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Fundamental and Molecular Mechanisms of Mutagenesis

Development and validation of alternative metabolic systems for mutagenicity testing in short-term assays

J. Rueff [d,*], C. Chiapella [a], J.K. Chipman [b], F. Darroudi [c], I. Duarte Silva [d], M. Duverger-van Bogaert [e], E. Fonti [f], H.R. Glatt [g,i], P. Isern [a], A. Laires [d,j], A. Léonard [e], M. Llagostera [a], P. Mossesso [f], A.T. Natarajan [c], F. Palitti [f], A.S. Rodrigues [d], A. Schinoppi [f], G. Turchi [h], G. Werle-Schneider [g]

[a] *Department of Genetics and Microbiology, Autonomous University of Barcelona, 08193, Bellaterra (Barcelona), Spain*
[b] *School of Biochemistry, University of Birmingham, P.O. Box 363, B 15 2TT, Birmingham, UK*
[c] *Department of Radiation Genetics and Chemical Mutagenesis, MGC – State University of Leiden-Wassenaarseweg 72, NL-2333 AL, Leiden, The Netherlands*
[d] *Department of Genetics, Faculty of Medical Sciences, UNL, R. da Junqueira 96, P-1300 Lisbon, Portugal*
[e] *Unit of Teratogenesis and Mutagenesis (UCL 7237), Catholic University of Louvain, Av. E. Mounier 72, B-1200 Brussels, Belgium*
[f] *Department of Agrobiology and Agrochemistry, University of Tuschia, Via san Camillo de Lellis sn, 01100 Viterbo, Italy*
[g] *Department of Toxicology, University of Mainz, Obere Zahlbacher Strasse 67, D-55131 Mainz, Germany*
[h] *ECVAM-European Centre for the Validation of Alternative Methods, I-21020 Varese, Italy*
[i] *German Institute of Human Nutrition, Arthur-Scheunert-Alleee 114-116, D 14558 Potsdam-Rehbrücke, Germany*
[j] *Faculty of Sciences and Technology, UNL, P-2825 Monte da Caparica, Portugal*

Abstract

We present here the results obtained within the framework of an EU funded project aimed to develop and validate alternative metabolic activating systems to be used in short-term mutagenicity assays, in order to reduce the use of laboratory animals for toxicology testing. The activating systems studied were established cell lines (Hep G2, CHEL), genetically engineered V79 cell lines expressing specific rat cytochromes P450, erythrocyte-derived systems, CYP-mimetic chemical systems and plant homogenates. The metabolically competent cell lines were used as indicator cells for genotoxic effects as well as for the preparation of external activating systems using other indicator cells. The following endpoints were used: micronuclei, chromosomal aberrations and sister chromatid exchanges, mutations at the *hprt* locus, gene mutations in bacteria (Ames test), unscheduled DNA synthesis and DNA breaks detected in the comet assay. All metabolic systems employed activated some promutagens. With some of them, promutagens belonging to many different classes of chemicals were activated to genotoxicants, including carcinogens negative in liver S9-mediated assays. In other cases, the use of the new activating systems allowed the detection of mutagens at much lower substrate concentrations than in liver S9-mediated

Abbreviations: BrdU, bromodeoxyuridine; CYP, cytochrome P450; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-methylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine; SCE, sister chromatid exchanges; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pirido[4,5-*b*]indol; UDS, unscheduled DNA synthesis

* Corresponding author. Fax: +351 1 362 20 18.

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assays. Therefore, the alternative metabolizing systems, which do not require the use of laboratory animals, have a substantial potential in in vitro toxicology, in the basic genotoxicity testing as well as in the elucidation of activation mechanisms. However, since the data basis is much smaller for the new systems than for the activating systems produced from subcellular liver preparations, the overlapping use of both systems is recommended for the present and near future. For example, liver S9 preparations may be used with some indicator systems (e.g., bacterial mutagenicity), and metabolically competent mammalian cell lines may be used with other indicator systems (e.g., a cytogenetic endpoint) in a battery of basic tests.

Keywords: Bioactivation of mutagen; Human Hep G2 cell; CHEL cell; V79-derived Chinese hamster cell; Erythrocyte; Plant activation; Cytochrome P450; Peroxidase activity; Ames test; Chromosome aberration; Sister chromatid exchange; Micronuclei; Cell survival; Gene mutation assay

1. General introduction

The presence of mutagens and promutagens in the environment constitutes a threat to the human health (Ames, 1979). Depending on the ontogenic stage of an organism when exposed, a mutagen might exert teratogenic effects, enhance the aging process, induce mutation in germinal cells or induce neoplastic transformation in somatic cells. Since most carcinogens are mutagens, it has been imperative to develop a suitable screening and monitoring system. Simple and sensitive bacterial test systems are available for detecting chemical mutagens (Ames et al., 1975). In addition, mammalian cells in culture are widely used for the detection of compounds which induce gene mutations or cytogenetical effects. However, many chemical carcinogens are not active per se, but require metabolic activation to reactive intermediates to exhibit their biological effects (Malling, 1966; Wright, 1980). In bacterial mutagenicity assays (Ames et al., 1973) and in cultured mammalian cells (Natarajan et al., 1976), the problem of metabolic activation of indirectly-acting mutagens has been partially solved by adding rodent liver microsomes to the culture plates. Therefore the majority of in vitro mutagenicity assays rely on exogenous activation system such as S9-fraction derived from rodent liver homogenate to detect promutagens. However, this test system does not meet all the requirements of a perfect system and has some disadvantages, such as follows:

1. A substantial number of animals are being used for the purpose of collecting liver subcellular S9-fractions.
2. Significant divergences have been observed in the profiles of the metabolites and in the DNA adducts produced when different metabolic activation systems are introduced in the assay (Bos et al., 1983). Substantial differences in the results were observed in the same indicator system, when cellular or subcellular hepatic activating systems were used (e.g., Utesch et al., 1987) or when subcellular preparations from rats and humans were compared (e.g., Glatt et al., 1994b).
3. The metabolites generated by such systems may differ from those generated by intact animals thus, many known carcinogens, for example hexamethylphosphoramide and safrole, which are detected as positive in in vivo tests (Purchase and Ray, 1981) are not found to be active in vitro in the presence of rat S9-mix (Natarajan and van Kesteren-van Leeuwen, 1981).
4. Externally generated metabolites may not reach the DNA in the target cells used (Glatt et al., 1995).

Therefore, there is an increasing demand for reducing or eliminating the use of vertebrate animals for conducting this type of experiments. Since the active metabolites can also be generated directly in the target cell, the use of epithelial cell strains from the liver of rodents and humans has recently received major emphasis in short-term screening tests and it would make the in vitro assay more reflective of the in vivo situation (Turchi et al., 1987; De Salvia et al., 1988; Kasper et al., 1988; Natarajan and Darroudi, 1991; Salissidis et al., 1991; Darroudi and Natarajan, 1994). Generally, intact cells of liver origin seem to satisfactorily mimic the metabolic activation/detoxification that occurs in vivo and they reflect the in vivo genotoxicity better than S9 extracts of rodent liver (Glatt et al., 1984). On the other hand, other metabolic activation systems may be

useful in gaining insight on the mechanisms by which mutagenic or carcinogenic agents may exert their genotoxic effects. In this perspective cell lines with well defined metabolic activities, such as cells genetically engineered to express certain enzymes, of human or rodent origin, may be a useful tool in in vitro toxicology studies (Glatt et al., 1987; Doehmer et al., 1988; Crespi et al., 1991; Langenbach et al., 1992).

2. Use of Hep G2 hepatoma cells as a source for metabolic activation

The Hep G2 cell line was initially derived from tissue minces from a liver biopsy of a child with primary hepatoblastoma. One of the most interesting aspects of this tumour derived cell line is that it retains many biosynthetic capabilities of normal parenchymal cells (Knowles et al., 1980). This epithelial cell possesses a greater spectrum of xenobiotic metabolising activities compared to fibroblasts and this might be related to the fact that, in man, carcinomas are more frequent than sarcomas. Indeed, epithelial liver cells are endowed with the maximal capacity for metabolic activation of several drugs (Huberman and Sachs, 1973).

Hep G2 cells are stable and have essentially an aneuploid chromosome number (in the range of 48–54 chromosomes per cell) with a modal number of 52, and a cell cycle duration of about 20 h (Natarajan and Darroudi, 1991). There are advantages in using this cell line: reproducible material is available, and the need for repeated isolation of fresh material or the preparation of primary cultures is eliminated.

Some studies illustrate the importance of defining and standardizing the culture conditions used for investigating factors affecting drug metabolism and cytotoxicity in cultured cells.

2.1. Effect of medium composition

When grown in the standard Dulbecco's medium, the Hep G2 cell line shows only 10–20% of the cytochrome P450 (CYP) -dependent monooxigenase activity of freshly isolated human adult hepatocytes (Doostdar et al., 1988). Culture in Williams E medium results in an approx. 2-fold increase in the monooxygenase activities.

Modified Earle's medium is more effective than Williams' E medium and increases markedly the *O*-dealkylation of ethoxyresorufin, benzyloxyresorufin and pentoxyresorufin (Doostdar et al., 1988).

Medium composition does not affect UDP-glucuronyltransferase and glutathione transferases activities as much as the monooxygenases, but the content of glutathione was 2-fold higher in cells grown in modified Earle's medium. The presence of L-cysteine in the modified Earle's medium may be a favourable factor in the expression of monooxygenase, glutathione transferase and UDP-glucosyltransferase activities (Doostdar et al., 1988).

Hep G2 cells cultured in Williams E or modified Earle's media contain levels of monooxygenase, UDP-glucuronyltransferase and glutathione transferases activities equivalent to those in freshly isolated human hepatocytes (Doostdar et al., 1988).

The presence of solvents such as dimethyl sulfoxide may influence the monooxygenase activities, however, this effect depends also on the growth medium selected (Doostdar et al., 1991).

2.2. Growth period of the cells

The activities of xenobiotic-metabolizing enzymes vary considerably with time in culture resulting in changes in the balance between activating and detoxifying pathways. As the rate of cell growth slows down, the activities of ethoxyresorufin deethylase, methoxyresorufin demethylase, NADPH cytochrome-*c* reductase and NADH cytochrome-b_5 reductase increase. While glutathione transferase remains constant, UDP-glucuronyltransferase show alterations and glutathione levels decline (Duthie et al., 1988; Doostdar et al., 1990). Higher cellular activities were reported during the first 48 h than later after the subcultivation (Herno et al., 1992).

