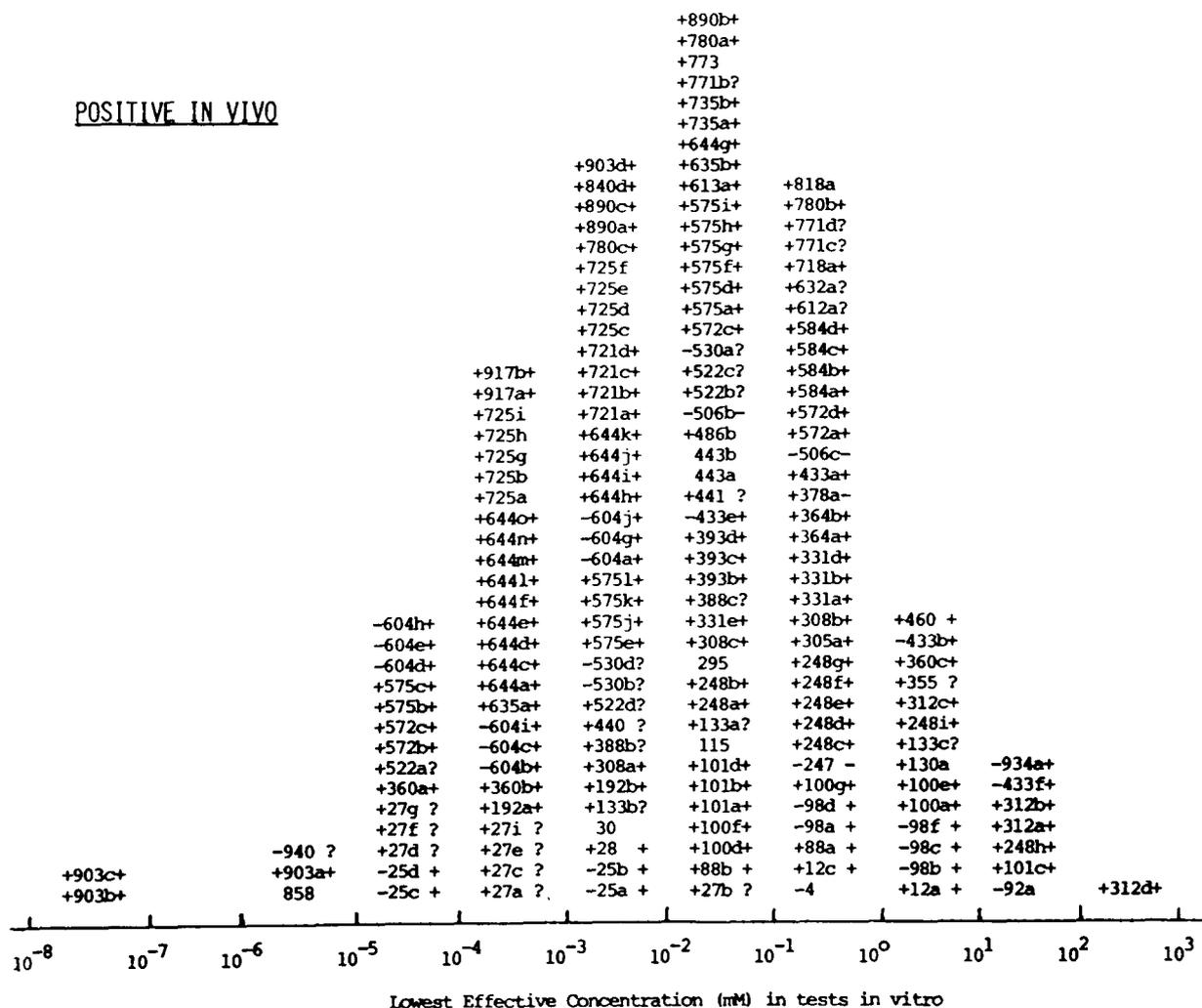


Fig. 1. Lowest effective concentrations in in vitro tests for those chemicals which induce micronuclei or metaphase aberrations in rodent bone marrow in vivo. Numbers refer to test substances in Table 3 which are clastogenic in vitro. Letters (a, b, c, etc.) refer to individual tests. Sign (+ or -) before the number indicates result of Ames test(s). Sign (+, - or ?) after the number indicates result of carcinogenicity test(s) in rodents. Where testing has been done with or without metabolic activation, the lower of the two LEC values has been plotted.

TABLE 5
DESCRIPTION OF THE CELLS USED IN STUDIES CITED IN TABLES 3 AND 4
Reproduced from Ishidate et al. (1988).

Abbreviation	Description	Abbreviation	Description
(1) B35M	<i>Human lymphoid cells</i> A line of cells derived from a patient with Burkitt's lymphoma (Minowada et al., 1967)	(4) CHO-K1	<i>Chinese hamster cells</i> (continued) A clonal derivative of CHO cells which requires proline for growth (Kao and Puck, 1967)
(1) B411-4	A cell line derived from lymphocytes of a healthy person	(4) CHO-K1-A	An ouabain-resistant clonal derivative of the CHO-K1 cell line
(1) CCRF-CEM	A T-lymphoblastoid cell line derived from a patient with acute lymphoblastic leukemia (Foley et al., 1965)	(4) CHO-K1-BH4	A clonal derivative of CHO cells isolated by selection in aminopterin-containing medium (O'Neill et al., 1977)
(1) HLCL	Cell lines derived from human lymphocytes by the investigator and/or not otherwise identified	(4) DON	A fibroblast cell line derived from lung tissue (Hsu and Zenzes, 1964)
(1) Hum lymph	Peripheral lymphocytes from normal human donors cultured for a short time after stimulation with phytohemagglutinin	(4) DON D6	A clonal derivative of the Don cell line
(1) P3J	A cell line derived from a patient with Burkitt's lymphoma (from Jiyoye, in Pulvertaft, 1964)	(4) HY	A line of fibroblast cells (Bauchinger and Schmid, 1972)
(1) RAJI	A cell line from a patient with Burkitt's lymphoma (Pulvertaft, 1964)	(4) V79	A line of fibroblast cells derived from lung tissue (Ford and Yerganian, 1958)
(1) RPMI-1788	A diploid cell line derived from lymphocytes of a normal human male donor (Huang, 1973)	(4) V79-4	A clonal derivative of V79 cells (Chu et al., 1969)
(1) SN1029	A cell line derived from a patient with chronic lymphocytic leukemia (Shiraishi et al., 1979)	(4) V79-CL-10	A clonal derivative of V79 cells
(2) Hum eue	<i>Human fibroblast cells</i> A heteroploid fibroblast cell line	(4) V79-CL-15	A clonal derivative of V79 cells
(2) Hum fibro	Fibroblast cultures from apparently normal humans (some fetal) which were established by the investigator, and/or not otherwise referenced	(4) V79-E	A clonal derivative of V79 cells (Thust, 1979)
(2) JA	Human fibroblast cells cultured from the skin of a normal human donor	(5)	<i>Syrian hamster cells</i>
(2) JHU-1	Human fibroblast cells cultured from foreskin explants (Lin et al., 1980)	(5) A(T1)CL-3	A line of pseudodiploid fibrosarcoma cells (Benedict et al., 1975)
(2) MRC5	A diploid fibroblast cell strain derived from a normal male human donor (Jacobs, 1970)	(5) SH fibro	Cultures of fibroblast cells established by the reporting investigator and/or otherwise not referenced
(2) WI-38	A diploid fibroblast cell strain derived from a normal female human donor (Hayflick, 1965)	(6)	<i>Rat cells</i>
		(6) MCT1	Cultures of cells derived from a chemically induced tumor in a Sprague-Dawley rat
		(6) Rat fibro	Low passage cultures of rat fibroblasts established by the reporting investigator
		(6) Rat lymph	Peripheral blood lymphocyte cultures established by the investigator
		(6) RL1	An epitheloid cell line which was derived from rat liver and which retains enzymatic activity (Dean and Hodson-Walker, 1979)
		(6) RL4	An epitheloid cell line which was derived from rat liver and which retains enzymatic activity

(3)	<i>Human cells, other types</i>	
(3) CA-1	A tumor cell line derived from a human donor	(7) C3H10T1/2
(3) FL	An epithelioid cell line derived from human amnion and having a modal chromosome number of 63 (Fogh and Lund, 1957)	(7) FM3A
(3) HeLa	A line of epithelioid cells derived from a cervical carcinoma in a human donor (Gey et al., 1952)	(7) L cells
(3) Hum amn	A culture of amniotic cells having a normal human karyotype	
(3) VUP-1	A cell line from a malignant melanoma of the choroid in a human donor	
(4)	<i>Chinese hamster cells</i>	(7) L-929
(4) B14F28	A quasi-diploid line of fibroblasts derived from a Chinese hamster (see Yerganian and Leonard, 1961 for information on FAF28 cells, and Hsu et al., 1962 for notes on the B14FAF28 cells)	(7) L5178Y
(4) B241	A near-diploid line of fibroblast cells	(7) M10
(4) CH Fibro	Chinese hamster fibroblasts cultured by the reporting investigator and/or not otherwise referenced	(7) Mus fibro
(4) CH1-L	A fibroblast cell line derived from the liver of a young male	(7) Q31
(4) CHL	A clonal sub-line of fibroblasts derived from lung tissue (Koyama et al., 1970)	
(4) CHMP/E	A near-diploid clonal derivative of a cell line established from the prostate	
(4) CHO	A cell line derived from an ovary (Puck et al., 1958)	
(4) CHO-A7	A clonal derivative of CHO that is deficient in arginase activity and requires ornithine or polyamines (Holtta and Pohjanpelto, 1982)	(8) DH/SV40
(4) CHO-AT3-2	A line derived from CHO in which the cells are heterozygous at both the apt and tk loci (Adair et al., 1980)	(8) MA61
(4) CHO-CL-10	A clonal derivative of CHO cells having a modal chromosome number of 23	(8) MUNT fibro
(4) CHO-CL-H	A clonal derivative of CHO cells	(8) PTK-1
		<i>Mouse cells</i>
		A cell line derived from the ventral prostate of C3H mouse embryos, then cloned (CL8) to yield a line of near-tetraploid cells (Reznikoff et al., 1973)
		A line of cells derived from a C3H mouse mammary carcinoma which grows in suspension (Nakano, 1966)
		Cells taken from normal subcutaneous areolar and adipose tissue of an adult C3H/An mouse were cultured, then transformed in vitro by methylcholanthrene, cloned, and the clones tested for tumorigenic growth in vivo. Strain L cells are derived from one of these tumorigenic clones (Earle, 1942)
		A clonal derivative of L cells (Sanford et al., 1948)
		A line of murine leukemic lymphoblasts (See the reference cited as well as Fischer and Sartorelli, 1964)
		A line of mutant L5178Y cells which is sensitive to ionizing radiation and 4-nitroquinoline-1-oxide (Sato and Hieda, 1979a)
		Low passage cultures derived from the skin of newborn mice by the reporting investigator
		A line of mutant L5178Y cells which is sensitive to ultraviolet light and 4-nitroquinoline-1-oxide (Sato and Hieda, 1979b)
		<i>Cells from other species</i>
		Cultures of Djungarian hamster fibroblasts transformed by the SV40 virus
		Fibroblast cultures derived from the lung of the European vole, <i>Microtus agrestis</i>
		Fibroblast cultures derived from a male <i>Muntiacus muntjak</i> (Wurster and Bemirschka, 1970)
		A cell line derived from the kidney of a rat kangaroo, <i>Potorous tridactylis</i>

For references see Ishidate et al. (1988). Where no reference is cited, the reference given in Tables 3 and 4 should be used.

TABLE 6
 CHEMICALS WITH LOWEST EFFECTIVE CONCENTRATIONS (LEC) OF > 10 mM FOR CLASTOGENESIS IN MAMMALIAN CELLS IN VITRO
 Extracted from Ishidate et al. (1988).

