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Characteristics of the activation of cyclophosphamide to a mutagen by rat liver

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Cyclophosphamide is used extensively as an antineoplastic agent for the treatment of various cancers and as an immunosuppressive agent for certain non-malignant conditions. It has also been implicated in producing primary and secondary cancer [1–3]. *In vitro*, it is the active metabolites of cyclophosphamide, rather than the parent compound, that have alkylating activity and cause cytotoxicity, oncogenic transformation and chromosomal aberrations. This activation of cyclophosphamide occurs primarily in the liver and is mediated by a mixed-function oxygenase system [4–17].

It is well established that there are multiple cytochromes P-450, each differing in substrate specificity, carbon monoxide difference spectrum, response to a variety of inducing agents, and sensitivity to different inhibitors [18–31]. The induction of sister chromatid exchanges by cyclophosphamide has been reported recently to be associated with metabolism by the phenobarbital-induced cytochrome P-450 rather than by the polyclic aromatic hydrocarbon-induced cytochrome P-448 (also called cytochrome P-446 or P_1-450) [32]. Sladek [10] demonstrated a greater increase in the alkylating activity of cyclophosphamide *in vitro* after incubation with phenobarbital-induced liver fractions than with control or polycyclic hydrocarbon-induced liver fractions.

In this study, the *in vitro* activation of cyclophosphamide, by male rat liver, to metabolites that are mutagenic to *Salmonella typhimurium* TA 1535 is characterized. Inducers and inhibitors of mono-oxygenase activity have been used in an attempt to determine which form(s) of cytochrome P-450 is (are) involved in activating cyclophosphamide to a mutagen or mutagens.

Phenobarbital was a gift from Merck, Sharp & Dohme, Kirkland, PQ; Aroclor 1254 (Monsanto Chemical Co.) from Dr. D. J. Ecobichon, McGill University, Montreal, PQ, and SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate HCL) from Dr. H. A. Sheppard, Smith, Kline & French, Canada Ltd., Montreal, PQ. Cyclophosphamide was purchased from Koch-Light Laboratories Ltd., Colnbrook, England. β-Napthoflavone was purchased from ICN Pharmaceuticals Inc., Plainview, NY, and metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) from Aldrich Chemical Co., Inc., Milwaukee, WI. α-Napthoflavone, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and bovine serum albumin were purchased from the Sigma Chemical Co., St. Louis, MO.

Male Sprague–Dawley rats (175–200 g), obtained from the Canadian Breeding Laboratories (St. Constant, Quebec), were housed on pine chips in the McIntyre Animal Center (McGill University, Montreal, Quebec) and given Purina rat chow and water *ad lib.* for at least 1 week before the initiation of treatments. Each group of animals consisted of five rats. Control rats were not treated. Induced rats were treated with phenobarbital, β-napthoflavone or Aroclor 1254 according to established procedures for induction of the hepatic mono-oxygenase system [33]. The phenobarbital-pretreated rats received 0.1% of the drug in the drinking water for 1 week before being killed. Other groups of animals received β-napthoflavone intraperitoneally, 40 mg/kg, in corn oil (10 mg/ml) on day 1 and on day 2 before being killed on day 5, or Aroclor 1254 intraperitoneally, 500 mg/kg, in corn oil (200 mg/ml) on day 1 before being killed on day 5.

Rats were decapitated. The excised livers from each group (five rats) were pooled and homogenized using a Potter–Elvehjem homogenizer in 3 vol. of ice-cold 0.15 M KCl (3 ml/g wet wt of liver). Homogenates were centrifuged for 10 min at 9000 *g* and the pellet was discarded. The supernatant fraction (S-9) was distributed in 3 ml portions in small sterile polypropylene tubes, frozen in liquid nitrogen, and stored at −80°. No loss of activity was observed after storage for up to 6 weeks; storage for longer time periods did result in a decrease in activity. To prepare the microsomal fraction, the S-9 fraction was centrifuged at 105,000 *g* for 60 min and the pellet was resuspended in the original volume of cold 0.15 M KCl.