2.2.1. Expression of phase I drug metabolizing enzymes

CYP is detectable in partially purified microsomes (Sassa et al., 1987; Roe et al., 1993) of Hep G2 hepatoma cells. Analysis by Western blotting showed that microsomes from Hep G2 cells contain CYPs of the 1A, 2C and 3A subfamilies (Fardel et

al., 1992); Dawson et al. (1985) observed a strong reaction with the antibody to the phenobarbital-inducible protein PB_2.

Aryl hydrocarbon hydroxylase activity was detected using benzo[*a*]pyrene as substrate (Limbosh, 1983; Sassa et al., 1987; Labruzzo et al., 1989). *O*-Deethylation of 7-ethoxycoumarin was observed (Dawson et al., 1985). Ethoxyresorufin deethylase and methoxyresorufin demethylase could be detected readily (Doostdar et al., 1988). Hep G2 cells metabolize acetanilide, phenacetin (Fardel et al., 1992) and bioactivate acetaminophen to a species forming protein adducts (Roe et al., 1993).

All these activities mentioned are catalyzed preferentially by the polycyclic aromatic hydrocarbon inducible CYP1A subfamily.

Aldrin was found to be converted into dieldrin (Limbosh, 1983). Benxyloxyresorufin dealkylase and pentoxyresorufin dealkylase activities were measured in the cells (Doostdar et al., 1988, 1990), while no benzphetamine *N*-demethylase (Dearfield et al., 1983) nor aniline hydroxylase nor ethylmorphine *N*-demethylase (Sassa et al., 1987) could be detected. The former are mainly catalyzed by the phenobarbital inducible P450 2B isoenzymes.

Microsomal NADH cytochrome-b_5 reductase (Dearfield et al., 1983; Grant et al., 1988) as well as NADH and NADPH cytochrome-*c* reductase (Grant et al., 1988; Duverger-van Bogaert et al., 1993) were readily measurable. Some other activities such as catalase, peroxidase and flavin-monooxygenase were measured in Hep G2 cell supernatant (Duverger-van Bogaert et al., 1993).

2.2.2. *Expression of phase II drug metabolizing enzymes*

Epoxide hydrolase activity was measured in Hep G2 microsomes; this activity depended on the substrate used (Dearfield et al., 1983; Grant et al., 1988). Glutathione transferase activity equals about that reported in freshly isolated human hepatocytes (Doostdar et al., 1988); the isoenzyme content was studied and the *alpha* class represents the major glutathione transferase as in the case of human liver (Dierick, 1989). Glutathione transferase of class *pi* was undetectable, but the *mu* class protein was present. At confluence, Hep G2 cells contain about 50% of the intracellular glutathione content of freshly isolated hepatocytes but have not retained the cystathionine pathway (Duthie et al., 1988). Sulfotransferase activity was low (Fardel et al., 1992). Nevertheless, this cell line permitted the study of enzymatic sulfation of chiral drugs (Walle et al., 1994) and the activation of benzylic alcohols to mutagens (Glatt et al., 1994a, b). Hep G2 cells possess low levels of *N*-acetyltransferase activities (Fardel et al., 1992). UDP-glucuronosyltransferase activity was similar to that of freshly isolated human adult hepatocytes (Grant et al., 1988).

2.2.3. *Enhancement of some drug metabolizing enzymes*

Xenobiotic exposure enhanced several drug metabolizing enzymes in Hep G2 cells. Aryl hydrocarbon hydroxylase was induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 3-methylcholanthrene (Labruzzo et al., 1989) and benzo[*a*]anthracene (Duverger-van Bogaert et al., 1993). Benzo[*a*]anthracene pretreatment of cells strongly increased ethoxyresorufin deethylase, pentoxyresorufin dealkylase and benxyloxyresorufin dealkylase activities (Grant et al., 1988) and enabled cells to metabolize 11-methyl 17-ketone (Bhatt, 1986).

Exposure of cells to 3-methylcholanthrene resulted in an increase in CYP1A content and rate of metabolism of acetanilide and phenacetin, while treatment with rimfamycin and dexamethazone increased CYP3A (Fardel et al., 1992).

Hep G2 cells cultured in a medium containing 0.1% acetone showed a 2–3-fold increase in microsomal CYP2E1-dependent activities as measured by acetaminophen metabolism, dimethylnitrosamine *N*-demethylase and 4-nitrophenol hydroxylase activities (Roe et al., 1993). Phenobarbital pretreatment increased pentoxyresorufin dealkylase and benxyloxyresorufin dealkylase activities (Grant et al., 1988).

Glucuronidation was induced by phenobarbital pretreatment, whereas benzo[*a*]anthracene and phenobarbital have been shown to increase the glutathione content of the cells (Duthie et al., 1988) like benzo[*a*]anthracene (Duverger-van Bogaert et al., 1993). *N*-Acetyltransferase activity was not sensitive to various pretreatments (Fardel et al., 1992).

2.2.4. Mutation assays

Hep G2 cells could be useful in genetic toxicology testing both as the target cells and means of endogenous bioactivation. Intact Hep G2 cells were used as a metabolic activation system in the Ames test. A positive mutagenic response was observed with 2-aminofluorene, but the response was lower than that mediated by rat hepatocytes (Zhuo et al., 1986).

Benzidine, acetylbenzidine and 2-aminoanthracene produced Hep G2 cell mediated mutagenic responses in Salmonella strain TA98, while aflatoxin B_1 produced a weak positive response (Zhuo et al., 1986).

Hep G2 supernatant from control and benzo[*a*]anthracene-treated cells were used for activation of mutagens in the Ames test. Benzidine, 2-aminofluorene and 2-acetylaminofluorene were activated to mutagens and the supernatant from benzo[*a*]anthracene treated cells together with bacterial strains possessing high levels of *O*-acetyltransferase increased strongly the sensitivity of the assay (M. Duverger: in preparation). Using similar experimental conditions, heterocyclic amines were activated into mutagens in the Ames test (M. Duverger: in preparation). Hep G2 supernatant from benzo[*a*]anthracene treated cells activated benzo[*a*]pyrene into mutagens toward *Salmonella typhimurium* TA98 (Duverger-van Bogaert et al., 1993).

Cyclopenta[*a*]phenanthrenes are potent mutagens in cell-mediated mutation assays with V79 Chinese hamster cells as targets and Hep G2 cells as mediators of activation. Cyclophosphamide induced sister chromatid exchanges (SCEs) in Hep G2 cells; phenobarbital pretreatment did not modify significantly this effect (Dearfield et al., 1983).

For cytogenetic studies Human Hep G2 cells as a metabolic activation system and target for DNA damage by promutagenic carcinogens were established (Natarajan and Darroudi, 1991; Darroudi and Natarajan, 1994, 1995; Darroudi et al., 1995; Knasmüller et al., 1995). Furthermore, the abilities of isolated S9-fractions from Hep G2 cells to activate promutagenic carcinogens using CHO cells in vitro was investigated (Darroudi and Natarajan, 1993, 1994, 1995). Different cytogenetic effects, i.e., SCEs, micronuclei, cytotoxicity and/or gene mutation at the *hprt* locus, were used as biological endpoints.

For standardization and validation of these assays, a series of chemicals with known carcinogenic/non-carcinogenic potentials was se-

Table 1
Genotoxic effects of various chemicals in Hep G2 cells and in CHO cells in the presence of Hep G2 S9 fraction, using various endpoints

Test chemical	Hep G2			CHO + Hep G2 S9-fraction				
	Dose (μM) [a]	MN [b]	SCEs [c]	Dose (μM) [a]	MN	SCEs [c]	Survival [d]	HPRT − [e]
2-Acetylaminofluorene	45–90	1.5–2.5	1.5–2.6	4.5–70	2.0–8.0	1.2–3.0	12–88	7–40
4-Acetylaminofluorene	45–225	1.0	1.0	22.5–225	1.0	1.0	95–100	0
Benzo[*a*]pyrene	10–160	1.2–4.5	1.5–8.0	2–30	1.4–5.0	1.2–4.0	12–95	3–65
Cyclophosphamide	100–10000	1.3–3.5	1.2–5.0	200–5000	1.7–10.0	2.4–4.0		
Dimethylnitrosamine	500–60000	1.5–3.0	1.5–2.8					
Hexamethylphosphoramide	3–56	1.5–5.0	1.2–2.5	28–84	8.0–20.0	1.4–4.0	45–90	1–75
Pyrene	40–320	1.0	1.0	10–30	1.0	1.0	90–100	0
Safrole	100–1500	1.2–4.4	1.2–2.7	150–1000	1.2–4.8	1.2–2.4	30–95	3–45
IQ	25–300	1.0–3.0						
MeIQ	25–300	1.5–2.5						
MeIQx	25–300	1.4–2.4						
PhIP	25–300	1.5–2.3						
Trp-P-1	0.2–12.5	1.3–2.5						

[a] Dose range tested.
[b] Micronuclei. The results are presented as fold increase when compared to the control level.
[c] Sister chromatid exchanges. The results are presented as fold increase when compared to the control level.
[d] % of the control level.
[e] Gene mutations at the *hprt* locus, presented as number of induced TG^r mutants per 10^6 viable cells.

lected and comparison was made with data obtained in vivo and in vitro (using rat liver S9-fraction activation systems). The summary of results obtained with Hep G2 assays using different classes of chemicals (i.e., aromatic amines, polycyclic aromatic hydrocarbons, aminoimidazo arenes and pyridoimidazo arenes (cooked food mutagens, etc.) is presented in Table 1.

When Hep G2 cells were treated with cyclophosphamide, dimethylnitrosamine, hexamethylphosphoramide, safrole, benzo[*a*]pyrene for 1 and/or 28 h, the frequencies of micronuclei and SCEs increased in a dose-dependent manner. This increase was also dependent on the duration of treatment (Natarajan and Darroudi, 1991). However, when the putative non-carcinogen pyrene was tested negative data were obtained in both assays. In order to expand the utility of this assay in short-term mutagenicity test systems, we investigated the ability of microsomal fractions prepared from human hepatoma cells to activate procarcinogens (as mentioned above) in other mammalian cells in vitro (Darroudi and Natarajan, 1993). All promutagenic carcinogens tested were found to be effective only following metabolic activation by Hep G2 cell extracts. The non-carcinogen pyrene was not able to induce an increase in the frequencies of micronuclei or SCEs in CHO cells (Darroudi and Natarajan, 1993). Furthermore, the genotoxic potentials of several indirectly acting procarcinogens such as 2-acetylaminofluorene, benzo[*a*]pyrene, hexamethylphosphoramide, safrole and two non-carcinogens pyrene and 4-acetylaminofluorene were investigated in human hepatoma cells and in CHO cells in the presence of Hep G2 S9 fractions. In addition to micronuclei and SCE assays cell survival and point mutation at the *hprt* locus were used as biological endpoints.