No.	Test substance	CAS No.	Results	Cell type	MET ACT	LEC ($\mu\text{g}/\text{ml}$)	LEC (mM)	TRT (h)	REC (h)	A	m	C
9	Acetone	67-64-1	+	[4] CHL	-	40000	690	24	0	-	-	-
17	Acid red (C I acid red 52)	3520-42-1	+	[4] CHL	-	9000	15	48	0	-	-	-
22	Acrylic acid	79-10-7	+	[4] CHL	-	750	10	48	0	-	-	-
63	Aniline	62-53-3	+	[4] CHL	+	1000	11	3	21	-	-	-
92	Barbital	57-44-3	+	[4] CHL	-	2000	11	48	0	-	-	-
146	<i>N</i> -sec-Butyl- <i>N</i> -(methoxymethyl) nitrosamine	64005-63-6	+	[4] CHL	-	2000	14	48	0	-	-	-
238	Cochineal	1260-17-9	+	[4] CHL	-	12000	20	48	0	+	-	-
241	Creatinine	60-27-5	+	[4] CHL	-	10000	88	24	0	-	-	-
289	Diethanolnitrosamine	11116-54-7	+	[4] CHL	-	5000	37	24	0	+	-	+
291a	Diethylnitrosamine	55-18-5	+	[4] CHL	+	3000	29	3	21	+	-	+
b			+	[4] CHO	+	10200	100	3	24	-	-	-
343	8-Ethoxycaffeine	557-66-2	+	[4] CHO	-	4000	17	1	24	-	-	-
344	Ethyl acetate	141-78-6	+	[4] CHL	-	9000	100	48	0	-	-	-
354	Ethylene glycol	107-21-1	+	[4] CHL	-	26690	430	24	0	-	-	-
361	<i>N</i> -Ethyl- <i>N</i> '-nitroguanidine	39197-62-1	+	[4] CHL	-	2000	15	48	0	-	-	-
417	Glycodiazine	339-44-6	+	[4] CHL	-	8000	26	24	0	-	-	-
434	<i>N</i> -Hexane	110-54-3	+	[4] CHL	-	5150	60	3	21	-	-	-
526	Metformin hydrochloride	15537-72-1	+	[4] CHL	-	2000	12	48	0	-	-	-
534	<i>N</i> -Methyl- <i>N</i> -acetylaminoethyl nitrosamine	59665-11-1	+	[4] CHL	-	2000	15	48	0	-	-	-
537	<i>N</i> -Methyl- <i>N</i> '-acetylurea	623-59-6	+	[4] CHL	-	4000	34	48	0	-	-	-

540	4-Methylamino-antipyrène	519-98-2	+	[4] CHL	-	5 000	23	48	0
574	N-Methyl-N'-nitroguanidine	4245-76-5	+	[4] CHL	-	3 000	25	48	0
623	Nicotinamide	98-92-0	+	[4] CHL	-	3 000	25	48	0
637	Nitroguanidine	556-88-7	+	[4] CHL	-	2 000	19	24	0
689	L-Phenylalanine	63-91-2	+	[4] CHL	-	2 000	12	24	0
719	Potassium bromide	7758-02-3	+	[4] CHL	-	4 000	34	48	0
730 a	Potassium sorbate	24634-61-5	+	[4] CHL	-	4 000	27	48	0
b			+	[4] DON	-	3 000	20	26	0
738	Propylene glycol	57-55-6	+	[4] CHL	-	32 000	420	48	0
791	Saccharin sodium	128-44-9	+	[4] CHL	-	8 000	39	48	0
823	Sodium 5'-cytidilate		+	[4] CHL	-	30 000	82	24	0
827	Sodium D-tartrate	21 106-15-0	+	[4] CHL	-	15 000	87	48	0
833	Sodium 5'-inosinate	4691-65-0	+	[4] CHL	-	10 000	25	48	0
838	Sodium nitrate	7 631-99-4	+	[4] CHL	-	4 000	47	24	0
849	Sodium 5'-uridilate		+	[4] CHL	-	32 000	87	24	0
857	Streptomycin A	57-92-1	+	[7] FM3A	-	5 800	10	48	0
933 a	Urea	57-13-6	+	[1] Hum lymph	-	3 000	50	72	0
b			+	[4] CHL	-	12 000	200	24	0
934	Urethane	51-79-6	+	[4] CHL	-	8 000	90	48	0
946	Xylitol	87-99-0	+	[4] CHL	-	16 000	110	24	0

No. The number of the test substance used by Ishidate et al. (1988) in their listing of all substances tested in vitro. Substances with LEC values of > 10 mM in some cell types but < 10 mM in others are not included.

Cell type

See Table 5.

MET ACT + - with or without metabolic activation.

TRT duration of treatment (h).

REC recovery time (h).

A Ames test.

m micronucleus test in mice.

C carcinogenic in rodents.

TABLE 7
 CHEMICALS WHOSE LEC VALUES FOR CLASTOGENICITY IN VITRO IN PARTICULAR CELL TYPES ARE > 1, 5, 10, 20 or 50 mM
 All chemicals induce micronuclei or metaphase aberrations in vivo in the bone marrow (see Table 3) and have been tested for clastogenicity in vitro.

	Lowest effective concentration (mM)				Comments
	> 1.0	> 5	> 10	> 20	
2-Acetylaminofluorine				> 50	
CHL + (LEC = 2.2)					Difficult to activate? LEC = 0.13 mM in RL1
Barbital		Barbital	Barbital		
DON - (> 8)		DON -	CHL -		Not tested with activation or in other cell lines. Lowest effective dose (LED) in vivo = 330 mg/kg (Manna & Das, 1973)
CHL - (11)		CHL -			LEC = 0.11 mM in HL. Probably requires metabolic activation. Floats on medium; cultures must be agitated during treatment. LED in vivo = 0.125 mg/kg (Heddle et al., 1983)
Benzene		Benzene	Benzene		
CHO + (2.6)		CHL +	RL4 -		Detectable at 0.22 mM in RL4
CH1-L - (> 3.2)		RL4 -			Detectable at < 0.04 mM in HL
CHL + (7.6)					
RL4 - (> 12.8)					
Benzidine		Benzidine			
HL + (1.1)		CHO +			
CHO + (9.0)					
Busulphan		Busulphan			
CHL - (8.1)		CHL - (8.1)			
Cyclophosphamide					
RL1 - (3.8)					Activation required. Positive in several cell types at < 0.2 mM with activation.
Dimethylaminoazobenzene		Dimethylaminoazobenzene	Dimethylaminoazobenzene	Dimethylaminoazobenzene	LEC = 0.11 mM in CH1-L. Difficult to activate. LED in vivo = 185 mg/kg (Heddle et al., 1983)
CHO + (> 55)		CHO +	CHO +	CHO +	

Dimethyl nitrosamine CHL + (6.7) CHO + (27.0) HL + (50.0) SHF + (100.0)	Dimethyl nitrosamine CHO + HL + SHF +	Dimethyl nitrosamine CHO + HL + SHF +	Dimethyl nitrosamine SHF +	Difficult to activate? LED in vivo = 18.5 mg/kg (Heddle et al., 1983)
Ethyl methane-sulphonate CHL - (4.0)				LEC < 2.4×10^{-4} mM in other cell types
Ethylene oxide FL - (5.0)				
Hexamethyl-phosphoramide RL4 - (> 11.2) CHL - (33.5) CHO + (> 56)	Hexamethyl-phosphoramide RL4 - CHL - CHO +	Hexamethyl-phosphoramide CHL - CHO +	Hexamethyl-phosphoramide CHO +	LEC = 0.56 mM in HL and 0.01 mM in CH1-L. LED in vivo = 0.4 mg/kg.
Tetramethyl-benzidine CHO + (> 21)	Tetramethyl-benzidine CHO +	Tetramethyl-benzidine CHO +		Not tested in other cell lines. LED in vivo = 113 mg/kg (Heddle et al., 1983). In vivo data require confirmation
Tolbutamide CHL - (> 1.8)				Not tested in other cell lines
Urethane DON - (> 80) CHL - (90)	Urethane DON - CHL -	Urethane DON - CHL -	Urethane DON - CHL -	LEC = 225 mM in CHL + (Ishidate personal communication). LED in vivo = 180 mg/kg (Heddle et al., 1983)

The + or - sign after the designation of the cell type indicates + or - metabolic activation. LEC values for each cell type are given in brackets in the first column. Where LEC values are given, for example, as '> 10', 10 mM was the highest dose used and was not clastogenic. Where testing has been done with and without metabolic activation, the lower of the two LEC values has been tabulated. Where more than one test has been done with the same cell type the lowest LEC value has been tabulated. Lowest effective doses (LED) in vivo (micronuclei or metaphase aberrations) are given under 'Comments' for those chemicals with LEC values of > 10 mM in vitro in one or more cell types. In Table 3 the LEC for cyclophosphamide in CHL - is 11.0 mM; when tested with activation LEC = 0.04 mM (Ishidate, personal communication).

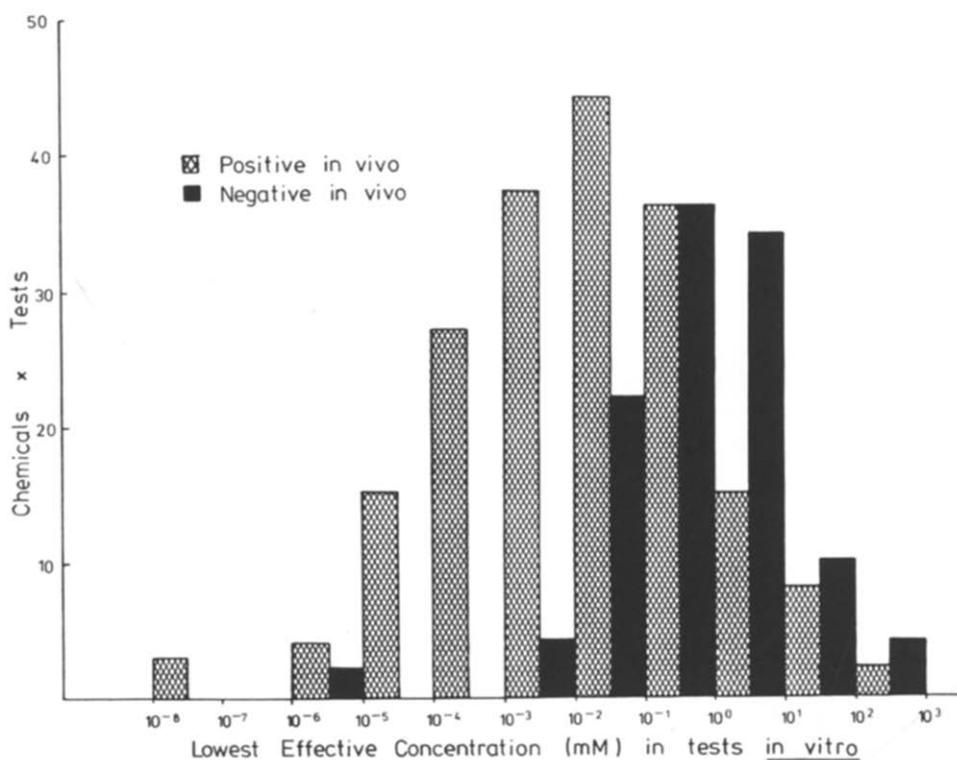


Fig. 3. Lowest effective concentration in *in vitro* tests for those chemicals which are clastogenic *in vivo* and those which are not. This diagram is simply a combination of data in Figs. 1 and 2 shown in histogram form to represent the number of tests in each LEC range.

trosamine and diethylnitrosamine) are DNA-reactive after metabolic activation. The clastogenicity of the remaining 5 chemicals (acid red, cochineal,

ethyl acetate, potassium sorbate and propylene glycol) may be related to medium osmolality (although this was not measured) but the positive

TABLE 8

CHEMICALS WHICH ARE CLASTOGENIC IN VITRO AT >10 mM IN AT LEAST ONE CELL TYPE AND NON-CLASTOGENIC IN VIVO

(extracted from Table 4)

Number	Name	Cell type	LEC (mM)	Ames test	Carcinogen	Comment
17	Acid Red	CHL	15	-		
162	Caffeine	WI-38	10			LEC = 1.3 mM in human lymphocytes
238	Cochineal	CHL	20	+		Complex mixture
289	Diethanolnitrosamine	CHL	37	+	+	DNA-reactive
291	Diethylnitrosamine	CHL	29	+	+	DNA-reactive
		CHO	100			
344	Ethylacetate	CHL	100	-		Non DNA-reactive
482	Isoniazid	CHL	15			LEC = 3.2 mM in FM3A cells
687	Phenobarbital	CHO	15			LEC <10 mM in other tests with CHO and in CH1-L
730	Potassium sorbate	DON	20	-		Non DNA-reactive
		CHL	27			
738	Propylene glycol	CHL	420	-		Non DNA-reactive

Ames test with cochineal (Ishidate, 1988) suggests some other mechanism, and the LEC of 15 mM for acid red is probably too low to induce osmotic stress (see Table 2). Thus, only a small part (perhaps around 5%; 3/68) of the discrepancy between *in vivo* and *in vitro* results can possibly be attributed to osmotic effects *in vitro*. Other reasons must be sought for this large discrepancy.

A further possible contribution to this discrepancy which relates to the concentrations used for *in vitro* testing is that even when clastogenesis is detected at relatively low concentrations *in vitro* these concentrations may not be achievable *in vivo* because they exceed the tolerance of the test animals. The clastogenicity of fluoride is an example. In extensive tests for the genotoxicity of fluoride *in vitro*, the lowest effective concentration (4.5 $\mu\text{g}/\text{ml}$) was for clastogenicity in human fibroblasts exposed to sodium fluoride for 48 h; a fairly clearcut threshold response was found at this concentration (Scott and Roberts, 1987). The 24 h oral LD_{50} dose in rats (30–50 mg/kg) gives a maximum plasma fluoride concentration of about 10 $\mu\text{g}/\text{ml}$ which is maintained for less than 4 h; by 24 h the concentration is only about 1.0 $\mu\text{g}/\text{ml}$ (DeLopez et al., 1976). Mice are more sensitive than rats to the acute lethal effects of fluoride (Lim et al., 1978) so maximum achievable plasma concentrations are likely to be lower. It is unlikely therefore that a clastogenic concentration of fluoride could be reached in a bone marrow metaphase or micronucleus test in mice; it is perhaps not surprising that such tests have been negative (e.g. Martin et al., 1983). If we assume that *in vivo*, in man, there are no cells which are more sensitive than the most sensitive cells *in vitro* then for this chemical there will be a large safety margin for human exposure because even in areas with fluoridated water supplies the steady-state plasma level is only around 0.05 $\mu\text{g}/\text{ml}$ (Singer and Ophawagh, 1979), some 100 times lower than the LEC in cultured human fibroblasts. There appears to be a similar high safety margin for human exposure to caffeine which also shows a threshold response for clastogenicity *in vitro* (Kihlman, 1977).