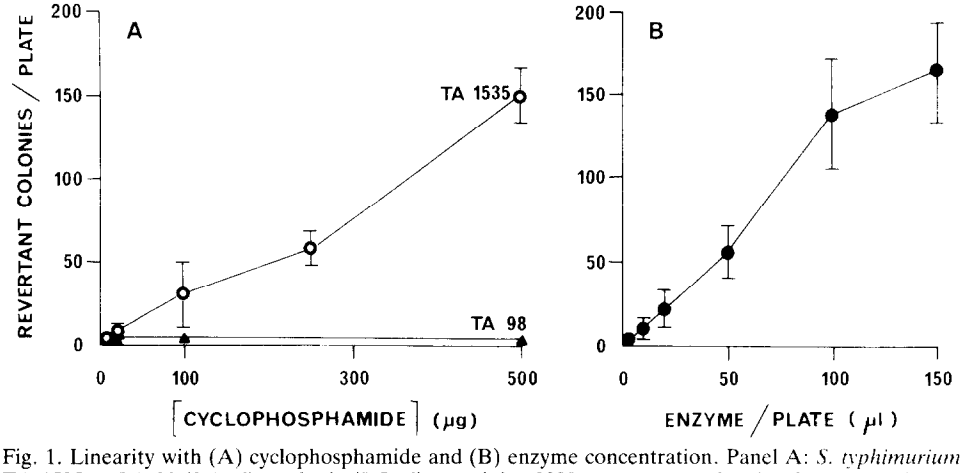

Fig. 1. Linearity with (A) cyclophosphamide and (B) enzyme concentration. Panel A: *S. typhimurium* TA-1535 or TA-98 (0.1 ml), and mix (0.5 ml) containing 9000 *g* supernatant fraction from control male rat liver (0.1 ml) and an NADPH-generating system were mixed rapidly with 0.1 ml of cyclophosphamide solution (of the appropriate dilution) and molten top agar and plated. Values are means ± S.E.M. for three separate experiments performed in duplicate (N = 3). Panel B: Experiments with varying amounts of control male rat liver microsomes/plate were done at a cyclophosphamide concentration of 500 μg/plate.

Cytochrome P-450 was assayed by its carbon monoxide difference spectrum after reduction with dithionite according to the procedure of Omura and Sato [34] with use of a Beckman Acta III spectrophotometer. An extinction coefficient of 91 mM^{-1} cm^{-1} between 450 and 490 nm has been assumed. The protein concentration was determined by the method of Lowry *et al.* [35] using bovine serum albumin as a standard.

S. typhimurium strains TA 1535 and TA 98 (provided by Dr. Bruce Ames) were used to assay for mutagenic activity. TA 1535 is a histidine-requiring auxotroph which is reverted to prototrophy by mutagens that cause DNA base-pair substitutions, and TA 98 is sensitive to frame shift mutagens. The plate incorporation assay was performed as recommended by Ames *et al.* [33]. The NADPH-generating system contained (per ml) NADP (4 μmoles), glucose-6-phosphate (5 μmoles) and glucose-6-phosphate dehydrogenase (2 units). Assays were done in duplicate on three separate occasions. After incubation for 2 days at 37°, the colonies in both test and control plates (no cyclophosphamide) were counted. Spontaneous revertant colonies on control plates (no cyclophosphamide, approximately 20 colonies/plate) were subtracted from test plate colonies. This spontaneous reversion rate without mutagen is in agreement with that reported by Ames *et al.* [33] for TA 1535.

Without the NADPH-generating system or control liver S-9 fraction, cyclophosphamide (500 μg/plate) induced some mutations in TA 1535. This rate was not changed by the addition of an NADPH-generating system alone or S-9 fraction alone; however, the number of revertants per plate was significantly increased by the addition of both an NADPH-generating system and the S-9 fraction from control male rat liver. The mutagenic response in TA 1535 induced by cyclophosphamide in the absence of metabolic activation may represent spontaneous hydrolysis of cyclophosphamide under these assay conditions. This background response has also been subtracted in subsequent experiments to permit measurement of the enzymatic activation of cyclophosphamide.