A dose dependent increase in cytotoxicity and the frequencies of SCEs, micronuclei and point mutations was found in Hep G2 cells and in CHO cells in the presence of Hep G2 S9 fractions, with 2-acetylaminofluorene, hexamethylphosphoramide, benzo[*a*]pyrene and safrole, but with 4-acetylaminofluorene and pyrene no increase was found (Darroudi and Natarajan, 1994, 1995).

Mutagens such as 2-acetylaminofluorene, hexamethylphosphoramide and safrole which are known to be carcinogens in vivo (Purchase and Ray, 1981) always gave negative results for induction of chromosomal aberrations and/or point mutations (at the *hprt* locus) in CHO, V79 and/or mouse lymphoma (L5178Y) cell lines with the standard rodent S9-activation system (Natarajan and van Kesteren-van Leeuwen, 1981; Utesch et al., 1985; Fox and Delow, 1985; Lane et al., 1985; Lee and Weber, 1985). The results indicate that human Hep G2 cells themselves and their S9 fractions appeared to be more efficient in activating different classes of indirectly-acting promutagens than the activation system derived from rat liver S9-fractions. The reasons for the difference between Hep G2 and rat liver activation systems could be that: (a) rat liver S9 may not possess all of the enzymes necessary to activate diverse classes of procarcinogens (b) the activity of some detoxification enzymes may be higher in rat liver S9 than in Hep G2 cells. This could possibly explain why benzo[*a*]pyrene is more active in the induction of SCEs and micronuclei in Hep G2 cells (Natarajan and Darroudi, 1991; Darroudi and Natarajan, 1995) when compared to cultured rat hepatoma cells (Kasper et al., 1988; Rocher and Wiebel, 1988), and in CHO cells in the presence of S9-fractions isolated from Hep G2 cells than with rat liver S9-fractions (Darroudi and Natarajan, 1993).

In addition, the genotoxic potentials of different cooked food mutagens, namely 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-methylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP) and 3-amino-1,4-dimethyl-5*H*-pirido[4,5-*b*]indol (Trp-P-1) were investigated in Hep G2 cells using the micronucleus assay. All caused clear dose response effects in Hep G2 cells, and Trp-P-1 was the most potent inducer of micronuclei (Knasmüller et al., in preparation). This may indicate that cooked food mutagens are genotoxic in humans.

Among different short-term assays, the Salmonella/microsome assay (Ames et al., 1975; McCann et al., 1975) plays a dominant role in the field of mutagenicity testing. However, when the Ames test was used to assess the mutagenic potentials of hexamethylphosphoramide and safrole in the presence and absence of rat liver S9-fractions no evidence of mutagenic activity was found (Ashby et al., 1985; Baker and Bonin, 1985). However, when

we employed Hep G2 S9 fractions as an exogenous activation system, positive effects were found with hexamethylphosphoramide and safrole in the Salmonella assay using strains TA98 and TA100 (Darroudi et al., in preparation).

The Hep G2 assay appears to be the only system so far reported to be capable of detecting the mutagenic potentials of hexamethylphosphoramide and safrole in vitro (Natarajan and Darroudi, 1991; Darroudi and Natarajan, 1993, 1994). The use of S9-fractions from Hep G2 cells opens up the possibility of investigating cytotoxicity and the production of point mutations (i.e., *hprt*) by using the standard mutation assay in an in vitro mammalian system (i.e., CHO cells) and in the Ames Salmonella assay. This will obviously also make it possible to sequence the mutants produced by these chemicals, and this can also be used for the detection of the mutagenic potential of other human carcinogens in vitro.

In addition, Hep G2 supernatant from control and benzo[*a*]anthracene-pretreated cells were used for activation of mutagens (i.e., benzidine, 2-aminofluorene and 2-acetylaminofluorene) in the Ames test. These chemicals were activated to mutagens and the supernatant from benzo[*a*]anthracene-pretreated cells together with bacterial strains showing high levels of *O*-acetyltransferase activity increased significantly the sensitivity of the assay (Duverger, in preparation).

In the in vitro assays used (Hep G2 cells and CHO cells in the presence of Hep G2 S9 fractions) positive and negative results were found with carcinogens (i.e., benzo[*a*]pyrene, 2-acetylaminofluorene) and chemically related non-carcinogens (i.e., pyrene and 4-acetylaminofluorene) respectively. Further experiments are in progress in order to investigate the ability of the human Hep G2 cell system for discriminating between carcinogens and non-carcinogens of different classes.

3. Use of an established Chinese hamster epithelial liver (CHEL) cell line to activate promutagens in cytogenetic short-term assays

We used a near diploid established CHEL cell line which retains some metabolic competence to activate different classes of procarcinogens/promutagens into biologically active intermediates in cytogenetic assays (De Salvia et al., 1988; Mosesso et al., 1994) and in the *hprt* gene mutation assay (Turchi et al., 1992).

Table 2
Genetic damage induced by various chemicals, in CHEL cells, using chromosomal aberrations and sister chromatid exchanges

Test chemicals	CA	SCE
2-Aminofluorene	+	+
2-Acetylaminofluorene	+	+
4-Acetylaminofluorene	0	0
2-Aminoanthracene	0	0
IQ	+	+
Benzidine	+	+
p-Phenylenediamine	+	+
Phenacetin	+	+
Benzo[*a*]pyrene	+	+
Pyrene	0	0
7,12-dimethylbenzo[*a*]anthracene	+	+
Cyclophosphamide	+	+
Alachlor	+	+
Thiram	+	0
Ziram	+	0
Quinoline	0	0

0, no significant increase compared to control.
+, significant increase compared to control.

The test chemicals employed have been selected as indirect promutagens or procarcinogens, since they are effective in the short-term cytogenetic assays only in the presence of S9 metabolism (Ishidate et al., 1988).

The results for both chromosomal aberrations and SCEs are presented in Table 2 as positive or negative.

Aromatic amines: 2-Acetylaminofluorene, 2-aminofluorene, benzidine and *p*-phenylenediamine induced dose-related increases of aberration-bearing cells and SCEs. After treatment with the non-carcinogen 4-acetylaminofluorene the frequencies of aberration-bearing cells and SCEs were within the control level. 2-Aminoanthracene was expected to be positive, but came out negative in both assays.

Polycyclic aromatic hydrocarbons: 7,12-Dimethylbenz[*a*]anthracene induced marked and significant increases in the frequencies of aberration-bearing cells and SCEs. Significant increases in aberration-bearing cells and SCEs were also observed when

CHEL cells were treated with benzo[*a*]pyrene. On the other hand the non-carcinogen pyrene did not increase significantly the frequencies of aberration-bearing cells and SCEs.

Other compounds: Treatments with cyclophosphamide, IQ and phenacetin significantly increased the frequencies of aberrant cells and SCEs, while quinoline came out negative in both assays.

Treatments with the pesticides alachlor, thiram and ziram induced significant increases in the frequencies of aberration-bearing cells; this effect was more pronounced for alachlor. For the SCEs assay, positive results were only observed after treatment with alachlor.

The results showed that CHEL cell line is able to activate a wide range of indirect mutagens/carcinogens. For the chromosomal aberration assay 12 compounds out of 14 expected were positive while for the SCEs assay only 10 out of 14 expected (86%) were positive (Table 2). Combining both chromosomal aberration and SCEs assays the number of positive compounds was 12 out of 14 expected (86%). Furthermore, discriminated chemical carcinogens from non-carcinogens (e.g., benzo[*a*]pyrene/pyrene and 2-acetylaminofluorene/4-acetylaminofluorene).

On the other hand, compounds like 2-aminoanthracene and quinoline are negative in the CHEL cells probably due to the lack of enzymes needed for the activation of these compounds.

Furthermore, the positive results found in the literature for the in vivo micronucleus test correlate well with the positive results observed for the chromosome aberration and SCEs assays in the CHEL cells (9 positive out of 12).

4. Use of V79-derived indicator cells expressing defined rat cytochromes P450 (CYPs)

4.1. Gene mutation tests

In the papers in which the construction of V79-derived cell lines expressing rat CYPs was described, single examples of their use in gene mutation assays

Table 3
Induction of gene mutations by various mutagens in V79 cells and in V79-derived cell lines expressing rat cytochromes P450

Test compound	Response in gene mutation assay							
	V79-MZ	V79-MZ +S9	V79-MZ-r1A1	V79-MZ-r1A2	V79-MZ-r2B1	V79-NH	V79-NH-r1A2	others
Benzo[*a*]pyrene	0	++	+++ [a]	0	0	nt	0	
(±)-Benzo[*a*]pyrene-*trans*-7,8-dihydrodiol	+	++++	++++ [a]	+++ [a]	+	(+)	+++ [a]	+ [b]
7,12-Dimethylbenz[*a*]anthracene	0	++++	(+)	nt	0	nt	0	
(±)-Chrysene-*trans*-1,2-dihydrodiol	0	+++	+++ [a]	0	0	nt	(+)	
(±)-Chrysene-*trans*-3,4-dihydrodiol	0	(+)	0	0	nt	nt	0	
2-Aminoanthracene	0	(+)	0	(+)	0	0	+++	
2-Acetylaminofluorene	0	0	0	0	0	0	+	+++ [c]
Aflatoxin B_1	0	++	++	nt	++	nt	(+)	(+) [b]
N-Nitrosodibutylamine	0	+	0	nt	++ [a]	nt	(+)	

Acquisition of resistance toward 6-thioguanine was used as a marker for gene mutations. Some experiments with the parental V79 line were carried out using rat liver S9 mix from Aroclor-1254-treated male Sprague-Dawley rats (column headed V79-MZ + S9). In all other experiments, no external metabolizing system was added. V79-NH and V79-NH-r1A2 cells differ from all other cell lines used by the expression of an endogenous acetyltransferase. All compounds were tested over wide concentration ranges, usually in at least 2 independent experiments. At the optimal concentration, allowing for survival of > 10% of the cells, they increased the mutant frequency above the spontaneous levels (usually about 5×10^{-6}) as follows: 0, no reproducible nor concentration-dependent increase observed; (+), weak increase (in all or most experiments $< 25 \times 10^{-6}$); +, $25–60 \times 10^{-6}$; ++, $60–150 \times 10^{-6}$; +++, $150–400 \times 10^{-6}$; ++++, $> 400 \times 10^{-6}$; nt, not tested.