In spite of the fluoride and caffeine examples it is not always wise to disregard a positive *in vitro* response on the basis of dose dependency, using the argument that 'This concentration could never

be achieved *in vitro*', because:

(a) it would be necessary to demonstrate a true threshold response. For DNA-reactive agents there are very few examples of concentration thresholds for genotoxicity and indeed '... the central mechanism of chemical attack on DNA should in principle be a non-threshold process...' (Ehling et al., 1983). On the other hand, some genotoxic effects which result from mechanisms not involving direct DNA interaction might be expected to be of the threshold type. For example, chemicals which inhibit the enzymes involved in DNA synthesis and DNA repair may be clastogenic. If the inhibition is not rate-limiting at low concentrations, there being an excess of enzyme, the dose-response will be of the threshold type. Threshold responses may also occur when clastogenesis results from cellular energy depletion or from the production of active oxygen species when concentrations necessary to overwhelm cellular antioxidant defences may be required before DNA damage occurs. Examples of 'indirect' genotoxicity including those that might be expected to show threshold responses are given in Table 9. The extent of the dose-response studies required to satisfactorily demonstrate a threshold response for a particular chemical is very considerable (see Scott and Roberts, 1987). Confidence in the existence of a threshold must come primarily from an understanding of the mechanisms involved.

(b) the sensitivity of cultured mammalian cells may not adequately reflect the sensitivity of cells *in vivo* particularly when metabolic activation is required. The insensitivity of some *in vitro* tests relative to responses *in vivo* is clearly seen in Table 7. In addition, Ishidate et al. (1988) cite some striking examples of differences in sensitivity to particular chemicals between different types of cultured cells and between different protocols using the same cell type (see also Tables 3, 4 and 7 in this Report).

In the light of these considerations, from the viewpoint of the concentration dependency of *in vitro* clastogenesis it would appear a wise precaution to follow up all *in vitro* positive results, regardless of the doses required, with an *in vivo* assay. Possible exceptions are non-DNA-reactive agents which appear to be clastogenic only at concentrations > 10 mM and where there is a

TABLE 9
INDIRECT MECHANISMS OF GENOTOXICITY ^a

Mechanisms	Cellular target	Examples
Enzyme inhibition	Enzymes of DNA synthesis	Hydroxyurea, fluorodeoxyuridine, aphidicolin, 2-deoxyadenosine (Kihlman and Natarajan, 1984), methotrexate (Benedict et al., 1977)
	Enzymes of DNA repair	Cytosine arabinoside, aphidicolin, 3-aminobenzamide (Kihlman and Natarajan, 1984), arabinofluranosyladenine (Nichols et al., 1980)
	Topoisomerase I	Camptothecin (Degrassi et al., 1989)
	Topoisomerase II	m-AMSA ^b (Deaven et al., 1978), formaldehyde (Gaulden, 1987), VP-16 ^c (Palitti et al., 1990)
	Na ⁺ /K ⁺ ATPase	Ouabain (Wangenheim and Bolesfoldi, 1988)
Imbalance of DNA precursors	DNA precursors	DNA bases and nucleosides (MacPhee et al., 1988)
Energy depletion	Energy metabolism systems	Dinitrophenol (Mitchell and Simon-Reuss, 1952), cyanide (Kihlman, 1957; Umeda and Nishimura, 1979)
Production of active oxygen species	Oxygen and superoxide radicals	Paraquat (Nicotera et al., 1985), hydrogen peroxide (Tsuda, 1981)
Lipid peroxidation	Membranes	Phorbolmyristate acetate (Cerutti et al., 1983), asbestos (Cerutti et al., 1983), chromium chloride (Friedman et al., 1987)
Sulphydryl depletion	Sulphydryls	Diethylmaleate (Wangenheim and Bolesfoldi, 1988)
Nuclease release from lysosomes	Lysosomes	N-Dodecylimidazole (Bradley et al., 1987), hypotonic medium (Nowak, 1987)
Inhibition of protein synthesis	Nuclear proteins	Cycloheximide (Wangenheim and Bolesfoldi, 1988), Galloway et al. (unpublished data)
Protein denaturation	Nuclear proteins	Calcium hypochlorite (Isnidate et al., 1984), N-chloropiperidine (Ashby et al., 1987)
Ionic imbalance	Chromatin? Enzymes?	Ethylenediaminetetraacetic acid, salts of saccharin, nitrotriacetic acid, secalonin acid (Ashby, 1985), hypertonicity (Section 2.1)
?	?	Fluoride (refs. in Scott and Roberts, 1987)
?	?	Low pH (Section 5)

^a The examples given are for clastogenesis except for ouabain, cycloheximide and diethylmaleate which induce 'mutations' at the thymidine kinase locus in L5178Y mouse lymphoma cells which may be point mutations or clastogenic events (Applegate and Hozer, 1987). Genotoxicity from hypertonic treatment (Table 2), low pH (Table 14) and fluoride (references in Scott and Roberts, 1987) also includes endpoints other than clastogenesis.

^b m-AMSA, N-[4-(acridinylamino)-3-methoxyphenyl]methanesulphonamide.

^c VP-16, an epipodophyllotoxin.

measured increase in the osmolality of the culture medium of > 50 mOsm/kg.

2.3. Extrapolation from *in vitro* genotoxicity for endpoints other than clastogenesis

Only for clastogenesis is there a sufficient database *in vivo* and *in vitro* to seriously address the question of upper concentration limits for *in vitro* testing which will detect all *in vivo* clastogens. Although the accumulated data on mutations at the HPRT locus in Chinese hamster cells is now considerable (Li et al., 1988) very few of these chemicals have been tested for HPRT mutagenesis *in vivo*. An alternative, indirect, approach has been to compare *in vitro* mutagenesis with rodent carcinogenicity but it is important to bear in mind that there are mechanisms of carcinogenicity that do not involve mutation or indeed any form of genotoxicity (Butterworth and Slaga, 1987). In comparing dose-response data for mutations at the thymidine kinase (TK) locus in mouse lymphoma cells with rodent carcinogenicity for the same chemicals, Wangenheim and Bolcsfoldi (1988) noted that virtually all rodent carcinogens were detected *in vitro* at less than 20 mM and recommend this as the upper concentration limit.

2.4. Summary and recommendations

There is increasing evidence of genotoxicity at very high concentrations as a consequence of the elevated osmolality of the culture medium. Such effects are unlikely to be of relevance to human exposure. To avoid this problem in clastogenicity tests an upper concentration limit of 10 mM has been suggested. However, some *in vivo* clastogens are only detectable *in vitro* at high concentrations; this may in part reflect the inadequacy of metabolic activation systems. Nevertheless, we conclude that if an upper concentration limit of 10 mM is used for testing, very few chemicals which are capable of *in vivo* clastogenesis in rodent bone marrow will be missed. We therefore recommend the use of an upper concentration of 10 mM in clastogenicity tests *in vitro* to avoid the artefacts associated with higher concentrations. Of the 50% of tested chemicals which are clastogenic *in vitro* but not *in vivo* probably less than 5% are clastogenic through osmolality effects. Other reasons

must be sought for the large discrepancy between *in vivo* and *in vitro* responses. Even relatively low LECs *in vitro* may be higher than can be achieved *in vivo* with toxic chemicals. However, to be sure that an *in vitro* clastogen would be ineffective *in vivo* it is necessary to demonstrate a threshold response at a concentration well above that which could be achieved *in vivo*. Confidence in the existence of a true threshold must come primarily from an understanding of the mechanisms involved.

3. Genotoxicity and cytotoxicity

3.1. Introduction

Various guidelines for *in vitro* genotoxicity testing recommend inclusion of cytotoxic dose levels (see Table 1 for clastogenicity tests), with the intention of demonstrating a biological response in the system. This raises the question of whether genotoxic effects observed *in vitro* only at cytotoxic concentrations are relevant to the situation *in vivo* where cytotoxicity is likely to be less well tolerated and, in particular, to human experience where exposure concentrations will usually produce no, or minimal cytotoxic effects. If some degree of cytotoxicity is acceptable, can we define upper limits for *in vitro* test which would allow detection of all (or most) known *in vivo* genotoxins, as we have attempted for concentration dependency in Section 2? The aim would be to exclude chemicals which are genotoxic only above a certain level of cytotoxicity which could not be achieved *in vivo*.

3.2. Direct and indirect genotoxicity and cytotoxicity

The relationships between genotoxicity and cytotoxicity are complex. Apart from the wide spectrum of genotoxic endpoints (e.g. gene mutation, chromosome aberrations, sister-chromatid exchanges, DNA-strand breaks) there are many different *in vitro* assays of cytotoxicity. Some are measurements of cell death such as loss of colony-forming ability or cell lysis, whereas others are not necessarily associated with lethality, e.g. growth inhibition, cell-cycle delay, reduction in mitotic index and metabolic changes. These are dealt with in detail in Section 3.3.

RELATIONSHIPS BETWEEN DIRECT AND INDIRECT GENOTOXICITY AND CYTOTOXICITY

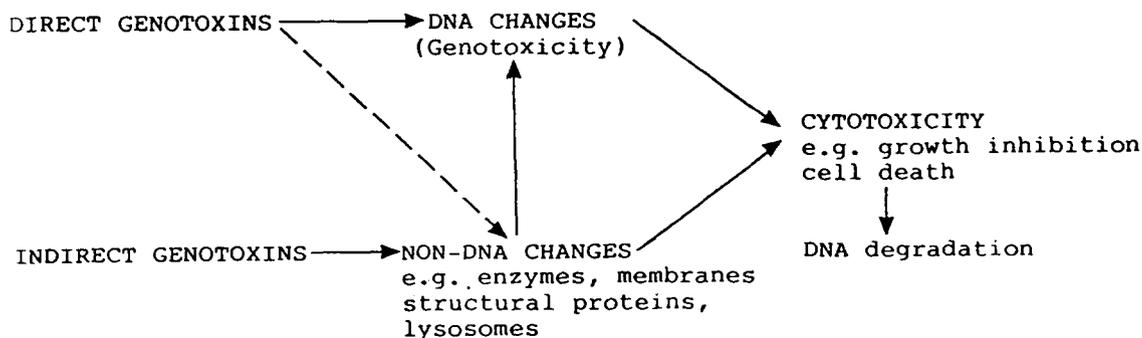


Fig. 4. Relationships between direct and indirect genotoxicity and cytotoxicity.

Genotoxic effects can result from *direct* interaction of chemicals with DNA through covalent binding or intercalation. These chemicals can induce a spectrum of DNA lesions that differ in their propensity to cause different genotoxic effects and in their susceptibility to repair (Swensen et al., 1980; Heflich et al., 1982; Liu-Lee et al., 1984; Natarajan et al., 1984). Gene mutations arise during the repair (misrepair) or replication (misreplication) of DNA carrying such lesions. More extreme alterations to the genome such as DNA-strand breakage and structural chromosome aberrations can also arise in this way. In addition, however, such gross changes can also be brought about without direct DNA interaction *via* a wide variety of *indirect* mechanisms (Table 9, Fig. 4). These include interference with the processes of DNA replication and repair; interaction with specific chromosomal non-histone proteins such as topoisomerase II, and with peripheral proteins; nuclease release from lysosomes; protein denaturation and the production of active oxygen species. Other methods of inducing these indirect effects by less well-understood mechanisms are through cellular energy depletion, pH changes (Section 5), tonicity changes (Section 2) and hyperthermia.

Since a significant proportion of chemicals that are genotoxic *via* indirect mechanisms probably exhibit threshold responses (Section 2.2.2), indirect genotoxins are likely to be of less importance than direct genotoxins from the point of view of human risk. Nevertheless, there are some indirect

genotoxins that are active *in vivo*; for example, cycloheximide induces micronuclei in rodent bone marrow (Basic-Zaninov et al., 1987; Gulati et al., personal communication) and methotrexate is clastogenic in mice (Maier and Schmid, 1976) and in man (Jensen and Nyfors, 1979).

Agents which are genotoxic by either direct or indirect mechanisms are also cytotoxic. Cytotoxicity will result from the damage to the DNA itself (e.g. DNA-strand breakage and structural chromosome aberrations) and to other cellular targets (e.g. enzymes, membranes). For some agents the cytotoxicity will be expressed mainly through effects on DNA, whether induced directly (e.g. alkylating agents) or indirectly (e.g. DNA synthesis inhibitors) whereas for others the toxicity will be mediated mainly through damage to non-DNA targets (e.g. agents which induce lipid peroxidation, energy depletion, protein denaturation or ionic imbalance). A simplified scheme of the relationships between direct and indirect genotoxicity and cytotoxicity is shown in Fig. 4.

A mechanism of genotoxicity which can be considered as intermediate between direct and indirect mechanisms is when base analogues become incorporated into DNA at the time of DNA synthesis and induce chromosomal aberrations (e.g. bromodeoxyuridine; Hsu and Somers, 1961). For simplicity this mechanism is not shown in Fig. 4.

DNA changes that take place whilst cells are dying will not, of course, constitute a genetic hazard. DNA degradation in association with

necrosis in cultured mammalian cells has been reported after treatment with a variety of agents both genotoxic and non-genotoxic (Williams et al., 1974; Afanas'ev et al., 1986). A distinction must be made between induced DNA damage which is not necessarily cell lethal and is therefore potentially mutagenic (in the broadest sense) from DNA degradation which occurs in dying cells. This distinction is made in Fig. 4. The highly fragmented chromosomes in mitotic cells sometimes seen in clastogenicity assays at doses which induce significant cell death may be a manifestation of DNA degradation although it is important to note that several chemicals tested up to high levels of toxicity by Galloway et al. (1987b) were non-clastogenic, so chromosome aberrations are not an inevitable consequence of toxicity.

3.3. Assays of cytotoxicity

In genotoxicity tests various methods are used to assess the associated cytotoxicity induced by the test chemicals. As indicated above, some of these measure cell death, but other endpoints are also used.

The manifestations of cell death include:

(a) Loss of membrane integrity

This can be detected by using vital dyes and enzyme assays to detect membrane leakage (Roper and Drewinko, 1976). There are, however, problems of accurately quantifying cell death by this method because cells which are destined to die do not necessarily exhibit membrane damage immediately after treatment so the proportion of

TABLE 10

CYTOTOXICITY AND CHROMOSOME ABERRATIONS IN CHO CELLS TREATED WITH MITOMYCIN C OR ADRIAMYCIN

Armstrong et al., in preparation.