The mutagenicity assay was linear with both cyclophosphamide and enzyme concentration (Fig. 1). In the presence of the S-9 fraction, increasing doses of cyclophosphamide produced proportional increases in mutation rate in *S. typhimurium* TA 1535 (sensitive to base substitution mutagens) but not in strain TA 98 (sensitive to frame shift mutagens) (Fig. 1A). The ability of the S-9 fraction to activate cyclophosphamide to a mutagen was localized in the microsomes. At a cyclophosphamide concentration of 500 μg/plate, increasing amounts of control liver microsomal fraction caused a linear increase in the number of revertant colonies per plate (Fig. 1B).

To characterize the enzyme system activating cyclophosphamide to a bacterial mutagen, liver microsomes from control male rats were compared to microsomes prepared from rats pretreated *in vivo* with three drugs that induce hepatic mono-oxygenase activity (Fig. 2). Phenobarbital induces predominantly cytochrome P-450, whereas

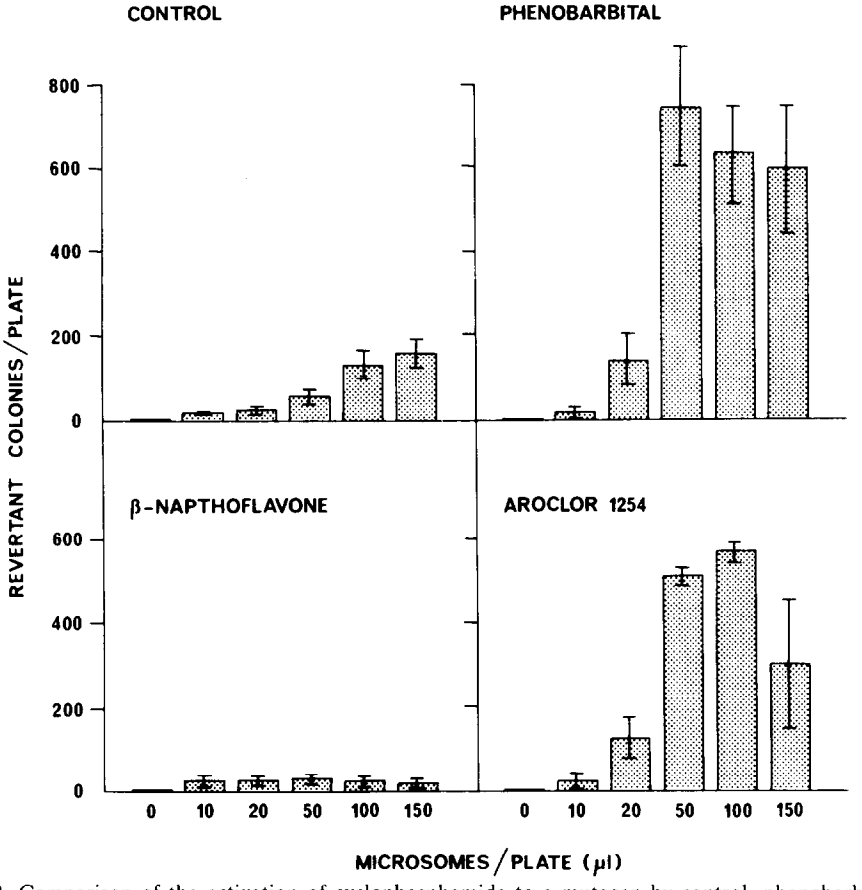

Fig. 2. Comparison of the activation of cyclophosphamide to a mutagen by control, phenobarbital-, β-napthoflavone- and Aroclor 1254-pretreated rat liver microsomes. *S. typhimurium* TA-1535 (0.1 ml), cyclophosphamide (500 μg in 0.1 ml) and mix (NADPH-generating system and varying amounts of resuspended microsomes from control or induced rat liver, 0.5 ml) were mixed rapidly with molten top agar at 45° and plated. Induced rats had been pretreated as described in the text. Values are means ± S.E.M. for three separate experiments performed in duplicate (N = 3).