[a] For equal mutagenic effects, lower substrate concentrations were required than in liver S9 mix-mediated experiments (1/3 to 1/500, depending on the test compound and the expressed cytochrome P450 form).

[b] V79-MZ-r2B2 (generously obtained from T. Friedberg).

[c] V79-MZ-r1A2-rST1C1, a cell line expressing rat sulfotransferase 1C1 in addition to rat cytochrome P450 1A2.

using the *hprt* locus were given (Doehmer et al., 1988; Dogra et al., 1990; Wölfel et al., 1991). We have now systematically applied this test system, using the cell lines expressing different CYPs, to a series of representative mutagens and have compared the results with those obtained in rat liver S9 mix-mediated tests with parental V79 cells (Table 3). The methods of the gene mutation assay were the same as used in previous studies (Glatt et al., 1987; Glatt, 1993). In addition, we have studied the reproducibility of the mutagenicity results in the new cell lines.

V79-NH cells and V79-NH-r1A2 (old name: XEMd-NH) cells express an endogenous acetyltransferase, which is absent in V79-MZ cells and cell lines derived from them [V79p, V79-MZ-r1A1 (old name: XEM2), V79-MZ-r1A2 (old name: XEMd-MZ), V79-MZ-r2B1 (old name: SD1) and V79-MZ-r2B2] (H.R. Glatt, C. Wölfel, F.J. Wiebel and J. Doehmer, manuscript in preparation). V79p cells were genetically engineered for puromycin resistance, without concomittant introduction of a mammalian xenobiotic-metabolizing enzyme (Czich et al., 1994). The other cell lines express the rat CYPs indicated in their name.

Benzo[*a*]pyrene was mutagenic in parental V79 cells only in the presence of liver S9 mix. No external activating system was, however, required for mutagenic activity in V79-MZ-r1A1 cells (Table 3). The maximal response was stronger, and for equal effects about 500-fold lower substrate concentrations were sufficient, than in the S9-mediated test with parental V79 cells. Benzo[*a*]pyrene showed no mutagenicity in the genetically engineered cells expressing CYP1A2 or CYP2B1. The proximate mutagen, benzo[*a*]pyrene-*trans*-7,8-dihydrodiol (B[*a*]P-7,8-diol), showed weak direct mutagenic effects in parental V79 cells, when used at very high concentrations. Addition of liver S9 mix strongly enhanced the effect. Similarly, strong effects were observed in V79-MZ-r1A1 cells, without the need of an external activating system, at about 1/500 of the concentrations required in the S9-mediated test with V79 cells. B[*a*]P-7,8-diol was also activated by expressed CYP1A2, but not by CYP2B1 or CYP2B2. 7,12-dimethylbenzo[*a*]anthracene is commonly used as a positive control compound in V79-mediated tests with V79 cells, as it shows strong effects which are completely dependent on the presence of S9 mix. However, in the genetically engineered cell lines used, it was virtually inactive, although its proximate mutagen, 7,12-dimethylbenzo[*a*]anthracene-*trans*-3,4-dihydrodiol was detected as a potent mutagen in V79-MZ-r1A1 cells at about 1000-fold lower concentrations than in the S9-mediated test with V79 cells (Glatt et al., 1993). These results suggest that CYP1A1 is inefficient in the oxidation of 7,12-dimethylbenzo[*a*]anthracene to 7,12-dimethylbenzo-[*a*]anthracene-3,4-oxide and/or that the relatively low level of epoxide hydrolase present in V79 cells (Glatt et al., 1987) is inefficient in the hydrolysis of 7,12-dimethylbenzo[*a*]anthracene-3,4-oxide. Among the chrysene-dihydrodiols tested, the carcinogenic isomer, (±)-chrysene-*trans*-1,2-dihydrodiol was strongly mutagenic in CYP1A1-expressing cells, whereas its 3,4-regioisomer, which was negative in the carcinogenicity studies performed, showed no mutagenicity in any CYP-expressing cell line used.

The aromatic amine 2-aminoanthracene demonstrated only marginal mutagenic activity and the aromatic amide 2-acetylaminofluorene was completely inactive in V79-MZ and V79-NH cells, when rat liver S9 mix was used as a metabolizing system. Similar negative or marginally positive results were obtained in V79-MZ derived cell lines expressing CYP1A1, 1A2 or 2B1. However, 2-aminoanthracene showed very strong mutagenic effects in V79-NH-r1A2 cells. This result supports the view that *N*-oxidation has to be followed by *O*-acetylation for the formation of the ultimate mutagen. In contrast to 2-aminoanthracene, 2-acetylaminofluorene was only weakly mutagenic in V79-NH-r1A2 cells. However, it induced a high frequency of gene mutations even at very low substrate concentrations in a new V79-MZ-r1A2-derived cell line expressing rat CYP1A2 as well as rat hydroxamic acid sulfotransferase (ST1C1) (I. Bartsch, Y. Yamazoe, J. Doehmer and H.R. Glatt, manuscript in preparation). Hence, the final activation of 2-acetylaminofluorene involves sulfonation rather than acetylation. These examples demonstrate the importance of conjugating enzymes in the activation of mutagens, an aspect which is ignored in the use of S9 for metabolic activation.

Aflatoxin B_1 was inactive in parental V79 cells in the direct test, but was activated to a mutagen by liver S9 mix as well as by expressed CYPs. All expressed CYP forms studied showed some activity,

Table 4
Reproducibility of the induction of gene mutations in V79-Mz-r1A2 cells by 2-aminoanthracene (2-AA) and (±)-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol (BP-7,8-diol)

Experiment No.	Experimentator	Cells (passage No.)	Serum		$10^6 \times$ mutant frequency		
			Batch No.	Conc. (%)	Untreated	BP-7,8-diol	2-AA
1	A	2	1	5	18 ± 2	–	298
2	A	2	1	10	7 ± 1	–	285
3	A	5	1	10	15 ± 0	–	409
4	A	7	1	10	19 ± 3	–	206
5	A	3	2	10	15	177	386
5	A	3	3	10	5	364	144
5	A	3	4	10	12	396	209
5	A	3	5	10	18	208	198
5	A	3	6	10	20	415	279
5	A	3	7	10	5	418	267
6	A	4	2	10	6	202 ± 14	–
7	A	10	2	10	1 ± 0	–	151
8	B	21	2	5	7 ± 4	–	182
9	C	6	8	5	2 ± 1	–	154 ± 10
10	C	9	8	5	3 ± 1	–	125
11	C	11	8	5	3 ± 1	–	108
12	C	13	8	5	5 ± 1	–	173
13	C	19	8	5	18 ± 1	122	271 ± 48

The results of all experiments in which 2-AA (0.1 μM) and or BP-7,8-diol (3 μM) were used at the indicated concentrations are shown. They were carried out over a period of 4.5 years. Experiments designed with different numbers were conducted on different occasions. Values were obtained from a single exposed culture, or are means and SE from 2 independent cultures.
–, not done.

Table 5
Mutant frequencies in cytochrome P450-proficient and -deficient V79-derived cell lines exposed separately or as mixed cultures to (±)-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol (BP-7,8-diol)

Cells	Exposure to BP-7,8-diol	Results					
		Standard selection		Selection A		Selection B	
		CE	$10^6 \times$ MF	CE	$10^6 \times$ MF	CE	$10^6 \times$ MF
V79-MZ	0	0.52	1	0.003	–	0.000	–
V79-MZ	+	0.73	3	0.003	–	0.000	–
V79p	0	0.75	2	0.000	–	0.77	1
V79p	+	0.67	1	0.000	–	0.65	2
V79-MZ-r1A1	0	0.85	2	0.78	2	0.000	–
V79-MZ-r1A1	+	0.48	310	0.42	288	0.000	–
V79p + V79-MZ-r1A1 [a]	+	0.30	348	0.23	275	0.23	146
V79p + V79-MZ-r1A1 [b]	+	0.36	480	0.22	484	0.23	215
V79p + V79-MZ-r1A1 [c]	+	0.24	528	0.32	272	0.22	173
V79p + V79-MZ-r1A1 [d]	+	0.42	334	0.30	278	0.23	190
V79p + V79-MZ-r1A1 [e]	+	0.79	220	0.42	280	0.40	180

In the incubations with a single type of cells, 1.5×10^6 cells were seeded with 30 ml medium in a 15-cm Petri dish. In the experiments with mixed cultures, 0.75 [a], 1 [b], 1.5 [c], 2 [d] and 3 [e] $\times 10^6$ cells of each type were used. After 18 h, the cultures were exposed to BP-7,8-diol (0.1 μM), or to the solvent only, for 24 h. After the expression period, the frequency of 6-thioguanine-resistant cells was determined either by the standard procedure, or with additional selection for V79-MZ-r1A1 cells (using G418, selection A) or V79p cells (using puromycin, selection B).
CE, cloning efficiency; MF, mutant frequency; –, not applicable.

with the strongest effects being induced in V79-MZ-r1A1 cells (already at relatively low concentrations) and V79-MZ-r2B1 cells (only at rather high concentrations). The mutagenicity of *N*-nitrosodibutylamine was readily detected in V79-MZ-r2B1 cells, more efficiently than in S9-mediated tests with parental V79 cells. Weak mutagenicity was detected in V79-NH-r1A2 cells as well (Table 3).

The stability and expression of heterologous genes may be influenced by the culture conditions (e.g., cell density, serum batch, handling of the cells). It is therefore important to use appropriate positive controls. We usually included benzo[*a*]pyrene or B[*a*]P-7,8-diol in experiments with V79-MZ-r1A1 cells, B[*a*]P-7,8-diol in experiments with V79-MZ-r1A2 cells, 2-aminoanthracene or B[*a*]P-7,8-diol in experiments with V79-NH-r1A2 cells, and aflatoxin B_1 or *N*-nitrosodibutylamine with V79-MZ-r2B1 cells. In Table 4, the results of all experiments are listed in which 2-aminoanthracene and/or B[*a*]P-7,8-diol were tested in V79-NH-r1A2 cells. They were carried out over a period of 4.5 years by 3 persons, using different batches and concentrations of fetal bovine serum and cells from different passages after the transfection. In spite of these experimental variables, the results varied in a rather small range. A similarly small variation was observed with the positive controls used in the V79-MZ-r1A1 cells, whereas those with the V79-MZ-r2B1 and V79-MZ-r1A2 cells were somewhat more larger. It is not clear whether this difference indicates a higher variability of the latter cell lines or simply reflects the fact that the concentration-mutagenicity and concentration-toxicity relationships of the positive control compounds used were not equally ideal in all cell lines.