Chemical	Concentration (μ M)	Sampling time										CFE% (7d)
		10 h					24 h					
		Cell count ^a cell ^b	ATP/ cell ^b	MI ^c	Aberrations ^d		Cell count	ATP/ cell	MI	Aberrations		
			Abnormal cells (%)	per 100 cells				Abnormal cells (%)	per 100 cells			
Mitomycin C	0	100	100	15.9	3	3	100	100	10.5	4	4	100
	0.10	103	100	11.6	6	6	101	101	11.1	6	6	109
	0.25	106	105	7.4	11	13	93	106	13.8	14	15	96
	0.50	99	107	5.4	9	9	96	97	10.5	40	63	89
	0.75	105	98	7.5	16	20	78	127	16.2	74	140	75
	1.00	89	125	5.2	26	28	69	139	16.1	88	340	48
	2.00	99	112	4.2	31	38	57	177	10.0	100	530	10
	4.00	97	104	3.9	37	58	67	135	6.4	-	-	2
Adriamycin	0	100	100	20.9	2.0	2.0	100	100	13.3	3.0	3.5	100
	0.10	88	100	19.1	17.5	20.0	91	101	15.8	12.5	13.5	91
	0.25	80	106	8.9	70.0	114.0	82	99	16.7	20.5	35.0	92
	0.50	71	102	5.2	90.0	196.0	66	96	15.4	66.0	146.0	49
	0.75	71	98	1.6	100.0	580.0	55	91	10.7	82.0	240.0	9
	1.00	68	91	0.3	-	-	38	87	12.1	-	-	4
	2.00	66	93	0.4	-	-	33	91	11.1	-	-	0

Cells were treated for 3 h and sampled at 10 h and 24 h.

^a Total viable cells as % of controls.

^b ATP per cell as % of controls, measured by luminescence.

^c Mitotic index based on 1000 cells scored.

^d Aberration yields based on 200 cells scored except at high yields.

damaged cells will be different at different post-treatment sampling times. Also, membrane-damaged cells that lyse and disintegrate will not be included in the cell sample tested for viability; cell death will thus be underestimated unless a total cell count is compared to controls. Nevertheless, this method is useful when test cells are non-proliferating (as in some DNA damage/repair assays) or, for other reasons, cannot be evaluated for colony-forming efficiency.

(b) Loss of colony-forming efficiency (CFE)

This method is routinely used in mammalian cell mutation assays and sometimes in conjugation with other tests on clonogenic cells. It has the merit that, provided sufficient time elapses between treatment and observation to allow for colony-formation by surviving cells (usually 1 week or more), the surviving fraction remains constant thus giving a single numerical value.

Other assays, which do not necessarily measure cell death are:

(a) Reduction in cell numbers

Typically, cell counts in control and treated samples are made at 1 or 2 days after treatment. Reduction in cell number relative to controls will result both from cell lysis and, in proliferating cell populations, from a decreased growth rate. The ratio of cell numbers in control and tested samples will vary with sampling time (see Table 10). If cell counts are continued, in proliferating populations, well beyond the time when dead cells have been eliminated, a true estimate of cell killing can be obtained from back extrapolation of growth curves (Alexander and Mikulski, 1961) but this method is seldom used in genotoxicity tests because of its time-consuming nature.

(b) Mitotic index (MI)

In cytogenetic assays, doses are often chosen which induce some degree of inhibition of MI as an indication of biological responses. With typical control MI values of 5–10%, a 75–80% reduction in MI defines the upper concentration limit since at higher doses insufficient cells would be available for analysis. MI values can vary markedly at different times after treatment and may even in-

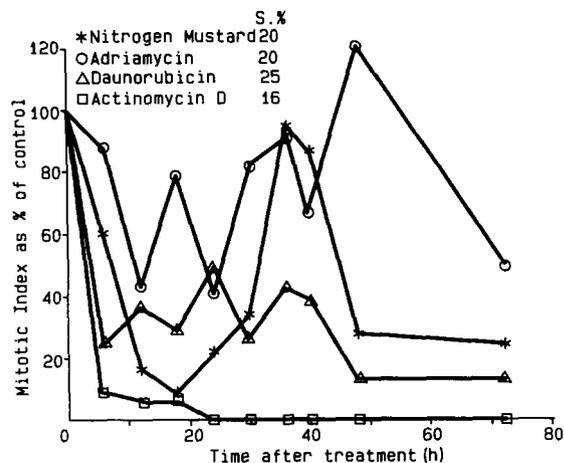


Fig. 5. Mitotic index as a percent of the untreated controls after treatment of asynchronous human fibroblasts with the clastogens, nitrogen mustard (0.2 $\mu\text{g/ml}$), adriamycin (0.25 $\mu\text{g/ml}$), daunorubicin (0.1 $\mu\text{g/ml}$) or actinomycin D (0.2 $\mu\text{g/ml}$) for 1 h. These doses induced a similar degree of loss of colony-forming efficiency which was assayed in parallel. S = survival (Data from Parkes and Scott, 1982)

crease above control levels (see Fig. 5 and Table 10) if chemicals induce partial synchronisation or cause arrest of cells in mitosis.

Depression of the MI is usually a consequence of a reduced rate of cell proliferation (mitotic delay) but there may also be a contribution from cells which have permanently lost their proliferative capacity.

(c) Reduction in metabolic activity

This may be detected by measurements of ATP levels (e.g. Garrett et al., 1981; Garewal et al., 1986) or changes in dyes which require mitochondrial energy production (e.g. thiazolyl blue; Carmichael et al., 1987). Again, the extent of the reduction will vary as a function of time after treatment. The ATP content per cell can actually increase (Table 10) so that alterations are difficult to interpret. Suppression of ATP levels does not necessarily indicate cell death, as cells can recover from such depletion.

(d) Quantitative relationships between various endpoints of cytotoxicity

These relationships are complex (see Weisenthal et al., 1983; Roper and Drewinko, 1976). For example, for a series of chemicals, the dose levels

gens in rodents. The induction of CA was measured at 10 and 24 h after treatment of Chinese hamster ovary (CHO) cells and concurrent measures of cytotoxicity were made in terms of cell counts, mitotic index, ATP content and CFE. We will use some of these data to illustrate the complexity of relationships between clastogenicity and cytotoxicity and the difficulty of using this information to predict *in vivo* response.

These investigations show that, typically, for a given chemical, clastogenicity/cytotoxicity relationships differ not only according to sampling time, but also according to the particular endpoint of cytotoxicity. For example (Table 10) at 10 h after mitomycin-C (MMC) treatment CA are observed without any reduction in cell count or ATP levels (e.g. at 0.25–0.50 μM), but with a substantial suppression in MI and some reduction in CFE (measured at 7 days). In contrast, at 24 h (Table 10, Fig. 6) very high aberration yields are seen at concentrations (e.g. 0.75–1.00 μM) at which there is actually an *increase* in MI, following the suppression at 10 h. Cell counts are also reduced at 24 h. At high concentrations of MMC the clastogenicity/cytotoxicity ratios are very different for the different cytotoxic endpoints. For example, at 1.00 μM (see left hand column in Fig. 6), CFE was reduced by 52% compared with untreated cells, cell counts at 24 h by 32% and ATP levels by 4% whereas the MI at 24 h *increased* by 53% (shown as negative cytotoxicity on the abscissa). These ratios are, unusually, less variable with 2-aminobiphenyl (2-ABP), which is a weaker clastogen than MMC at any given level of cytotoxicity (Fig. 6). Incidentally, although there appears to be a threshold in the clastogenesis/cytotoxicity ratio for 2-ABP this was not confirmed in subsequent experiments using a series of sampling times for CA (Bean and Galloway, personal communication).

For the reasons given previously, the most meaningful comparison of clastogenicity and cytotoxicity is when cell killing, assayed as loss of CFE, is used as the measure of the latter. This relationship is shown in Fig. 7 for 7 chemicals studied by Armstrong, Galloway et al. The 24-h CA data are used for all chemicals other than adriamycin (Table 10) because for the other 6 chemicals the yields were higher at 24 h than at 10

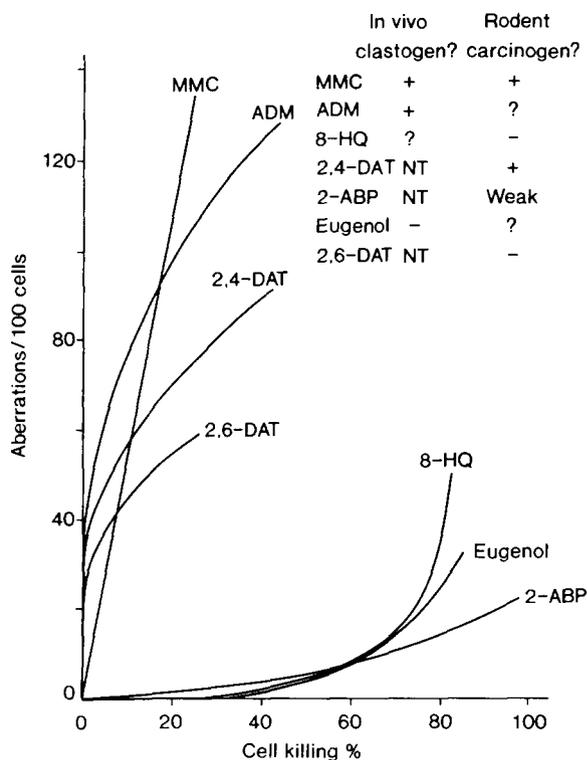


Fig. 7. Relationship between clastogenicity and cell killing (loss of CFE) in CHO cells exposed to mitomycin C (MMC, 0.25–4.00 μM), 2,4-diaminotoluene (2,4-DAT, 1.0–10.0 mM), 2-aminobiphenyl (2-ABP, +S9, 1.0–1.2 mM) or 2,6-diaminotoluene (2,6-DAT, 10–18 mM). Aberration yields are for cells fixed at 24 h and control frequencies have been subtracted. CFE was measured at 7 days. (Armstrong, Galloway et al., personal communication)

h at most concentrations. It should be borne in mind, however, that accurate quantification of CA yields requires multiple sampling times when an asynchronous cell population is used since the patterns of yield versus time may differ for different chemicals (see Parkes and Scott, 1982). The yields at 24 h (or 10 h for adriamycin) are not necessarily the maximum frequencies induced by these chemicals; the peaks may be at different times. Bearing in mind this limitation it appears that these chemicals differ markedly in potency (Fig. 7) which, in this context, is the ratio of clastogenesis to cell death. Four of the chemicals [MMC, adriamycin (ADM), 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT)] were very potent in that they induced substantial

aberration frequencies (> 20 per 100 cells) at concentrations which produced little or no reduction in CFE. Clearly, CHO cells can tolerate a certain amount of structural chromosome damage without loss of viability. In contrast, the remaining 3 chemicals [8-hydroxyquinoline (8-HQ), eugenol and 2-aminobiphenyl (2-ABP)] were weak clastogens, inducing very low aberration frequencies at concentrations which reduced CFE by up to 50%.

Another notable difference among the clastogens was in the distribution of aberrations between cells. Whereas MMC and ADM produced a general increase in the proportions of cells with aberrations, only relatively low percentages of cells had aberrations after treatment with 2,4- and 2,6-DAT, or 8-hydroxyquinoline, but many of the cells that were damaged had multiple aberrations. This is important for several reasons. In testing chemicals for clastogenicity, many investigators report data only for the percentages of cells with aberrations. Clearly, since cells with multiple aberrations are rare in controls, the existence of such cells even when the percentage of aberrant cells is modest, is supporting evidence that the test compound is clastogenic. Also, a delayed harvest time is required to detect these cells (in the case of CHO cells, this was 24 h from the beginning of the 3-h treatment). Finally, this type of chromosome damage may be produced by indirect mechanisms (Fig. 4), perhaps in a subset of cells in a particular phase of the cell cycle. If this is the case then, at least for some chemicals, indirect clastogenesis is not necessarily associated with extreme cytotoxicity to the whole cell population; for example, the survival level at which cells with multiple aberrations were detected was 80% or more after treatment with 2,4- or 2,6-DAT. Cells suffering extreme chromosome damage are likely to lose their proliferative capacity but less severely damaged cells may not, and could constitute a long-term genetic hazard.

Can *in vitro* potency (clastogenicity with respect to cytotoxicity) be used to predict *in vivo* response? Of the 7 chemicals studied by Armstrong et al., only 4 have been tested for clastogenicity in rodent bone marrow. MMC is widely used as a positive control because of its clastogenicity in bone marrow cells of the mouse; it also induces aberrations in bone marrow cells of monkeys

(Michelmann et al., 1978). ADM also induces aberrations in mouse bone marrow (e.g., Au and Hsu, 1980) and in human lymphocytes *in vivo* (Nevstad, 1978). MMC and ADM are both potent clastogens in CHO cells (Fig. 7). Eugenol induced aberrations *in vitro* at doses that reduced CFE and was reported negative *in vivo*, in a rat bone-marrow micronucleus assay (Maura et al., 1989). CA induction by 8-hydroxyquinoline (8-HQ) was only detectable at concentrations that caused a 75% reduction in CFE and a 50% reduction in cell count at 24 h. *In vivo* results with 8-HQ are marginal; McFee (1989) observed a small but non-significant increase in chromosome aberrations in mouse bone marrow, but Hamoud et al. (1989) detected small, but significant increases in bone-marrow micronuclei, particularly in normochromatic erythrocytes. 8-HQ is not a rodent carcinogen (Ashby and Tennant, 1988). If 8-HQ is truly clastogenic *in vivo*, it implies that weak clastogenicity *in vitro* detected at doses which are substantially cytotoxic cannot necessarily be discounted. Support for these conclusions comes from another study (Galloway et al., 1987b) in which at least 8 out of 24 clastogens were detectable in CHO cells only at dose levels that caused measurable cytotoxicity, assayed as a reduction in cell numbers ('reduced monolayer confluence') at 10–20 h after treatment, which was the time of harvesting for aberration analysis. *In vivo* data are available for only 2 of these 8 chemicals. Of these two, 2,4,5-T did not induce micronuclei *in vivo* (Davring and Hultgren, 1977; Jenssen and Ramel, 1980) whereas malathion reportedly induced aberrations in mouse bone marrow (Doulout et al., 1983) although this was not reproduced in another study using similar test conditions (Degraeve and Moutschen, 1984). The reduction in cell count/confluence *in vitro* for malathion was approximately 50%. Malathion is not carcinogenic in rodents (Ashby and Tennant, 1988). In summary, concentrations that are quite cytotoxic *in vitro* (e.g. $\geq 50\%$ cell killing, Fig. 7) are sometimes required to detect chemicals that are clastogenic *in vivo*.