β-napthoflavone induces cytochrome P-448 [18–22]. Aroclor 1254 is a mixed-type of inducer, inducing both cytochrome P-450 and cytochrome P-448 [23, 24]. Pretreatment with phenobarbital increased cyclophosphamide activation by 1360 per cent (at a microsome concentration of 50 μl/plate) and cytochrome P-450 concentration by 294 per cent. Pretreatment with β-napthoflavone decreased the activation of cyclophosphamide to a mutagen(s) by 50 per cent while increasing cytochrome P-448 concentration by 187 per cent. Pretreatment with Aroclor 1254 increased the activation of cyclophosphamide by 1030 per cent (at a microsome concentration of 50 μl/plate) and cytochrome P-450 and P-448 concentration by 338 per cent. When added *in vitro* at concentrations ranging up to 10^{-4} M, none of the three inducers had any effect on cyclophosphamide mutagenicity with control microsomes. Unlike control microsomes, neither phenobarbital-induced nor Aroclor 1254-induced activation of cyclophosphamide to a mutagenic metabolite was linear with microsome concentration. This decline in activation at high enzyme concentrations (150 μl of resuspended microsomes/plate) was especially noticeable with Aroclor 1254-induced microsomes.

The results of the induction studies suggest that activation of cyclophosphamide to a mutagen is catalyzed by a phenobarbital-responsive form of cytochrome P-450. For this reason, liver microsomes from phenobarbital-pretreated rats were used to test the effect of SKF 525-A, metyrapone and α-napthoflavone on the activation of cyclophosphamide to a mutagen. Inhibition by SKF 525-A or metyrapone has been interpreted as evidence for the involvement of a phenobarbital-inducible cytochrome P-450 [25–28]. Conversely, inhibition by low concentrations of α-napthoflavone has been interpreted as evidence for the involvement of a cytochrome P-448 mono-oxygenase [28–30]. Activation of cyclophosphamide to a mutagen could be inhibited by SKF 525-A, metyrapone or α-napthoflavone; however, significant differences in I_{50} were noted (Fig. 3). SKF 525-A was an extremely potent inhibitor with an I_{50} of 4×10^{-7} M. Metyrapone and α-napthoflavone had I_{50} values of 6×10^{-6} M and 5×10^{-5} M respectively. In the absence of cyclophosphamide, none of these inhibitors altered the spontaneous reversion rate of TA 1535.

The data presented above suggest that the production of mutagenic metabolites of cyclophosphamide is enhanced by a phenobarbital-inducible form of cytochrome P-450. Previous studies have demonstrated that, except for acrolein, all cyclophosphamide metabolites were mutagenic [36–38]. Thus, the mutagenic activity of cyclophosphamide generated by biotransformation probably represents the cumulative effect of a variety of metabolites involving different metabolic reactions. A decrease in cyclophosphamide mutagenicity is observed in the presence of β-napthoflavone-induced microsomes. A decrease in the alkylating activity of metabolites of cyclophosphamide has also been reported when 3-methylcholanthrene-induced microsomes were compared with control [10]. This decrease in both mutagenicity and alkylating activity may indicate the existence of alternate metabolic pathways, mediated preferentially by cytochrome P-448, that compete with the mutagenic metabolite producing cytochrome P-450 pathway(s). This hypothesis can be tested because a cytochrome P-448-dependent pathway should be sensitive to inhibition by low concentrations of α-napthoflavone (10^{-9}–10^{-8}M) [31]. However, 10^{-7} M α-napthoflavone does not alter the activation of cyclophosphamide by β-napthoflavone or by Aroclor 1254-induced microsomes (data not shown).

The characteristics of inhibition and induction of the activation of cyclophosphamide to a mutagenic metabolite(s) are concordant with the conclusion that this activation is catalyzed primarily or solely by a phenobarbital-inducible cytochrome P-450. Phenobarbital-inducible cytochrome P-450 is also involved in the activation of cyclophosphamide to metabolites promoting alkylation [10]