4.2. Use of V79-derived, CYP-proficient cells as external activating systems in mammalian and bacterial gene mutation tests

Many in vitro genotoxicity test systems require the use of external activating systems. Moreover, many discrepancies between the results in different genotoxicity test systems may have their reason in differences in the activating system used. We therefore examined the suitability of V79-derived, CYP-expressing cell lines for external activation.

In a first series of experiments, we co-exposed mixed cultures of CYP-deficient V79p and CYP1A1-proficient V79-MZ-r1A1 cells to B[*a*]P-7,8-diol. V79p are resistant to puromycin, but sensitive to the antibiotic geneticin (G418), whereas the reverse is true for V79-MZ-r1A1 cells (Table 5). These properties allowed for separate determination of the frequencies of the 6-thioguanine mutants in both cell lines from mixed cultures. B[*a*]P-7,8-diol was strongly mutagenic in V79p cells when the exposure took place in the presence of V79-MZ-r1A1 cells, but not in their absence (Table 5). The mutagenic effects observed in V79p cells were about half as strong as in the co-exposed V79-MZ-r1A1 cells. The results indicate that the ultimate mutagen formed was capable of diffusing from one cell to the other, although no full equilibration appeared to be achieved. The strong effect in V79p cells under these conditions was surprising, as much higher concentra-

Table 6
Mutagenicity of (±)-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol (BP-7,8-diol) in Chinese hamster V79p cells in the presence of NADPH-fortified homogenate of V79-derived cell lines expressing rat cytochromes P450

Enzyme preparation	$10^6 \times$ frequency of 6-thioguanine-resistant cells			
	Control	0.3 μM	3 μM	30 μM
V79p homogenate	3, 6	7	4,7 [a]	17 [a], 25,29
V79-NH-r1A2 homogenate	4	nt	nt	15
V79-MZ-r2B1 homogenate	6	nt	nt	27
V79-MZ-r1A1 homogenate	1, 9	20	11 [a], 54	29 [a], 57,95 [b]
Liver S9, untreated rat	6	nt	nt	252

The concentration of BP-7,8-diol used was 0 to 30 μM as indicated. The subcellular preparation was added to a concentration of 10 mg of protein per incubation (18 ml) unless specified otherwise ([a] 2 mg, [b] 20 mg). Each value is based on 6 selection plates derived from one treated culture.
nt, not tested.

tions of B[*a*]P-7,8-diol were required in liver S9 mix-mediated assays in V79-MZ cells for the induction of equal mutagenic effects (see preceding section).

In other experiments, homogenate of CYP-proficient V79-derived cells, supplemented with the same cofactors as used in liver S9 mix, were added to V79p cells during the exposure to B[*a*]P-7,8-diol (Table 6). The presence of homogenates of V79p, V79-NH-r1A2 or V79 did not enhance the mutagenicity of B[*a*]P-7,8-diol above the level observed in the absence of an external activating system. V79-MZ-r1A1 homogenate led to activation, but its efficiency was substantially weaker than that of liver S9 mix from control rats (Table 6) or Aroclor 1254-treated rats (data not shown). Moreover, a clear-cut activation required the use of large amounts of V79-MZ-r1A1 homogenate (10 mg protein, equivalent to 10^8 cells per incubation). This method, therefore, is not feasible for routine purposes, because costs and labor are excessive. It is striking that the use of V79-MZ-r1A1 homogenate rather than intact cells decreased its efficiency for the activation of B[*a*]P-7,8-diol from much superior to much inferior to rat liver S9 mix. The reasons for these differences are not clear, although the duration of the exposure (2 h with the subcellular preparations versus 24 h with intact cells) and the geometry (3-dimensional for the subcellular preparations versus 2-dimensional with the intact cells, which were attached together with the V79p target cells to the culture vessel) may have played a role.

Several promutagens were tested in *Salmonella typhimurium* TA98 in the presence of cofactor-supplemented homogenate of CYP-proficient V79-derived cells (Table 7). None of them was activated by homogenate of V79p control cells, whereas the presence of V79-MZ-r1A1 homogenate led to the activation of B[*a*]P-7,8-diol and the aromatic amines 2-aminoanthracene and IQ. The presence of CYP1A2-expressing cells led to the activation of the aromatic amines, as well. In all these cases, the number of induced mutants was increased with an increase in the amount of homogenate used. Although 2-aminoanthracene was much more mutagenic in V79-NH-r1A2 than V79-MZ-r1A2 cells when tested in

Table 7
Mutagenicity of benzo[*a*]pyrene (BP), (±)-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol (BP-7,8-diol), 2-aminoanthracene (2-AA) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in *S. typhimurium* TA98 in the presence of NADPH-fortified homogenate of V79-derived cell lines expressing rat cytochromes P450

Activating system		Revertants per plate				
Type	Amount, mg protein	control	BP(10 μg)	BP-7,8-diol(2 μg)	2-AA(1 μg)	IQ(1 μg)
V79p homogenate	0.2	44 ± 5	40 ± 3	48 ± 2	36 ± 7	50 ± 7
	2	24 ± 1	30 ± 5	69 ± 4	44 ± 5	55 ± 3
V79-MZ-r1A1 homogenate	0.2	39 ± 83	35 ± 8	58 ± 6	64 ± 11	50 ± 9
	2	38 ± 1	43 ± 4	208 ± 11	664 ± 123	181 ± 21
V79-MZ-r1A2 homogenate	0.2	25 ± 1	29 ± 3	42 ± 9	89 ± 10	196 ± 4
	2	29 ± 2	34 ± 4	84 ± 5	612	1970 ± 10
V79-NH-r1A2 homogenate	0.2	31 ± 0	31 ± 3	37 ± 7	58 ± 3	133 ± 12
	2	33 ± 9	35 ± 1	43 ± 2	325 ± 32	1590 ± 110
V79-MZ-r2B1 homogenate	0.2	28 ± 1	30 ± 4	64 ± 7	nt	52 ± 6
	2	31 ± 2	36 ± 10	86 ± 14	nt	51 ± 1
Liver S9, untreated rat	0.2	34 ± 6	47 ± 4	99 ± 4	308 ± 27	741 ± 35
	2	31 ± 7	331 ± 21	589 ± 43	2170 ± 40	4100 ± 200
Liver S9, Aroclor 1254-treated rat	0.2	47 ± 2	266 ± 4	395 ± 75 [a]	3450 ± 200	> 10000
	2	37 ± 5	762 ± 15	147 ± 1 [a]	3090 ± 140	> 10000

A 20-min liquid preincubation assay was used. Total cell homogenate of the cells was supplemented with the same cofactor mix as used with liver S9 fraction. Liver S9 was prepared from adult male Sprague-Dawley rats. Values are means ± SE from 3 plates (incubations with 0.2 mg protein) or 2 plates (incubations with 2 mg protein). All test compounds were also tested at lower dose levels, at which they showed lower or equal mutagenic effects, unless indicated. ([a] On decreasing part of the dose-response curve, probably due to bacteriotoxicity). nt, not tested.

the endogenous *hprt* locus (*see* preceding section), its mutagenicity in Salmonella was very similar with the homogenate of both cell lines. These results are reasonable, as similar levels of CYP1A2 protein and enzyme activity were observed in both cell lines (H.R. Glatt, C. Wölfel, F.J. Wiebel and J. Doehmer, manuscript in preparation), the acetyltransferase of the V79-NH-r1A2 cells may be inactive in the homogenate due to the lack of cofactor, and the final activation may be carried out by bacterial acetyltransferases. Likewise, it is interesting that 2-aminoanthracene was activated even somewhat more efficiently by homogenate of the CYP1A1-expressing cell line than by that of the CYP1A2-proficient cell lines, whereas IQ was activated with high superiority by the latter preparations. While the use of subcellular preparations of genetically engineered cell lines appears to be useful for the elucidation of critical enzymes, it may be inefficient for routine questions, since liver S9 mix from Aroclor 1254-treated rats, and even from control rats, led to stronger effects with all promutagens investigated.

4.3. Unscheduled DNA synthesis (UDS)

Additional provisional studies in Chinese hamster V79 cells expressing rat CYP1A2 and acetyltransferase (V79-NH-r1A2) (Doehmer, 1993) were aimed at assessing DNA repair (unscheduled DNA synthesis, UDS) and DNA strand breakage. To assess UDS it was necessary to remove the complication of S-phase cells which interfere with the measurement of incorporation of [^{3}H]thymidine. The various approaches discussed by Waters (1984) for inhibition of cell division were investigated. Hydroxyurea was not employed to inhibit cell division since there was evidence of DNA damage induced by this agent at the concentration (10 mM) required. Rather, the approach of Benigni et al. (1983) to minimise cell division was employed. Briefly, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing glutamine, foetal calf serum and geneticin for 24 h. Fresh medium was then applied and the cells were grown to confluence with medium changes every 48 h. Cell cultures were then treated with arginine-free, serum-reduced medium for a period of 72 h prior to treatment with pro-genotoxic agent for 6 h and analysis of UDS based on an autoradiographic technique (Chipman and Davies, 1988). Cell division was found to be inhibited sufficiently to allow UDS to be assessed (cell numbers per incubation were retained at 5×10^4 during incubation with arginine-free, serum -reduced medium compared to an elevation to 5×10^5 cells in control incubations).

A positive effect (6.36 net nuclear grain count compared to -0.76 in controls) was seen in response to the direct acting genotoxicant *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.05 μM). However, for genotoxicants that require metabolic activation (2-acetylaminofluorene and IQ) no positive response was seen at concentrations (up to 1 and 100 μM, respectively) which give marked effects in rat and human hepatocytes (Chipman and Davies, 1988; Paterson and Chipman, 1987). This lack of response was found to be influenced by the critical need for cell division in order for the CYP1A2 gene to be well expressed. The activity of this enzyme was approx. 10-fold lower in quiescent cells (arginine-free) compared to the activity in cells in logarithmic growth phase. This phenomenon was emphasised by the results of mutagenicity assays in *S. typhimurium* strain TA98 employing S9 obtained from quiescent and logarithmic growth phase V79-NH-r1A2 cells. S9 from V79-NH-r1A2 cells in the growth phase was clearly able to activate 2-aminoanthracene and IQ albeit at a level substantially lower than found with an equivalent number of hepatocytes from Wistar rats.