A further difficulty in attempting to extrapolate from *in vitro* tests to the situation *in vivo* is that clastogenesis/cytotoxicity ratios *in vitro* may differ considerably between different cell types. For

TABLE 11
RELATIONSHIPS BETWEEN CHROMOSOME ABERRATION YIELDS AND CELL KILLING (LOSS OF CFE) IN HUMAN SKIN FIBROBLASTS TREATED *IN VIVO* WITH ANTI-TUMOUR AGENTS^a

From Parkes and Scott (1982).

Chemical	Concentration ^b (μ M)	Aberrations per 100 cells			CFE %
		Peak ^c	24 h ^d	Mean ^e	
Nitrogen					
mustard	1.0	10.2 (36 h)	0	4.6	20
Adriamycin	0.45	11.0 (6 h)	3.0	3.9	20
Daunorubicin	0.15	8.5 (6 h)	2.0	4.4	45
Daunorubicin	0.20	44.0 (6 h)	2.0	11.8	25
Daunorubicin	0.30	100.0 (6 h)	16.0	15.2	13

^a Mitotic index data given in Fig. 5.

^b Treatments were for 1 h.

^c The time of maximum aberration frequency is given in brackets.

^d Yield at 24 h given because this is a sampling time regularly used in clastogenicity tests.

^e The mean yield between 6 and 48 h post-treatment.

example, Parkes and Scott (1982) examined in detail the relationship between chromosome aberration yields, CFE and MI in cultured human skin fibroblasts treated with anti-tumour agents which are clastogenic in human lymphocytes or bone-marrow cells *in vivo* (Table 11). Aberration yields were determined at 6 hourly intervals from 6 to 48 h after treatment. Although cell killing levels ranged from 55 to 87%, the average aberration yield over the entire sampling period did not exceed 15 aberrations per 100 cells. At 24 h, a time commonly used in testing, a direct comparison is possible between human fibroblasts (Table 10) and CHO cells (Table 11) in their response to adriamycin. The aberration yield at 20% survival in human fibroblasts was only 3% and the MI was reduced to about 40% of the control value (Fig. 5). In striking contrast, at the lowest survival level achieved in the assay in CHO cells, 32% CFE, the CA yield was 85 per 100 cells and there was actually an increase in MI relative to the control value. In human fibroblasts even at the peak frequency, at 6 h, the CA yield was only 11% at 20% survival. In another study in human fibroblasts exposed to MMC, only a 20% CA yield was observed at 80–90% cell killing and a 50% reduc-

tion in MI after a continuous 48-h exposure (Scott and Roberts, 1987). This level of chromosome damage was achieved with virtually no loss of viability in CHO cells (Table 10, Fig. 7) and with no reduction in MI (at least 24 h, Table 10). Clearly, if human fibroblasts are used in cytogenetic testing, then for these classes of chemicals highly cytotoxic concentrations must be used to detect clastogenic activity. In fact, human fibroblasts are rarely used, but human lymphocytes are, and it would be of value to examine clastogenesis/cytotoxicity ratios *in vitro* in this system for chemicals which are clastogenic *in vivo*.

One aim of the investigation of Armstrong, Galloway et al. was to examine clastogenesis/cytotoxicity relationships *in vitro* for chemicals whose rodent carcinogenicity is known (see key to Fig. 7). MMC is a rodent carcinogen (Ikegami et al., 1967) as is 2,4-DAT (cited by Ashby and Tennant, 1988). 2-Aminobiphenyl is a 'weak' carcinogen, classed as questionable by Lewis and Tatken (1979), and as negative in rats but positive in female mice and equivocal in male mice, by Hasemann et al. (cited in Ashby and Tennant, 1988). Eugenol is an equivocal carcinogen (rodent liver) and 8-HQ is a non-carcinogen (Tennant et al., 1987). As discussed above, for the rodent carcinogens MMC and 2,4-DAT, *in vitro* clastogenicity was detected with little or no cell killing, whereas for the weak carcinogen 2-ABP, the equivocal carcinogen eugenol and the non-carcinogen 8-HQ, concentrations that caused substantial acute killing or reductions in CFE were required to detect chromosome aberrations *in vitro*. At first glance, it might seem that the stronger carcinogens induced aberrations *in vitro* at less toxic doses than the weak or non-carcinogens. However, the non-carcinogen 2,6-DAT (Tennant et al., 1987) was only mildly cytotoxic at clastogenic doses *in vitro*. [Note that this was a case where concentrations above 10 mM were required *in vitro* to detect aberrations at the 24-h sampling time; the osmolality was not markedly increased. Gulati et al. (1989) were able to detect higher frequencies of aberrations with 2,6-DAT·HCl at 7.7 mM, at a 17-h sampling time in CHO cells.] It is clear, therefore, that clastogenic non-carcinogens can exert their effect without overwhelming toxicity, and cannot be distinguished

from clastogenic carcinogens on the basis of cytotoxicity alone.

There is need to examine, in detail, the relationships between the *type* of chromosome damage induced by chemicals in *in vitro* tests and the *in vivo* response. In preparing their review of *in vitro* clastogens, Ishidate et al. (1988) found that such information was often missing from published reports so they attempted such an analysis using only data for CHL cells from their own studies. In general they found that chemicals which were most efficient in inducing exchange-type aberrations were more likely to be rodent carcinogens than chemicals which induced predominantly gaps and breaks. [This may be because chromosomal deletions are more likely to be cell lethal than exchange events, since exchanges of the symmetrical type involve no loss of chromosomal fragments at mitosis and have now been clearly implicated in malignancy (Heim and Mitelman, 1987). Also, gaps may not reflect genomic damage relevant to carcinogenicity.] There was also a tendency for substances which were active only at high concentrations to induce more gaps and breaks than exchanges. However, there are exceptions; urea has an LEC value of 200 mM (12 mg/ml) in CHL cells but induces a high frequency of exchanges (Ishidate, 1988) as does sodium chloride in CHO cells at concentrations above 200 mM (Galloway et al., 1987a). Further evaluation of aberration type in relation to *in vivo* response is needed in different cell types and at different sampling times, with various types of clastogen (direct and indirect) before any general conclusions can be drawn.

From our consideration of the very limited quantitative data relating clastogenicity to cytotoxicity *in vitro*, it follows that, in our present state of knowledge, this relationship cannot be used to predict *in vivo* response. There is simply insufficient information to determine if there should be an upper limit of cytotoxicity for *in vitro* testing and, if so, what the upper limit should be. However, in the two examples discussed above, malathion and 8-HQ, for which positive results in CHO cells were obtained only at toxic levels and for which *in vivo* clastogenicity had been demonstrated, a reduction in cell growth of up to 50% (at the time of harvest for aberration analysis) was required to detect aberrations. For 8-HQ, CFE

was reduced by 75% at clastogenic doses. In practice, the upper limit of testing in fibroblasts or lymphocytes is determined by the degree of reduction in the mitotic index at the chosen sampling time(s). With a typical MI of 5–10% a reduction of 75% is probably the maximum tolerable to give sufficient cells for analysis.

3.6. Cytotoxicity and mammalian cell mutation assays

For mutation assays, published guidelines generally recommend that the assay extend into the cytotoxic range. The OECD guidelines (1983) specify that mutation assays should extend to 'very low survival'. In the U.S. E.P.A. Gene-Tox Committee reports on mutation assays, upper limits of doses giving not less than 10% survival were recommended for mutations at the TK locus in L5178Y mouse lymphoma cells (Clive et al., 1983) and HPRT mutations in Chinese hamster ovary (CHO) cells (Hsie et al., 1981). For the HPRT mutation assay in Chinese hamster V79 cells (Bradley et al., 1981) a lower survival limit of 1–10% was suggested, but the authors pointed out the problems of trying to detect mutations in the small samples of cells remaining at survival levels below about 10%. Clearly, estimation of mutation frequency can become unreliable at very low survival (e.g. < 10%), because of the small sample size and increasing variability. Also, pre-existing mutants may be selected because very slight differences in growth efficiency between mutant and wild type cells can have disproportionately large effects on final mutation frequency because by chance alone one can obtain an entirely spurious increase in mutations if a clone of mutant cells outgrows the rest of the depleted culture.

The reproducibility of results at high toxicity in the TK-locus mutation assay in mouse lymphoma cells has been discussed recently by Caspary et al. (1988). Several authors of papers on this system argue that results are unreliable with relative total growth (RTG) at less than 5% (Oberly et al., 1984), 10% (Clive et al., 1983) or 20% (Amacher et al., 1980). RTG is the product of the relative growth in suspension in the two-day expression period, and the subsequent cloning efficiency in soft agar which is done in parallel with the mutant selection (see Mitchell et al., 1988). Caspary et al.

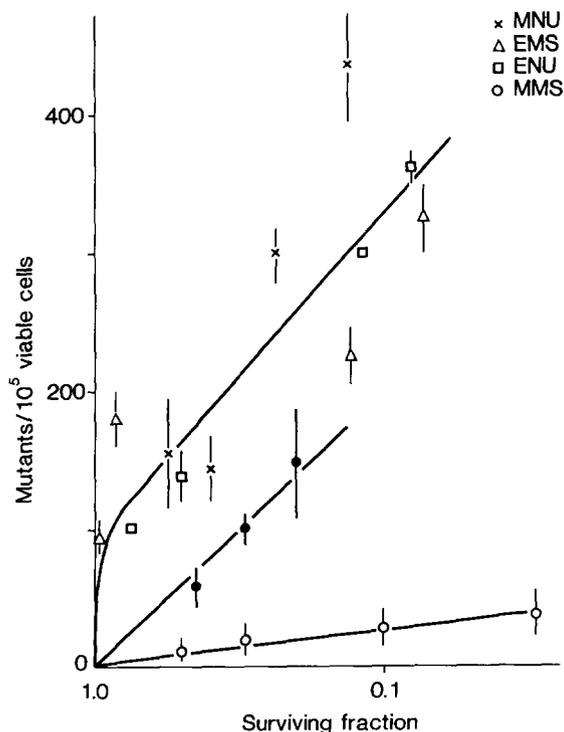


Fig. 8. Relationship between induced frequency at the HPRT locus and cell survival in V79 Chinese hamster cells. (Fox, 1980)

(1988a) analysed the variability in their data on 800 experiments with L5178Y cells, and examined the effect of toxicity on variability between replicate cultures, by comparing the coefficients of variation at all levels of toxicity using the three measures available; growth at one day and at two days, and cloning efficiency (CE). They concluded that under their protocol it was statistically valid to use results of mutation assays with RTG as low as 1%, provided that the CE was not less than 10% and that the observed increase in mutants per survivor was supported by an absolute increase in the number of mutants.

Mutagenicity/cytotoxicity ratios differ greatly for different mutagens (e.g. Carver et al., 1979, 1983; also Fig. 8). For example, methyl methane-sulphonate (MMS) is known as a 'toxic mutagen' and ethylnitrosourea (ENU) as a potent mutagen with low cytotoxicity. A database is lacking that would allow comparison of in vitro and in vivo mutation data, since in vivo measurement of

somatic mutations, e.g. at the HPRT locus, has been applied to very few compounds. For in vivo mutation assays, mutagens that have proven most effective are specifically chosen for lack of toxicity, e.g. ENU, which is a potent mouse germ cell mutagen (Russell et al., 1979) and the only chemical mutagen for which in vivo mouse lymphocyte HPRT mutation data are available (Burkhart-Schultz et al., 1989). Some assessments have been made, however, of in vitro mutagenic potency (in terms of cytotoxicity) compared with carcinogenicity in rodents, for the thymidine kinase locus (TK mutations) in L5178Y cells which detects both point mutations and clastogenic events (Aplegate and Hozier, 1987).

Data were examined from a study of a large series of compounds tested under the U.S. National Toxicology Program many of which are described by Mitchell et al. (1988) and Myhr and Caspary (1988) (Figs. 9 and 10, data kindly provided by W. Caspary, N.I.E.H.S.). Fig. 9 shows data for positive mutation assays; the RTG at the lowest dose that gave a detectable mutation response is plotted, and it is clear that there is a whole spectrum, from compounds that are detectable as mutagenic only at low survival, to those that are mutagenic at non-toxic levels. For the majority of compounds however, there is a degree

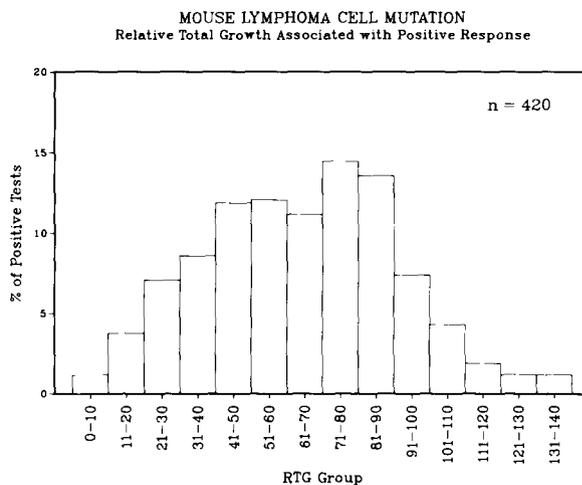


Fig. 9. Level of cytotoxicity (relative total growth, RTG) at which mutations at the TK locus in mouse lymphoma cells were detectable. Data are for 420 chemicals (kindly provided by W. Caspary, NIEHS).

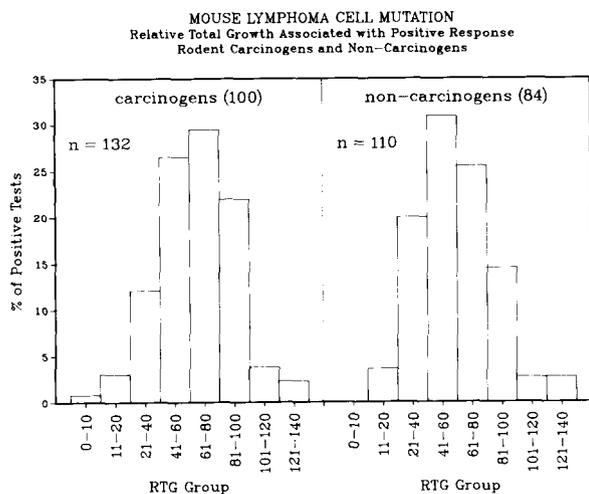


Fig. 10. As for Fig. 9 but chemicals are divided into rodent carcinogens and non-carcinogens.

of cytotoxicity associated with the concentrations of chemicals required to produce a detectable mutagenic response. In Fig. 10, the data for positive mutation assays have been broken down into rodent carcinogens and non-carcinogens, and there is no obvious difference between the patterns of association of toxicity with mutagenicity. In particular, some carcinogens were detectable only at RTG of < 20%. Similarly, Wangenheim and Bolcsfoldi (1988) tested 50 compounds in the mouse lymphoma (L5178Y) cell mutation assay, and examined their own data and published data for 105 compounds for the relationships between mutagenicity, toxicity and carcinogenicity. Of 33 compounds that were positive at less than 20 mM, 8 were positive only at quite high toxicity, i.e. when the RTG was in the 10–20% range. Two of these 8 were carcinogens, and the other 6 were weak, non-carcinogenic, or untested. Similarly, in the literature that they reviewed, 20 of the 105 compounds had 2–4-fold increases in mutation frequencies in the 10–20% RTG range, and of these 20, 8 were known carcinogens.

The data of Wangenheim and Bolcsfoldi and those of the NTP (Mitchell et al., 1988; Myhr and Caspary, 1988) therefore agree on the observation that some rodent carcinogens are detected as *in vitro* mutagens in the L5178Y TK-locus assay only when the RTG is less than 20%.

In the work of Mitchell et al. (1988) and Myhr and Caspary (1988) there were some positive results at very low survival, but also some very toxic non-mutagens. These included lithocholic acid with S9 activation, 4,4'-methylene-bis-2-chloroaniline without S9 and *p*-rosaniline HCl with S9 (Myhr and Caspary, 1988). This demonstrates that mutation is not an inevitable consequence of cytotoxicity. These toxic-but-negative tests were in the minority; of 29 negative assays without S9 and 12 with S9, only 2 and 3, respectively, were classed as negative and toxic.

In another *in vitro* mutation system, HPRT mutations on V79 Chinese hamster cells, some carcinogens (e.g. MMS, Fig. 8) are detectable only at highly cytotoxic concentrations, the latter measured as loss of CFE (reviewed by Fox, 1980). In this review Fox also draws attention to the different quantitative relationships between induced mutation frequency and cell killing in different cell types treated with the same chemical, and between different genetic loci.

In conclusion, the upper limits for cytotoxicity for *in vitro* mutation assays must be set to take account of the variability at small sample sizes, and the data examined to see if there is a real increase in the number of mutant colonies. The data discussed suggest, however, that mutation assays can and should be carried out at quite low survival. Although mutations can be induced by indirect mechanisms (e.g. cycloheximide, see Table 9 and footnote) evidence is lacking that these occur *only* above a certain level of cytotoxicity.

3.7. Cytotoxicity and DNA-strand breaks

DNA-strand breakage is sometimes used as a measure of genotoxicity (e.g. Williams et al., 1985) because, although double-strand breaks may be cell lethal (Leenhouts and Chadwick, 1984; Bryant, 1985; Radford, 1986) they are also believed to be the precursors of chromosome aberrations (Natarajan et al., 1980; Bryant, 1984).

Sina et al. (1983) have addressed the question of whether the quantitative relationships between DNA-strand breakage and cytotoxicity *in vitro* can be used to predict the carcinogenicity of chemicals. They measured DNA single-strand breaks (ssb's) in freshly isolated rat hepatocytes at the end of a 3 h treatment and at the same time

measured cytotoxicity by loss of membrane integrity (Section 3.3a). They found that all of 51 'relatively strong (rodent) carcinogens' induced ssb at minimally cytotoxic doses (i.e. less than 30% cytotoxicity) and were designated Class I compounds. Of 16 chemicals which caused ssb's concomitant with a significant reduction in viability (> 30%, designated Class III chemicals) 12 were weak carcinogens (i.e. tumourigenicity was dependent upon specific conditions such as species, strain, sex, high doses, route of administration, etc.) and 4 were non-carcinogens. In later studies these investigators found that DNA double-strand breaks (dsb's) induced by Class I and Class III compounds were only detectable at doses inducing considerable cytotoxicity (Bradley et al., 1987). They argued that the breaks detected as ssb in the alkaline elution assay of Class III compounds were actually the dsb detected at highly toxic doses in the subsequent assay by neutral elution. They suggest that these dsb's arise as a consequence of 'toxic cellular damage' which results in disruption of lysosomes, releasing DNA nucleases. Possibly, relatively non-specific 'damage' such as ionic imbalance, energy depletion or protein denaturation (Section 3.2) could lead to lysosomal leakiness. The unscheduled release of lysosomal enzymes other than DNA nucleases would be expected to contribute to the observed cytotoxicity.

On the scheme presented in Fig. 4, the Class I chemicals of Bradley and colleagues would produce 'DNA changes' (in this case strand breakage) predominantly by 'direct' mechanisms of DNA interaction, inducing ssb's at relatively non-toxic doses. However, a minor 'indirect' pathway for strand breakage (dashed line with arrow), detectable at high doses, would be *via* 'non-DNA changes' leading both to dsb's and cytotoxicity. This latter pathway would be the mechanism whereby Class III chemicals are effective i.e. on this scheme they would be classified as 'indirect genotoxins' and, the authors argue, might show a threshold in some cases. Bradley (1985) has suggested that this indirect genotoxicity may be important in rodent carcinogenicity assays at high doses, where limited double-strand DNA breakage in sublethally damaged cells could be a mechanism for oncogenic transformation *in vivo*.

Some support for the proposed role of lysosomal nucleases in indirect DNA breakage has come from the demonstration that the lysosomotropic detergent *N*-dodecylimidazole can induce dsb's and chromosomal aberrations (Bradley et al., 1987). Certainly bacterial restriction endonucleases can induce chromosomal aberrations in mammalian cells (e.g., Natarajan and Obe, 1984; Winegar and Preston, 1988), and DNAase I, when supplied to cells enclosed in liposomes to allow active enzyme to reach the nucleus, can induce cytotoxicity, morphological transformation, mutations and chromosome aberrations (Zajek-Kaye and Ts'o, 1984; Nuzzo et al., 1987). Under conditions of 'unbalanced growth', where protein synthesis continues but DNA synthesis is inhibited by exposure to hydroxyurea or excess thymidine, Sawecka et al. (1986) reported an increase in enzyme activity of DNAase II both in cells and in the medium, and postulated that the nuclease activity was responsible for the associated DNA breakage. Ayusawa et al. (1988) also presented evidence that the DNA breaks in thymidylate-starved cells result from endonuclease activity.

The general applicability of the nuclease theory to other cytotoxic compounds is not yet known. It is also unknown whether *limited* endonuclease damage is possible, such that some damaged cells are able to survive — a crucial point, since there are no mutagenic or carcinogenic consequences if the cells die or cannot reproduce.

3.8. *In vivo* cytotoxicity, indirect genotoxicity and carcinogenesis

There is evidence that cytotoxicity *in vivo* may itself play a role in carcinogenesis (Swenberg and Short, 1987; Zeise et al., 1985, 1986), perhaps by inducing chronic cell proliferation or inflammation. We have asked the question whether potentially toxicity-mediated tumours are associated with 'non-genotoxic' or genotoxic chemicals, and if genotoxic *in vitro*, is there any evidence that they might be directly genotoxic? It has been postulated that in rodent carcinogenicity assays at high doses limited double-strand breakage in sublethally damaged cells, e.g., as a result of lysosomal leakiness, could be a mechanism for oncogenic transformation (Bradley, 1985). The possible

TABLE 12
GENOTOXICITY DATA IN VITRO FOR CARCINOGENS INDUCING TARGET-ORGAN TOXICITY

'Toxicity associated carcinogen' ^a	Genotoxicity in vitro ^b			
	Ames	Mut	CA	SCE
1,4-dichlorobenzene	neg ^c	ND	ND	ND
ethyl acrylate	neg	pos	pos	pos
isophorone	neg	pos	neg	pos
melamine	neg	neg	neg	equivocal
pentachloroethane	neg	pos	neg	pos
1,1,1,2-tetrachloroethane	neg	pos	neg	pos
2,6-xylydine	ND	ND	pos ^d	ND
allyl isothiocyanate	equiv.	pos	pos	pos
11-aminoundecanoic acid	neg	neg	neg	pos
chlorodibromomethane	neg	pos	neg	pos
3-chloro-2-methylpropene	neg	pos	pos	pos
C.I. Disperse Yellow 3	pos	pos	neg	pos
C.I. Solvent Yellow 14	pos	pos	neg	pos
D and C Red 9	pos	neg	neg	neg
diglycidyl resorcinol ether	pos	pos	pos	pos
dimethyl hydrogen phosphite	pos	pos	pos	pos
monuron	neg	equiv.	pos	pos
pentachloroethane	neg	pos	neg	pos
polybrominated biphenyl mixture	neg	neg	neg	neg
propylene oxide	pos	pos	pos	pos

^a Hoel et al. (1988); Chemicals inducing either hyperplasia or toxic target-organ lesions for all species and both sexes at dose levels showing increased tumour incidence. The chemicals above the line were those with toxic lesions in all target organs showing chemically induced neoplasia, and likely to have been responsible for the types of tumours observed.

^b Tennant et al. (1987); Ames, Salmonella test; Mut, mutation (TK) in L5178Y cells; CA, chromosome aberrations in CHO cells; SCE, sister-chromatid exchanges in CHO cells.

^c Ashby et al. (1989).

^d Galloway et al. (1987).

ND, no data.

association between chronic toxicity and tumour induction for 53 rodent carcinogens has been analyzed by Hoel et al. (1988). Of the 53 carcinogens considered, they identified 7 compounds which exhibited the types of target organ toxicity that might have been involved in induction of the observed tumours (Table 12). All 7 were negative in the Ames test. Of the 6 for which in vitro chromosomal aberration data are available, 2 were positive. Hoel et al. (1988) also listed 15 compounds that caused tumours and also caused pre-neoplastic responses such as hyperplasia apparently associated with chronic toxicity. The 13 for which genotoxicity data are available are listed in Table 12. Of these, about half were positive in the aberration test in vitro. Clearly these 'toxic carcinogens' are not necessarily 'non-genotoxic' in

vitro; insufficient data exist to determine whether any of these might have induced genotoxicity indirectly in vitro, and whether toxicity might have been responsible for both the genotoxicity and carcinogenicity of any of these chemicals. It will be interesting to obtain in vivo clastogenicity data on these.

3.9. Conclusions and recommendations

Since human exposure to environmental genotoxins is unlikely to be at concentrations which induce much cytotoxicity, the relevance of in vitro genotoxicity at cytotoxic doses can be questioned. However, in using the rodent models for human genotoxicity it is clear that some rodent genotoxins can only be detected in in vitro tests at relatively high levels of cytotoxicity (Section 3.5).

Genotoxicity/cytotoxicity ratios in vitro cannot discriminate between chemicals that are positive or negative for genotoxicity or carcinogenicity in vivo. This is not surprising in view of the severe limitations of the quantitative data relating genotoxicity to cytotoxicity in vitro. We have seen that most measurements of cytotoxicity and some genotoxic endpoints are markedly dependent upon sampling time, that genotoxicity/cytotoxicity ratios may differ very considerably between cell types even for the same chemical (Tables 10 and 11) and between different gene loci in mutation assays (Section 3.6).

Nevertheless, if a chemical is found to be genotoxic without much cytotoxicity in an in vitro test it should be regarded as potentially active in vivo. On the other hand, to designate a chemical as being unlikely to have in vivo activity by virtue of associated cytotoxicity would require extensive investigations of genotoxicity/cytotoxicity relationships in a large number of in vitro tests. To further designate a chemical as being without human risk it would be necessary to establish that there was a true threshold in the relationship between genotoxicity and cytotoxicity at a level of cytotoxicity which could not be achieved in man. In vivo testing for genotoxicity would be important in assessing the results. If thorough in vivo testing gave a negative result, and genotoxicity in vitro was detectable only at highly cytotoxic concentrations (perhaps in only one assay and not in others) it would not be unreasonable to conclude that human risk would be very low.