These results highlight the dilemma regarding attempts to detect UDS in V79-NH-r1A2 cells; the optimum conditions for CYP enzyme expression are not compatible with the optimum conditions (non-proliferation) for the measurement of DNA repair. Two other techniques were found to be potentially able to overcome this problem. Firstly the complementary technique of employing caesium chloride equilibrium gradients for the separation of parental and daughter DNA strands was used based on that reported by Andrae (1984). This procedure allows the separation of parental DNA strands by their non-incorporation of bromodeoxyuridine (BrdU) and UDS is then measurable in the parental DNA strands through incorporation of [^{3}H]cytidine. The second approach was to exploit a fluorescence activated cell sorter to select cells that had not entered S-phase during the period of incubation with genotoxicant.

By detecting BrdU incorporation with the use of anti-BrdU fluorescein antibody followed by propidium iodide staining of DNA, cells in G1 could be enriched from cells in S or G2 phase. The quenching by BrdU of Hoechst 33258 dye can also be exploited to select cells that incorporate BrdU (Poot et al., 1989). Although these more sophisticated techniques might allow the effective measurement of UDS in a dividing population of V79-NH-r1A2 cells the approach is not realistic for routine testing of genotoxicants.

4.4. Measurement of DNA strand breakage

In contrast to UDS, the measurement of DNA strand breakage is not hampered by cell division. DNA strand breakage was assessed in V79-NH-r1A2 cells and in parental V79 cells by single cell gel electrophoresis (the Comet assay, Singh et al., 1988). Cells were cultured (initial seeding density, $2 \times 10^3/cm^2$ in 75 cm flasks) for a total of 48 h in DMEM containing 10% foetal calf serum, glutamine (2.0 mM) and geneticin (0.04%). The medium was then changed to serum-free DMEM and the cells were treated with IQ as indicated, for 1 h. Cells were then lysed and electrophoresed at high pH. The migration of DNA from the nucleus (comets) is indicative of DNA strand breakage and was measured by microscopy and image analysis (Zeiss Axiovert 10, Germany, and Comet software, Kinetic Imaging, UK). V79-NH-r1A2 cells responded to IQ at a concentration of 2.5 μM and above whereas there was no evidence of DNA strand breakage by IQ in the V79 cells devoid of CYP activity (Davies et al., 1995). The proportion of cells that responded to IQ was also concentration dependent; 90% of V79-NH-r1A2 cells showed no (63%) or minimal (27%) DNA strand breakage, whereas at 25 μM, 67% of cells exhibited high or medium damage (based on > 20% DNA in the comet tail, Anderson et al., 1994). The effect was not associated with any evidence of cytotoxicity as measured by leakage of lactate dehydrogenase. At 25 μM, the % cell viabilities after 1 h incubation were 96.2 (V79-NH-r1A2) and 95.7 (V79).

These results demonstrate that of the two techniques investigated (UDS versus Comet assay) the latter is technically more applicable to the measurement of DNA damage in V79-NH-r1A2 cells expressing CYP1A2. DNA strand breakage can be produced not only by direct chemical induced scission but also from alkali-labile sites and indirectly via excision repair enzymes. DNA strand breaks are therefore produced by a variety of chemical mutagens and carcinogens (Sina et al., 1983; McKelvey-Martin et al., 1993). It therefore represents a sensitive, non-specific assay which, when coupled to the use of cells expressing specific CYP enzymes, can indicate the extent and mechanism of metabolic activation of potentially a wide range of pro-genotoxic agents. The assay is also applicable to other cell lines (e.g., Hep G2 cells).

4.5. Sister chromatid exchanges

Further studies were carried out with V79 derived CYP proficient cells, using cytogenetic endpoints as indicators of DNA damage. The methods have been described elsewhere (Rodrigues et al., 1994). The carcinogenic aromatic amines and amides 2-aminoanthracene, 2-aminofluorene and 2-acetylaminofluorene and IQ, as well as the non-carcinogen 4-acetylaminofluorene were studied for their capacity to induce SCEs in CYP1A2 expressing cells (V79-MZ-r1A2). A further cell line expressing CYP1A2 as well as endogenous acetyltransferase activity (V79-NH-r1A2) was studied to ascertain the importance of this activity in the metabolic activation of the compounds studied. After 27 h treatment with the chemicals, 2-aminofluorene and 2-acetylaminofluorene induced a marked increase in SCEs, in the V79-MZ-r1A2 cell line, when compared to the parental V79-MZ cell line. After a 45 h treatment 2-aminoanthracene also induced a marked increase in SCEs in this cell line. The presence of acetyltransferase activity enhanced the increase in SCEs for 2-aminoanthracene, 2-aminofluorene and 2-acetylaminofluorene, once again supporting the view that acetylation following *N*-hydroxylation is an important step in the production of ultimate metabolites of these compounds. In contrast to results obtained with mutation at the *hprt* locus (see above), the induction of SCEs by 2-acetylaminofluorene is not dependent on the presence of sulfotransferase activity. The non-carcinogen 4-acetylaminofluorene was essentially negative in the

CYP proficient cells, which when compared to the results obtained with 2-acetylaminofluorene, suggest a specificity of the system studied. However, the potent heterocyclic amine and carcinogen, IQ did not elicit a positive response in either CYP proficient cell line studied, which is in contrast to its mutagenicity demonstrated in various short-term tests. The negative results obtained may be due to a lack of sulfotransferase, on one hand, and a lack of specificity of the acetyltransferase expressed for metabolites produced by CYP1A2, on the other hand (Rodrigues et al., 1994) (Table 8).

4.6. Chromosomal aberrations

A second cytogenetic endpoint, the induction of chromosomal aberrations, was studied, also with the aromatic amines and amides mentioned in the preceding section. The induction of chromosomal aberrations by 2-aminoanthracene (tested at 5 to 50 μM), 2-aminofluorene (5.5 to 55 μM) and 2-acetylaminofluorene (4.5 to 45 μM) was negative with all test compounds studied, which is in contrast to the other indicators used with these cells. 4-Acetylaminofluorene (4.5 to 45 μM) was also negative, as was IQ (10 to 90 nM) (Rodrigues et al., 1994). Taken as a whole, the results obtained with this genetic endpoint show that it is remarkably insensitive to the mutagenic activity of the compounds studied, in the conditions tested. The lack of induction of chromosomal aberrations may be due to differences in the metabolites produced, capable of inducing different genetic effects.

Other carcinogens (aflatoxin B_1, cyclophosphamide, benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]-anthracene and dimethylnitrosamine) induced chromosomal aberrations in V79-derived, CYP-proficient cell lines (Kulka et al., 1993).

Table 8
Frequency of SCEs per chromosome induced by aromatic amines and amides tested in the V79 cell lines expressing rat CYP 1A2 (V70-MZ-r1A2), CYP 1A2 and acetyltransferase (V79-NH-r1A2) and the control lines V79-MZ and V79-NH

Test compound	Dose(μM)	V79-MZ	V79-NH	V79-MZ-r1A2	V79-NH-r1A2
2-Aminoanthacene	0	0.33	0.40	0.43	0.44
	5	0.35	0.46	0.65	0.96
	12.5	0.36	0.42	0.71	1.43
	25	0.37	0.51	0.75	1.48
	50	0.35	0.56	0.83	1.63
2-Aminofluorene	0	0.31	0.38	0.42	0.50
	27.5	0.32	0.34	0.57	1.04
	55	0.29	0.46	0.73	1.09
	82.5	0.37	0.52	0.75	1.10
	164	0.36	0.31	0.82	1.22
2-Acetylaminofluorene	0	0.25	0.40	0.39	0.53
	4.5	0.28	0.34	0.54	0.96
	11.2	0.32	0.26	0.60	0.92
	22.5	0.26	0.33	0.68	1.07
	45	0.28	0.19	0.77	1.28
4-Acetylaminofluorene	0	0.17	0.28	0.32	0.32
	4.5	0.22	0.25	0.40	0.29
	11.2	0.29	0.31	0.34	0.31
	22.5	0.25	0.32	0.26	0.35
	45	0.25	0.36	0.32	0.38
IQ	0	0.22	0.31	0.34	0.31
	0.010	0.23	0.31	0.38	0.36
	0.030	0.20	0.28	0.38	0.36
	0.090	0.26	0.24	0.34	0.37

5. Red blood cells as a source for metabolic activation

The enzyme systems present in erythrocytes are involved in the protection of its main components haemoglobin and the cellular membrane from oxidative attack by a large range of compounds and maintain the energy supply of the cell. More recently, evidence has been accumulated that red blood cells are also capable of metabolizing certain drugs and chemicals.

5.1. Enzymatic activities of red blood cells

Erythrocytes are endowed with metabolic systems of defense against oxidants. Thus erythrocytes contain superoxide dismutase, that catalyzes the dismutation of two superoxide radicals to H_2O_2 and oxygen (Carrell et al., 1975), and catalase and glutathione peroxidase which are involved in the degradation of H_2O_2 (Rapoport and Müller, 1974). Erythrocytes contain high concentrations of reduced glutathione and glutathione transferases which participate in the local detoxification of various drugs and chemicals which otherwise might damage the red blood cell membranes or haemoglobin (Eckert and Eyer, 1986).

Acetylcholine esterases and arylesterases activities have been identified to play an important role in the metabolism of exogenous drugs such as aspirin or cocaine (Cossum, 1988 for review). Red blood cell membrane contains proteolytic enzymes capable of hydrolysing di-, tri- and polypeptides. These enzymes may be associated with the normal degradation of old red blood cells in vivo. They may also play a role in the metabolism of endogenous and exogenous peptides/proteins. Dehydrogenase activity probably intervenes in the interconversion of some androgens and estrogens (Cossum, 1988). The red blood cell also contains catabolic and anabolic pathways for purines and pyrimidines and may be the site of substantial metabolism of biogenic amines. A moderate acetylation activity was also detected (Cossum, 1988).

More recently, the potential of erythrocytes as a xenobiotic activating system has come into focus.

Human whole blood, as well as washed erythrocytes were shown to catalyze the production of *p*-aminophenol from aniline (Mieyal and Blumer, 1976; Mieyal, 1985).