In considering the relationship between genotoxicity and concentration (Section 2) we concluded that, at high concentrations of non-DNA-reactive chemicals, artefactual genotoxicity could arise because of osmolality changes in the culture medium and that such effects do not occur at lower concentrations i.e. there is a true threshold response. We also concluded that such effects are unlikely to be relevant to human exposure or human risk. We must ask whether similar artefactual responses occur at high levels of cytotoxicity but not at lower levels i.e. if there are circumstances in which there is a true threshold in the relationship between genotoxicity and cytotoxicity. If cell death is taken as the cytotoxic endpoint it is difficult to envisage mechanisms

leading to cell death which are accompanied by genotoxicity *only* at high levels of killing and not at lower levels. For direct genotoxins (Fig. 4) inducing, for example, CA or gene mutations, this would require that at lower concentrations the induced DNA lesions produce lethal but no clastogenic or mutagenic events and that the latter two are only produced when the burden of DNA lesions is above a certain level. If DNA strand breakage is the genotoxic endpoint it would require that cell death at lower concentrations be caused by mechanisms not involving DNA damage. For indirect genotoxins (Fig. 4) the non-DNA changes at lower concentrations would have to lead exclusively to cell death and, only at higher doses, to both cell death and DNA changes. A theoretical possibility for the latter is with chemicals for which there is a threshold in the genotoxicity/concentration relationship e.g. enzyme inhibitors or chemicals which produce active oxygen species or cause leakiness of lysosomes (Section 2). If, in addition to this mechanism such a chemical had a second independent mechanism of cytotoxicity, unaccompanied by genotoxicity, at lower concentrations then a true threshold in the genotoxicity/cytotoxicity relationship would be seen and could possibly be used to assess the likelihood of human risk. However, no chemical with these properties has yet been identified.

It follows that in our current state of knowledge we are unable to define upper limits of cytotoxicity for in vitro testing other than on purely practical grounds (e.g. having sufficient cells for analysis in clastogenicity testing, statistical accuracy in mutational assays) but we are not aware of any artefactual mutagenicity or clastogenicity occurring only above a threshold level of cytotoxicity, though there may be examples for DNA double-strand breakage. Further quantitative analyses of genotoxicity/cytotoxicity relationships are urgently required.

4. Genotoxicity of liver microsome activation systems (Table 13)

Most chemical mutagens are biologically inert unless metabolically activated. In in vitro tests, the capacity for metabolic activation is normally provided in the form of 'S9 mix'. S9 is the super-

nant after centrifugation of a liver homogenate (usually rodent) at 9000 g and comprises microsomes, which carry the enzymes required for metabolic activation and a cytosolic fraction, which can be removed after further centrifugation to isolate the microsomes. A source of NADPH is also required and this is generated in the S9 mix by cofactors, i.e. NADP and either glucose 6-phosphate or isocitrate as the energy source. It is becoming clear that under certain circumstances metabolic activation systems can themselves be genotoxic. It is important to determine the conditions under which mutagens in the activating systems can arise to avoid obtaining spurious positive results for chemicals undergoing genotoxicity testing.

4.1. Bacterial systems

Reports indicating that S9 preparations are mutagenic or cytotoxic in bacterial systems are rare. It has, however, been commonly observed that higher spontaneous reversion frequencies occur in the presence than in the absence of S9 (e.g. Ames et al., 1975). In the Salmonella/microsome assay this has been interpreted as a 'feeding effect' via the presence of histidine in the S9 preparation (Venitt et al., 1984). The phenomenon was considered, however, in some detail by Peak et al. (1982) who concluded that S9 either contained a mutagen or activated some component of the plating medium to a mutagen. The latter explanation was supported by the work of Dolora (1982) and of Maron et al. (1981) who both attributed the effect to the presence of indirect acting mutagens (i.e. agents metabolically activated to mutagens) carried over in the nutrient broth. This explanation, however, could not account for the results of Rossman and Molina (1983) who consistently found a small, but statistically significant increase in background reversion of *E. coli* WP2 induced by the presence of S9. The effect was not seen using *S. typhimurium* strain TA100. The result was shown not to be due to a feeding effect or the presence of indirect acting mutagens in nutrient broth. The mutagenic activity could be removed by dialysis of the S9 and it therefore appeared that the S9 itself contained a mutagen.

Although the toxic effects of S9 preparations in some mammalian systems are well documented,

such effects are not commonly associated with bacteria. This may be partly due to the fact that toxicity can only be detected in plate incorporation tests if it is pronounced. It is noteworthy, however, that S9 has been shown to be cytotoxic to Ames Salmonella strains in liquid suspension assays where cell survival is quantitatively and sensitively assessed (Rosenkranz et al., 1980).

That bacterial mutagens can be generated by isolated liver microsome preparations (and therefore, potentially by S9) was shown by Akasaka and Yonei (1985) who incubated *E. coli* WP2 *uvrA* (pKM101) cells in a preparation of microsomes from rat liver containing NADPH and Fe^{2+} . Mutation was measured following incubation periods of up to 60 min. Under these conditions the microsomes were both strongly cytotoxic and mutagenic. The mutagenicity was attributed to lipid peroxidation (Section 4.3) although the genotoxic product(s) of lipid peroxidation in this system was (were) not identified.

4.2. Mammalian systems

The cytotoxicity of S9 mix to human peripheral blood lymphocytes is well documented (Bimboes and Greim, 1976; White and Hesketh, 1980; Madle and Obe, 1977; Madle, 1981) although, again, the nature of the cytotoxic component has not been considered in depth. Tan et al. (1982) using Chinese hamster ovary (CHO) cells, compared the effects of two activation systems where the activating enzymes were supplied as either S9 or as purified microsomes. Whereas the former was not cytotoxic over a 5-h incubation period, the preparation utilising isolated microsomes reduced cell survival by approximately 60%. These results are comparable with those of Akasaka and Yonei (1985, cited above) using *E. coli*. Cytotoxicity was again attributed to the products of lipid peroxidation but it is noteworthy that in contrast to *E. coli* the microsome preparations did not induce mutation (at the HPRT locus) in the CHO cells.

Genotoxic effects associated with metabolic activation systems are summarised in Table 13. These isolated reports must, of course, be considered against a large volume of data indicating that, under most circumstances, S9 does not have a marked effect on spontaneous point mutation or chromosome aberration frequencies, although

slightly higher background frequencies of both these end-points are commonly observed in the presence of S9 mix (Caspary et al., 1988b; Margolin et al., 1986).

The first clear recognition of the mutagenic potential of S9 in mammalian cells was made by Myhr and Mayo (1987) in the mouse lymphoma L5178Y mutation assay. Although S9 alone or S9 mix induced no measurable increase in mutation following a 'normal' 4-h treatment period, striking increases were observed when the exposure time was extended to 8 or 24 h. Similar increases occurred when cells were exposed for 4 h to S9 preparations which had been preincubated with growth medium for 18 h prior to treatment, indicating that a process was occurring within the S9 itself to produce a mutagenic substance. McGregor et al. (1988) were able to show that background mutation frequencies in L5178Y cells could also increase if the proportion of S9 in the S9 mix was increased. S9 concentrations up to 10 mg whole liver equivalents/ml were not significantly toxic or mutagenic but 12.5 mg whole liver equivalents/ml increased the mutant fraction and re-

duced survival (concentrations of S9 up to 25 mg/ml are commonly used in this assay).

Myhr and Mayo (1987) observed that S9-induced L5178Y mutants were predominantly 'small colony' type. These are thought to arise as a result of chromosomal aberrations. This is significant in view of the findings of Kirkland et al. (1989) that certain batches of Aroclor 1254-induced S9 produced chromosomal aberrations in their clone of CHO cells. The effect appeared to be dependent on the presence of NADP and G-6-P and was observed following a short (2 h) incubation period. The clastogenic activity of the S9 mix could be reduced by co-incubation with catalase or vitamin E implying that active oxygen species were involved. Oxygen radicals are an initial product of lipid peroxidation (Vaca et al., 1988). It was assumed that microsomal lipid peroxidation was the cause of the clastogenicity of the S9 mix.

4.3. Lipid peroxidation

Metabolic activation systems utilizing isolated microsomes appear to be much more likely to generate toxic and mutagenic species than S9 mix.

TABLE 13
GENOTOXIC EFFECTS IN VITRO ASSOCIATED WITH METABOLIC ACTIVATION SYSTEMS

End point	Cell type	Activation system	Treatment duration	Comment	Reference
Point mutation	<i>E. coli</i> WP2	S9 mix	(Plate incorporation)	Increased mutation to Trp ⁺ . Effect not seen in <i>S. typhimurium</i> TA100	Rossmann and Molina (1983)
Point mutation	<i>E. coli</i> WP2 uvrA	Isolated microsomes NADPH + Fe ²⁺	up to 60 min	Increased mutation to streptomycin resistance	Akasaka and Yonei (1985)
Point mutation (HPRT locus)	CHO	Isolated microsomes + co-factors	5 h	Cytotoxicity but no mutagenicity observed	Tan et al. (1982)
Point mutation and/or CA	Mouse lymphoma	S9 mix	4-24 h	Mutant frequency increased after 8 h exposure	Myhr and Mayo (1987)
Point mutation and/or CA	Mouse lymphoma	S9 mix	4 h	Mutant frequency increased with increasing S9 fraction in mix	McGregor et al. (1988)
Chromosome aberrations	CHO	S9 mix	2 h	Marked increases in chromosome aberrations observed	Kirkland et al. (1989)

CA, chromosome aberrations.

This may be due to the presence of an inhibitor of microsomal lipid peroxidation found in the cytosolic fraction of liver (Kamatagi et al., 1974; Kotake et al., 1975; Player and Horton, 1978; Talcott et al., 1980). That microsomal lipid peroxidation can occur in S9 mix formulations, characteristic of those used in short-term testing, has, however, been clearly shown. Vaca and Harms-Ringdahl (1986) demonstrated that the rate of lipid peroxidation was higher in S9 mix prepared from rats fed diets rich in polyunsaturated fatty acids, could be stimulated by the presence of Fe^{2+} ions and was higher in S9 mix from Aroclor 1254-induced rats than uninduced animals. In contrast Paolini et al. (1983) found that lipid peroxidation occurred more readily in S9 mix from uninduced animals than in S9 mix from rats induced with β -naphthoflavone and phenobarbitone. It appears that marked changes in membrane lipid composition occur following induction, and this depends on the inducing agent. This may provide some explanation for the results of Kirkland et al. (1989) which indicated that batches of S9 mix from rats induced with Aroclor 1254 were clastogenic whereas S9 mix from rats induced with β -naphthoflavone and phenobarbitone was not.

The potential of S9 preparations to undergo lipid peroxidation must vary considerably among the different S9 mix formulations and treatment conditions used by different laboratories in *in vitro* tests. Media components (e.g. Fe^{2+} content) and concentration of cofactors are known to be important (Vaca and Harms-Ringdahl, 1986). Factors that accelerate microsomal lipid peroxidation have also been recognised and these include radiation, hyperoxia, nitrogen oxides and radical initiators (Buege and Aust, 1978).

4.4. Recommendations

Possible means of reducing or eliminating the genotoxic effects of S9 preparations include the addition of agents such as catalase or vitamin E to the incubation medium (Kirkland et al., 1989). BHA and EDTA are likely to be effective also because they can inhibit lipid peroxidation (Tan et al., 1982; Vaca and Harms-Ringdahl, 1986; Paolini et al., 1988). Simply reducing the duration of treatment may not be appropriate however, be-

cause long incubations (several hours) in the presence of S9 are required to detect some promutagens in mammalian cells (Sbrana et al., 1984; Machanoff et al., 1981).

Sensitivity to the products of lipid peroxidation must vary between mutagenic endpoints and this could explain why S9 induced mutagenesis is not more commonly observed. For example, active oxygen species are not mutagenic to the most frequently used *S. typhimurium* tester strains (TA1535, TA1538, TA1537, TA98, TA100) (Levin et al., 1982). Kirkland et al. (1989) found that the batches of S9 which caused elevated frequencies of chromosome aberrations in CHO cells gave normal mutation frequencies in Ames tests and mammalian cell mutation tests and normal levels of repair in unscheduled DNA synthesis assays. These same batches showed no evidence of clastogenicity with lymphocytes in whole blood culture. This may well have been attributable to culture conditions rather than cell type because active oxygen species have been shown only to be genotoxic in human lymphocytes when cultured following isolation from whole blood (Mehnert et al., 1984). At present, however, it is not possible to recommend the use of insensitive cell types or protective culture conditions because the peroxidation of membrane lipids is recognised as an indirect mechanism of genotoxicity. Phorbolmyristate acetate (Cerutti, 1985) and chromium chloride (Friedman et al., 1987) are examples of compounds which are thought to act in this way (Table 9). It seems unlikely that chemicals exist which initiate lipid peroxidation in liver microsomal membranes but not in other cell membranes and it is important that such 'membrane-active' agents are detected. Firstly, it must be clearly established that the genotoxicity of liver-microsome activation systems that has been observed can be attributed to lipid peroxidation. The next step should then be to develop methods for S9 induction and S9 mix formulations which make this less likely to occur.

5. Genotoxicity induced by extremes of pH (Table 14)

Non-physiological pH can not only influence the mutagenicity of many compounds (Zetterberg

et al., 1977; Whong et al., 1985; LeBoeuf et al., 1989) but can be mutagenic per se.