Haemoglobin in a reconstituted system containing NADPH and CYP reductase, catalyzes *p*-hydroxylation of toluidine, *N*-demethylation of benzphetamine and *O*-demethylation of anisidine, anisole, *p*-methoxyphenol and *p*-nitroanisole (Starke et al., 1984). Under similar conditions, haemoglobin catalyzed the oxidation of cyclohexane to cyclohexanol derivates. All these are prototypic monooxygenase activities.

N-Oxidation of 4-chloroaniline was also effected by haemoglobin in a reconstituted system; the oxidative attack on vulnerable nitrogen centres is catalyzed, in mammalian cells, by CYP and/or flavin-containing monooxygenases (Golly and Hlavica, 1983).

2-Aminofluorene was converted into *N*-hydroxy-2-aminofluorene by cytosol of red blood cells and NADH and NADPH are effective electron donors. The activity is inhibited by carbon monoxide, suggesting that the active form of the catalyst is probably reduced haemoglobin (Duverger-van Bogaert et al., 1992). Red blood cell cytosol catalyzes *N*-methylaniline demethylation; this activity is linked to oxyhaemoglobin and is enhanced in the presence of NADH- and the NADH-methemoglobin reductase system. Haemoglobin in its oxygenated form is involved in this reaction (Stecca and Duverger-van Bogaert, 1989; Stecca et al., 1992). Styrene was metabolized to styrene oxide by oxygenated human erythrocytes in vitro; methemoglobin, in the presence of H_2O_2 was also able to support styrene oxidation to styrene oxide (Belvedere and Tursi, 1981; Cantoni et al., 1982; Tursi et al., 1983). All these activities are probably related to the fact that the mature red blood cell does not possess any CYP but contains most of the elements of an electron transport system which in other tissues forms part of a drug-metabolizing system. Red blood cells are equipped with two electron transport system that maintain haemoglobin in the ferrous oxygenated form. The primary pathway involves a NADH-dependent reductase associated with cytochrome b_5 (Kuma, 1981); the secondary NADPH-dependent pathway is of minor importance in normal conditions and can be recruited by the administration of methylene blue as exogenous electron carrier (Sass et al., 1969). It was not

known whether these components are organized in a manner which could facilitate the metabolism of endogenous and exogenous substrates of a CYP system. However, some studies suggest that mechanisms differ between the oxidation reactions catalyzed by haemoglobin and CYP (Ortiz de Montellano and Catalano, 1985; Kelder et al., 1991). These differences are probably a consequence of a structural difference within the fifth ligand to heme: the thiolate ligand of P450 differs from haemoglobin in that it consists of the imidazole nitrogen and is responsible for the especially low redox potential of P450 in contrast to haemoglobin (Dawson, 1988).

5.2. Mutagenicity tests

Erythrocytes are able to activate promutagens; this was first reported by Ray and Altenburg (1978) who compared SCEs frequencies induced by Na_2SeO_3 in whole-blood cultures and isolated lymphocytes. They observed that SCEs were induced only in the presence of erythrocytes or erythrocyte lysate. In two human cell lines (XP 12 RO and a lymphoblastoid line) Na_2SeO_3 similarly increased SCEs only after erythrocyte lysate was added to the culture. These results were confirmed by Mehnert et al. (1984).

A significant concentration-related increase in SCE frequency was induced by aniline in human whole blood culture and in the mononuclear leukocyte cultures in the presence of haemoglobin (Wilmer et al., 1984).

Styrene and styrene 7,8-oxide were able to induce SCEs in whole blood lymphocyte cultures of several donors. The induction of SCEs by styrene depended on the presence and the number of red blood cells in the cultures. This activation probably results from the conversion of styrene into styrene 7,8-oxide by oxyhemoglobin (Norppa et al., 1983). The metabolizing capacity of erythrocytes is not restricted to human cells: styrene increases SCEs also in whole blood lymphocyte cultures of rat (Norppa and Tursi, 1984). Several analogs of styrene, differing from the parent compound by 1 or 2 substitutions, can induce SCEs in human whole blood lymphocyte cultures without exogenous metabolite activation. The presence of the vinyl side chain and the presence of an aromatic nucleus appears to be needed for the SCE-inducing capacity (Norppa and Tursi, 1984). Styrene produced a clear dose-dependent increase in chromatid-type aberrations in whole-blood cultures and a weaker effect in cultures of isolated lymphocytes. A part of this activation occurs in the lymphocytes (Jantunen et al., 1986).

Human erythrocytes can metabolically activate benzo[*a*]pyrene and the resulting excess SCE and micronuclei depends in a linear manner on the dose. Quinone derivatives detected by high pressure liquid chromatography are thought to be responsible for the cytogenetic abnormalities observed (LoJacono et al., 1992).

In the presence of erythrocytes, cyclophosphamide induced an excess of mitotic recombination, mitotic gene conversion and reverse mutation in the diploid D_7 strain of *Saccharomyces cerevisiae*. The genotoxic response depended markedly on the amount of erythrocytes added to the assay (Corsi et al., 1985).

Purified mouse erythrocytes have the ability to activate 2-aminofluorene and thereby increase the mutation frequency of strain TA1538 and of strain TA98 of *S. typhimurium*. The genetic response is related to the amount of erythrocytes/plate (Cantelli-Forti et al., 1986). Using human red blood cell cytosol in the presence of methylene blue in the Ames test *S. typhimurium* strain TA98, 2-aminoanthracene, 2-aminofluorene and 2-acetylaminofluorene were activated to genotoxic derivatives. Negative results were reported with non-carcinogens such as 1-naphthylamine and 4-acetylaminofluorene, while intermediate response were reported with 4-aminobiphenyl and 2-naphthylamine. The identification of *N*-hydroxy-2-aminofluorene suggests that red blood cell cytosol may contribute to the *N*-hydroxylation of aromatic amines (Duverger-van Bogaert et al., 1991a) Under the same experimental conditions, heterocyclic compounds such as IQ, MeIQ, Trp-P-1 and Trp-P-2 were also activated to mutagens (Duverger-van Bogaert et al., 1991a, b).

While it is generally accepted that the liver is the primary organ of xenobiotic metabolism for many chemicals, other tissues such as red blood cells, display many properties in common with the liver microsomal CYP system (Starke and Mieyal, 1989). These similarities make this system useful as a model

for CYP and because erythrocytes are readily available, they could be incorporated into mutagenicity tests as in vitro metabolic activation system. Indeed, erythrocytes were demonstrated to be able to activate certain promutagens in in vitro genotoxicity studies. Moreover, the incorporation of this cell type into short-term assays should provide more insight into possible alternative mechanisms of activation. Obviously, one must be aware of the fact that there are limitations to any such system and the erythrocyte activation system in particular is not an exception.

6. CYP-mimetic chemical systems

Model systems using CYP-mimetic synthetic porphyrin complexes have been frequently used to study the mechanisms of cytochromes P450 catalyzed reactions. Iron tetraphenylporphyrins in the presence of the exogenous oxygen donor iodosylbenzene were shown to catalyze the epoxidation of alkenes and the hydroxylation of hydrocarbons (McMurray and Groves, 1986). Salmeen et al. (1988) used the TPP/Iodopsylbenzene system to activate benzo[*a*]pyrene and 2-aminofluorene to mutagens in the Ames assay. Wood et al. (1987) showed that this system could metabolise aflatoxin B_1 to aflatoxin B_1-8,9-diol, and that aflatoxin B_1 was converted to DNA-adduct forming products. We have used the tetraphenylporphyrin (TTP) system with different exogenous oxygen donors to activate various promutagens in the Ames test (Table 9) (Rueff et al., 1992). Benzo[*a*]pyrene, 3-methylcholanthrene, 7,12-dimethylbenz[*a*]anthracene, 2-aminofluorene, 2-acetylaminofluorene and IQ were activated to mutagens in the Ames assay. IQ could be activated using iodosylbenzene, *tert*-butylhydroperoxide, cumene hydroperoxide and H_2O_2 as the oxygen donors. However, a pattern of specificity for the oxygen donor could be observed. Benzo[*a*]pyrene, 3-methylcholanthrene and 7,12-dimethylbenz[*a*]anthracene were activated preferentially in the presence of iodosylbenzene, whereas the aromatic amines 2-aminofluorene, 2-acetylaminofluorene were activated preferentially by *tert*-butylhydroperoxide. For IQ the highest levels of mutagenic activity were observed when using cumene hydroperoxide as the oxygen donor. The non-carcinogen pyrene and the flavonoid quercetin were not activated irrespective of the oxygen donor used.

7. Use of cell-free homogenates from *Zea mays* and *Persea americana* as metabolic activation systems of promutagens in the Ames test

Since the first insight of the metabolism of xenobiotics by plants to give mutagens was reported (Veleminsky and Gichner, 1968), many efforts have been made to explore the impact of plants in the transformation of promutagens to mutagens. The early experiences were addressed to the study of pesticide metabolism by plants (Menn, 1978; Plewa and Gentile, 1982; Wildeman and Nazar, 1982), but now there are also data about the transformation by higher plants of xenobiotics other than pesticides (Velemínsky and Gichner, 1988; Sanderman, 1992;

Table 9
Mutagenic activities of various compounds in *S. typhimurium* TA98 tested with the activation system TPP using four oxygen donors

Test compound	Revertants/nmol			
	Iodosylbenzene	Cumene hydroperoxide	*tert*-Butylhydroperoxide	H_2O_2
Benzo[*a*]pyrene	3.8	0.2	0	0
3-Methylcholanthrene	0.8	0.4	0.2	0.2
7,12-Dimethylbenz[*a*]anthracene	2.1	1.2	1.8	2.4
Pyrene	0	0	0	0
IQ	275	5809	1562	189
2-Aminofluorene	0.2	1.5	2.6	0.2
2-Acetylaminofluorene	0	0	0.1	0
Quercetin	0	0	0	0

Plewa and Wagner, 1993). Moreover, it must be emphasized that for some xenobiotics the metabolites produced by plants are different from those of mammals (Sanderman, 1988; Higashi, 1988; Seo et al., 1993).

The term 'plant activation' was coined to define the processes by which a promutagen is transformed by plant metabolism into a mutagen (Plewa, 1978). Several experimental approaches have been designed in plant activation studies (Velemínsky and Gichner, 1988). One of these is the preparation of plant cell-free homogenates and their incorporation in in vitro assays instead of mammalian S9. This approach has been successful in plant activation of some xenobiotics, using the Ames test as the genetic endpoint (Gentile and Plewa, 1988).