5.1. Non-mammalian systems

Extremes of pH (3–10) have been shown not to induce mutation in the Ames test in the presence or absence of S9 using standard plate incorporation or pre-incubation methods (Tomlinson, 1980; Cipollaro et al., 1986). Prolonged incubation (at least 4 h) prior to plating under strongly alkaline conditions (pH 10) does however result in mutagenesis in certain bacterial strains (Musarrat and Ahmad, 1988). The effect was observed in *Salmonella* strains TA97, TA102, TA104 and *E. coli* K12 but not in TA98 or TA100. From the strain specificity it was inferred that alkali treatment caused damage preferentially at A–T-rich regions in the DNA and, from liquid holding experiments following exposure, that this damage could be repaired.

As yet, no evidence has been obtained for pH-related increases in point mutation in yeast (Tomlinson, 1980; Nanni et al., 1984; Whong et al., 1985) though effects have not been measured outside the range 3–9. Low pH has, however, been shown to induce gene conversion in *Saccharomyces cerevisiae* (Nanni et al., 1984) and high pH to increase point mutation in the fungus *Cladosporium cucumerinum* (but not in *Aspergillus nidulans*, Nirenberg and Speakman, 1981).

Sea urchin embryos appear to be very sensitive to the genotoxic effects of low pH (Cipollaro et al., 1986). A short exposure to pH 6.0 was sufficient to induce a variety of mitotic abnormalities (anaphase bridges, lagging chromosomes, multiple breaks and multipolar mitoses).

The clastogenic effects of low pH are now well documented in *Vicia faba* root tips although exposure to pH 4 is required before significant increases in aberrations are observed (Bradley et al., 1968; Zura and Grant, 1981).

5.2. Mammalian systems

In mammalian systems the genotoxic effects of pH appear to be strongly enhanced by the presence of S9. Low pH has been shown to induce chromosome aberrations in CHO cells after treatment with hydrochloric or acetic acid (Thilager et

al., 1984). No increase in aberrations was observed in the absence of S9 at pH 5.5 but, in its presence, large numbers of aberrations were induced. These data were interpreted to indicate that S9 could be broken down into clastogenic products. It is now clear, however, that the presence of S9 is not a prerequisite for clastogenesis. Thus Morita et al. (1989a) showed that at low pH (5.5 or less), aberrations were induced in CHO cells in both the absence and presence of S9 though the effect was enhanced by S9. Phosphate-buffered saline acidified to pH 5.2 was also clastogenic indicating that the effect was not due to decomposition products of the culture medium. In addition, although no clastogenic activity was observed over the pH range 7.3–10.9 without S9, aberrations were observed at pH 10.4 with metabolic activation. Further studies (Morita et al., 1989b) indicated that reducing the pH of the medium and then neutralising to pH 6.4 or 7.2 or using an organic buffering system removed the clastogenic effect.

Chromosomal effects are also induced in other cell types. Shimada and Ingalls (1975) exposed human lymphocytes for 3 h to pH levels over a range 6.5–8.8. A statistically significant increase in hyperdiploid cells was observed in cultures exposed to pH 6.5–6.9 providing evidence for pH-induced non-disjunction. Endoreduplication was also significantly increased at low pH (< 7) but not at high pH. The authors claimed that chromosome structural damage (including exchange aberrations) was observed at both low and high pH levels but this was not quantified. In the light of these results the results of Sinha et al. (1989) are unexpected. These authors found that exposure of rat lymphocytes to pH levels as low as 2.73 or as high as 9.97 for 4 h in the absence or presence of S9 did not result in clastogenesis. Mitotic inhibition (which, interestingly, was more marked in the presence of S9) was evident at extreme pH levels indicating that it would not have been possible to expose the cells to more severe conditions of pH. It is not clear why rat and human lymphocytes should respond differently because culture conditions and sampling times were not dissimilar in the two experiments (both were cultured as whole blood).

Clastogenicity was used to explain increases in spontaneous mutation frequency at the TK locus

TABLE 14
GENOTOXIC EFFECTS IN VITRO ASSOCIATED WITH EXTREMES OF pH

End point	Cell type	Chemical	S9	pH range studied	Treatment duration (h)	Effective pH	Comment	Reference
Point mutation	<i>Salmonella typhimurium</i> TA100, TA1535	H ₃ PO ₄ /KH ₂ PO ₄ / K ₂ HPO ₄	-	3-8	1-4	None	Pre-incubation	Tomlinson (1980)
Point mutation	<i>Salmonella typhimurium</i> TA98, TA97, TA100, TA120, TA1535	H ₃ PO ₄ /H ₂ SO ₄	-/+	4.0-9.0	1 (pre-incubation)	None	Pre-incubation or plate incorporation. Toxicity at 5.0 and less	Cipollaro et al. (1986)
Point mutation	<i>E. coli</i> K12, <i>Salmonella typhimurium</i> TA104, TA102 TA97	Tris/NaOH	-	8-10	6	10	Pre-incubation Little or no effect with TA98 or TA100	Musarrat and Ahmad (1988)
Point mutation	<i>Aspergillus nidulans</i>	Lactic acid/KOH	-	5.0-6.8	8	None		Nirenberg and Speakman (1981)
Point mutation	<i>Cladosporium cucumerinum</i>	Lactic acid/KOH	-	5.0-6.8	20	6.8	'physiological' pH is 5.0-5.2	Tomlinson (1980)
Point mutation	<i>Neurospora crassa</i>	H ₃ PO ₄ /KH ₂ PO ₄ / K ₂ HPO ₄	-	3-8	2-4	None		Nanni et al. (1984)
Gene conversion	<i>Saccharomyces cerevisiae</i>	HCl/NaOH	-	5-8	40	5.8		Nanni et al. (1984)
Point mutation	<i>Saccharomyces cerevisiae</i>	HCl/NaOH	-	5-8	2	None		Nanni et al. (1984)
Point mutation	<i>Neurospora crassa</i>	Na ₂ HPO ₄ / KH ₂ PO ₄	-	5-9	3	None		Whong et al. (1985)
Mitotic abnormalities	Sea urchin embryos	HCl, H ₃ PO ₄ , H ₂ SO ₄	-	5.5-8.5	1 (or more)	6.0 (approx)		Cipollaro et al. (1986)

Chromosome aberrations	<i>Vicia faba</i>	Sucrose/KOH/ HCl	-	3.0-7.5	4	4	Bradley et al. (1968)
Chromosome aberrations	<i>Vicia faba</i>	Citric-citrate buffer	-	4-7	-	4	Zura and Grant (1981)
Aneuploidy	Human ^a lymphocytes	Lactic acid/ NaOH	-	6.5-8.8	3	< 7.0 and > 8.4	Shimada and Ingalls (1975)
Endoredupli- cation	Human ^a lymphocytes	Lactic acid/ NaOH	-	6.5-8.8	3	< 7.0	Shimada and Ingalls (1975)
Chromosome aberrations	Rat lymphocytes ^a	NaOH/HCl	-	5.10-9.97	4	None	Sinha et al. (1989)
			+	4.17-10.01	4	None	Toxicity, but no clastogenicity
Chromosome aberrations	CHO	Acetic acid	+	5.28-7.24	2	5.5	No effect minus S9
		HCl	+	5.00-5.75	2	5.25	No effect minus S9
Chromosome aberrations	CHO	KOH	+	7.3-10.9	6	10.4	No effect minus S9
		NaOH	+	7.4-10.8	6	10.8	No effect minus S9
		HCl	-	5.3-7.4	24	5.5	
			+	5.5-7.4	6	6.0	
		H ₂ SO ₄	-	3.5-7.4	24	4.5	
			+	4.5-7.4	6	6.2	
		NaOH	-	6.0-7.0	24	6.2	
		HCl	-	5.8-6.6	24	6.2	
Chromosome aberrations	CHO	Formic acid, lactic acid, acetic acid	-	5.9-7.1	24	6.0 (approx.)	Morita et al. (1989b)
			+	5.9-7.1	6		
Point mutation and/or CA	Mouse lymphoma	Good's buffers	+	6.4-7.6	4	6.8	No effect minus S9
		Good's buffers/ HCl	-	6.3-7.5	4	6.3	2-fold increase
			+	6.3-7.5	4	6.5	2.6-fold increase
		Good's buffers/ NaOH	+	7.3-8.8	4	8.3	2-fold increase (no effect minus S9)

^a In whole blood culture.
CA, chromosome aberrations.

in mouse lymphoma L5178Y cells following exposure to low pH (Cifone et al., 1984, 1985, 1987). The induced colonies observed were typically of the 'small colony' phenotype which implied that they arose as a result of chromosomal aberrations. This was confirmed by chromosome aberration analysis of low pH-treated cultures. The effect was greatly enhanced by the presence of S9 mix.

5.3. Mechanisms

The mechanism by which non-physiological pH levels cause genotoxicity is not clear but it has been known for many years that pH can influence the level of depurination of bacterial or viral DNA (Strack et al., 1964). Brusick (1986) noted that the fidelity of the DNA replication and repair enzymes may be reduced by extremes of pH and this could produce genotoxic effects. The data of Thilager et al. (1984) and Cifone et al. (1987) indicate that biologically active species may be produced under certain conditions in S9 mix at low pH.

5.4. Recommendations

From the discussion above it is clear that agents which cause large pH shifts can give false-positive results. The genotoxicity of 2,4-D, for example, may well be attributable to its acidic properties (Zetterberg, 1979). One approach to avoid potential problems might be to conduct all in vitro assays at neutral pH. The results of Morita et al. (1989b) indicate that neutralisation of the treatment medium may prevent pH-related genotoxicity, though these authors caution that this should only be done if the solubility or stability of the test chemical is not affected and hence any genotoxic property masked. However, this recommendation would imply that all compounds be tested over a range of pH values and this would markedly increase the burden of testing. Also, except for absorption through the stomach, human exposure is unlikely to involve non-physiological pH levels.

It is clear that the effect of a test chemical on the pH of the treatment medium should always be measured and it is recommended that positive results associated with pH shifts in the test system of greater than 1 unit should be viewed with caution and confirmed in experiments conducted at neutral pH.

6. Overall summary and recommendations

We have addressed the question of whether genotoxicity can be generated under extreme culture conditions which are irrelevant to the situation in vivo. Most of the available data relate to clastogenesis. Four in vitro conditions have been considered.

(a) Excessively high concentrations

Chromosome aberrations, TK mutations in L5178Y mouse lymphoma cells, morphological transformation, DNA-strand breakage in mammalian cells and mutations in yeast can be induced at very high concentrations of non-DNA reactive chemicals which cause a significant increase in the osmolality of the culture medium. To avoid this problem upper concentration limits for testing have been suggested, e.g. 10 mM in clastogenesis tests. However, some chemicals that are clastogenic in rodent bone marrow are only detectable in vitro at high concentrations, in part reflecting the inadequacies of metabolic activation systems. We conclude that if an upper concentration limit of 10 mM is adopted, with a rigorous protocol, very few in vivo clastogens will be missed and we recommend the use of a 10 mM limit for clastogenicity tests.

Of the 50% of tested chemicals which are clastogenic in vitro but not in vivo, probably less than 5% are clastogenic through osmolality effects.

Genotoxicity is not an inevitable consequence of exposure to high concentrations of chemicals.

Other mechanisms of indirect genotoxicity in addition to hypertonicity (e.g. enzyme inhibition, production of active oxygen species) may show true threshold responses at concentrations above those that can be achieved as a result of human exposure.

(b) High levels of cytotoxicity

Unlike the artefactual genotoxicity that occurs at high concentrations of some chemicals we have not obtained evidence for similar artefactual increases in gene mutations or chromosomal aberrations at high levels of cytotoxicity, although there is some evidence that double-stranded breaks in DNA may occur only at highly toxic levels after treatment with some chemicals. For mutation as-

says, the upper limits of cytotoxicity are determined by practical concerns such as limiting the variability that occurs at low survival. For chromosomal aberration assays we do not have a database to compare *in vitro* results at varying levels of toxicity with *in vivo* clastogenicity or carcinogenicity, but it appears that some chemicals that are clastogenic *in vivo* may be detected only at quite toxic concentrations *in vitro*. Further analyses are urgently required.

Relationships between genotoxicity and cytotoxicity *in vitro* can vary markedly depending on the endpoints studied, sampling time, exposure time and cell type; quantitative extrapolation to the *in vivo* situation must be undertaken with caution.

(c) *Metabolic activation systems*

Under certain conditions S9 mix may itself be genotoxic (e.g. chromosome aberrations in CHO cells, mutation in *E. coli* and mouse lymphoma cells). It is important that the underlying mechanism be elucidated because there may be chemicals whose genotoxicity *in vitro* is simply the result of inducing these conditions; this could lead to spurious positive results in genotoxicity testing. There is some evidence that active oxygen species produced *via* lipid peroxidation of microsomes are responsible. Certain endpoints and test systems appear to be less susceptible than others to S9 induced genotoxicity. It is probably premature to advocate modifications to current test procedures (e.g. addition of radical scavengers) until the mechanism is better understood.

(d) *Extremes of pH*

Extremes of pH can induce chromosome aberrations in mammalian cells, mutation in *E. coli* and mouse lymphoma cells and gene conversion in yeast but the mechanism is not clear. The effect of a test chemical on the pH of the medium should always be measured and positive results associated with pH shifts of greater than one pH unit should be viewed with caution and confirmed at neutral pH.

The artefactual genotoxicity associated with excessive concentrations, metabolic activation systems and pH extremes probably accounts for only

a small part of the discrepancy between *in vitro* and *in vivo* results. Nevertheless, identification of these factors with appropriate modification of protocols should help to improve the credibility of *in vitro* tests in predicting *in vivo* response and, ultimately, human risk.

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