The major reactions that could be involved in plant activation seem to be monooxygenation, peroxidation and conjugation (Velemínsky and Gichner, 1988). Different authors have emphasized the role of plant peroxidases in metabolizing foreign compounds (Lamoureux and Frear, 1979; Sanderman, 1988), while the involvement of CYP-dependent reactions in plant activation seems doubtful (Higashi, 1988). The enhancement of the 4-nitro-*o*-phenylenediamine mutagenicity mediated by some purified peroxidases has been clearly shown (Gentile et al., 1985) as well as the role of plant peroxidases in the activation of aromatic amines using the plant cell coincubation assay (Seo et al., 1993). On the other hand, the metabolism of xenobiotics by CYP has recently been revised by using benzo[*a*]pyrene and Jerusalem artichoke as a model (Higashi, 1988). In this system, it was found that the number of TA98 revertants increased after the induction of microsomal CYP, but there is no direct evidence that Jerusalem artichoke CYPs were the only enzymes involved in the activation of this chemical. The little data that we have about the participation of CYPs in plant activation can be explained by the low content of CYPs found in plants, their instability and their narrow substrate specificity in comparison with mammalian CYPs (Higashi, 1988). These facts have also raised difficulties in the purification and biochemical characterization of plant CYPs. Nevertheless, there are recent reports which indicate that there is a multiplicity of CYP enzymes in plants involved in the metabolism of natural substrates and xenobiotics also. In addition, different plant CYPs have been isolated and some enzymatic activities have been studied (Donaldson and Luster, 1991; Sanderman, 1992). Recently, the amino acid sequences have also been deduced from a substantial number of plant CYPs (Bozak et al., 1990). From *Zea mays*, 13 CYP genes in 4 different families are known (David Nelson's World Wide Web Page). Therefore, in the coming years, a quick progress in the knowledge of plant CYP is expected.

Our work has been focused on developing cell-free homogenates with peroxidase and/or CYP activities with the capacity to metabolize promutagens, with the aim of contributing to the determination of the role of higher plants in the activation of xenobiotics. In our studies we have used the assay of reversion of the TA98 strain as genetic endpoint, incorporating the cell-free homogenates into the plates, instead of rat liver S9. From *Z. mays*, we have prepared different fractions (S2, S9 and S14) and, afterwards, their ability to activate promutagens as well as some biochemical parameters was determined. We showed that these three fractions had a similar protein level and peroxidase activity. Furthermore, these fractions were able to enhance the mutagenicity of 4-nitro-*o*-phenylenediamine and to activate 2-aminofluorene (Ysern et al., 1991). Using the enhancement of the 4-nitro-*o*-phenylenediamine mutagenicity by the *Z. mays* S2 as a model, we have recently shown that this homogenate has neither CYP nor any CYP dependent enzymatic activity, and also that the metabolization of xenobiotics by this plant activation system depends on a peroxidase activity, similar to the peroxidase of horseradish (Ysern et al., 1994).

On the other hand, we have analyzed the content of CYP in several microsomal fractions from different tissues of various plants, finding that the S117 fraction from *Persea americana* presented the highest content and also had peroxidase activity. This fraction was able to activate the promutagen 2-aminofluorene. Furthermore, using 2-aminofluorene as a model and inhibitors of both CYP and peroxidase activities, we have recently demonstrated that plant activation of 2-aminofluorene is mediated by CYP from *P. americana* and by a peroxidase activity present in this fraction as well as in the S2 fraction from *Z. mays* (Llagostera et al., submitted).

Therefore, we have developed a plant cell-free

Table 10
Plant activation of several chemicals by *P. americana* S117 and *Z. mays* S2

Test chemicals	Revertants in TA 98	
	S117	S2
Benzo[*a*]pyrene	−	−
2-Aminofluorene	+ + +	+ +
4-Nitro-*o*-phenylenediamine	− *	+
4-Aminobiphenyl	−	nt
7,12-Dimethylbenz[*a*]anthracene	+	nt
2-Aminoanthracene	+ +	−
2 − Acetylaminofluorene	−	−

−, no increase compared to control. − *, significant decrease compared to controls. +, 2-fold increase compared to control. + +, 3-fold increase compared to control. + + +, > 3-fold increase compared to control. nt, not tested.

homogenate (*P. americana* S117) that is able to activate promutagens through both peroxidases and CYP-dependent reactions and also we have a plant cell-free homogenate (*Z. mays* S2) available whose peroxidase activity is the main pathway of xenobiotic activation. Table 10 presents a summary of both plant systems in the activation of some chemicals. These results suggest that *P. americana* S117 will be more effective in the xenobiotic activation than *Z. mays* S2, probably due to the combination of peroxidase and CYP activities. More comparative studies are in progress to confirm this suggestion. In any case, the use of both systems in studies of plant activation will allow a better determination of the role of both peroxidases and CYP in the metabolization of xenobiotics.

8. Discussion

All metabolizing systems used led to the activation of some promutagens in short-term tests, and therefore brought some insight into activation processes. The positive results which were obtained in the presence of CYP-mimetic systems and subcellular preparations from plants and red blood cells indicate that activation of promutagens is not strictly bound to typical xenobiotic-metabolizing enzymes of the mammalian organism, but may also be catalyzed in some cases by abiotic factors, plants and mammalian proteins with endogenous physiological functions (such as hemoglobin). It is important to be aware of this possibility, since in individual situations, these activation processes may be of greater relevance for ecotoxicological and human toxicological effects than the classical bioactivation systems. Nevertheless, they are not considered as alternatives for liver S9 preparations in routine genotoxicity testing.

All other metabolizing systems employed involve the use of mammalian cell lines which express xenobiotic-metabolizing enzymes. The epithelial liver cells used appear to express many different enzymes. A few of the major types of enzymes were characterized on the activity level and also, in part, immunologically. Carcinogens from many different chemical classes were readily detected in genotoxicity tests using epithelial liver cells. Non-carcinogenic isomers of the carcinogens gave negative test results. Therefore, these cells may be used for routine testing with new chemicals.

Genetic engineering has the advantage that in principle any desired enzyme from any mammalian species can be expressed in the indicator cell, but has the shortcoming, at least at present, that the number of enzymes which can be heterologously expressed in the same cell is small. In this study, usually only one heterologous enzyme was expressed, since at the present stage we were primarily interested in the capabilities of an individual enzyme in the activation of mutagens. This was also one of the reasons for using V79 cells as recipient cells, since they appear to be completely deficient for endogenous CYP, and also for some conjugating enzymes, such as the sulfotransferases and UDP-glucuronosyltransferases (Glatt et al., 1990, 1994a). Therefore, the background problems are minimized. Moreover, the parental V79 cells can be used as an isogenic negative control, when the influence of the heterologously expressed enzymes is studied. The free choice of the recipient cell has the additional advantage that cells can be used which allow the efficient detection of many genetic endpoints and which grow rapidly. In the present study, the results from mutagenicity tests with genetically-engineered V79 cell lines showed high reproducibility, even when experiments were carried out by different persons and using different batches of serum. However, all these experiments were carried out in the same laboratory, and it still has to be studied how much effort is required to

achieve similar reproducibility between different laboratories. Moreover, it became evident that it is time- and cost-intensive to test several compounds in several cell lines under conditions which allow quantitative comparison, rather than simple yes/no answers. At present, we do not recommend the use of a series of engineered cell lines for routine testing. Nevertheless, with special classes of compounds, the use of individual cell lines may be recommended even in the basic testing. For example, the fact that 2-aminoanthracene and 2-acetylaminofluorene were practically inactive in rat liver-S9-mediated gene mutation assays in V79 cells, but were very potent in genetically-engineered cells which express CYP1A2 and a conjugating enzyme (acetyltransferase and sulfotransferase, respectively) may be a reason to use these cell lines when testing new aromatic amines and related compounds. Alternatively, the Hep G2 and CHEL cell lines may be used, as they also detected this class of carcinogens in the present study.

Metabolically competent cells can be used directly as indicator cells and as external activating system with other indicator cells. The first method has the advantage that the metabolites are formed in the immediate vicinity of the target structure and do not have to penetrate the plasma membrane. Therefore, it may be more sensitive. The latter method has the advantage that the same activating system can be used with different indicator cells. Both methods were used in the present study. In agreement with the expectations, the internal activation was usually more efficient. External activation required large amounts of cell preparations, and therefore were cost- and labor-intensive. Moreover, they not always gave the same results as the tests using an endogenous endpoint. For example, benzo[*a*]pyrene was a potent mutagen in V79-MZ-r1A1 cells, but was inactive in *S. typhimurium* in the presence of NADPH-fortified homogenate of V79-MZ-r1A1 cells. Therefore, the use of subcellular preparations from metabolically competent cells is not recommended for routine testing, but may be useful for answering special questions.

All cell lines used allowed the detection of several genetic endpoints. In most cases, the results were qualitatively the same when different endpoints were used. In some of the cases where different endpoints led to discrepant results, the explanation may involve the preferential induction of certain forms of damage by the test compound and/or a generally higher sensitivity of the assay used for this endpoint. However, the use of different genetic endpoints with isogenic V79-derived cell lines which express different enzymes led to additional, more differentiated conclusions. When 2-aminoanthracene was investigated in V79-NH-r1A2 cells, gene mutation at the *hprt* locus was a much more sensitive endpoint than SCE, as indicated by the substrate concentration required and the *n*-fold increase above the background. However, using V79-MZ-r1A2 cells, 2-aminoanthracene induced virtually no gene mutations, but still showed about half of the SCE-inducing activity, compared to V79-NH-r1A2 cells. We therefore suspect that a metabolite which does not require the presence of acetyltransferase is capable of inducing SCEs but not gene mutations, and that in the presence of acetyltransferase a second active metabolite is formed which is a potent inducer of gene mutations and is responsible for the increase in SCE induction from V79-MZ-r1A2 to V79-NH-r1A2 cells. It would be interesting to know whether only one or both metabolites are involved in the carcinogenicity of 2-aminoanthracene. Discrepancies in the results, using different endpoints, were also observed with 2-acetylaminofluorene, which required the presence of CYP1A2 as well as sulfotransferase for the efficient induction of gene mutations. However, 2-acetylaminofluorene induced SCEs even when only CYP1A2 was expressed (V79-MZ-r1A2 cells) or when CYP1A2 was expressed together with an endogenous acetyltransferase (V79-MZ-r1A2 cells).

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