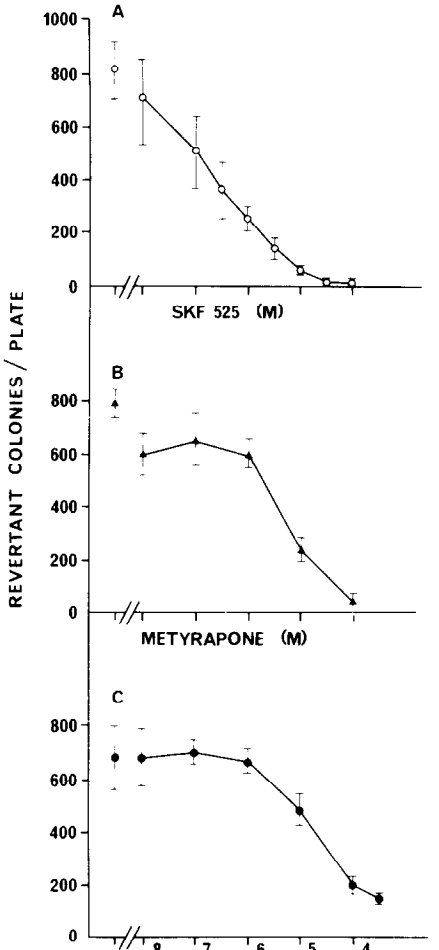

Fig. 3. Inhibition of the activation of cyclophosphamide to a mutagen by phenobarbital-induced rat liver microsomes. (A) SKF 525-A. (B) Metyrapone. (C) α-Napthoflavone. *S. typhimurium* (0.1 ml), cyclophosphamide (500 μg in 0.1 ml) and phenobarbital-induced microsome mix (containing 50 μl of resuspended microsomes/plate) were mixed rapidly with 0.1 ml of the inhibitor (of the appropriate dilution) and molten top agar at 45° and plated. Values are means ± S.E.M. for three separate experiments performed in duplicate (N = 3).

and sister chromatid exchange [32]. It would thus appear that the desirable effects of this drug as a cancer chemotherapeutic agent (alkylating activity) are not dissociable from its undesirable side-effects (mutagenicity and promotion of sister chromatid exchanges). Further research in this field should be aimed at developing alternative drugs (perhaps structural analogues of cyclophosphamide) which make this dissociation possible.

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Induction of both 3-methylcholanthrene- and phenobarbitone-type microsomal enzyme activity by a single polychlorinated biphenyl isomer

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Commercial mixtures of polychlorinated biphenyls (PCBs) are inducers of hepatic microsomal drug-metabolizing enzymes [1]. These enzymes, which include the microsomal mono-oxygenases, are involved in the metabolism of endogenous and exogenous chemicals and in metabolically mediated cellular toxicosis.

Chemicals which induce the microsomal mono-oxygenases have been divided into two categories: one is typified by phenobarbitone (PB) which induces the formation of cytochrome P-450 hemoproteins and the other by 3-methylcholanthrene (3-MC) which induces the formation of cytochrome P-448 hemoproteins [2, 3]. The two induced microsomal mono-oxygenase enzyme systems exhibit different spectral properties, substrate specificities and electrophoretic protein patterns [4].

The hepatic microsomal mono-oxygenases induced after pretreatment with a commercial PCB preparation, such as Aroclor 1254, exhibit properties consistent with a mixed induction pattern produced by the simultaneous administration of both PB and 3-MC. However, it has been shown recently that individual PCB isomers can be categorized on a structural basis into PB-type and 3-MC-type inducers of microsomal enzyme activity [5–7]. The structure–activity relationships indicate that PCB congeners, chlorinated in both the para (4,4′) and ortho (2,2′ or 2,2′,6,6′) positions, are PB-type inducers of cytochrome P-450 activity. Congeners chlorinated in both the para (4,4′) and meta (3,3′; 3,3′,5 or 3,3′,5,5′) positions are 3-MC-type inducers of cytochrome P-448 activity, and any alteration in this substitution pattern results in the loss of this induction activity.

Recent work in our laboratory using a 4-chlorobiphenyl hydroxylase assay system has indicated that some PCB isomers may be mixed inducers [8]. This possibility is reinforced by a report by Dannan *et al.* [9] which describes the simultaneous induction of cytochrome P-450 and cytochrome P-448 activity by 2,3′,4,4′,5,5′-hexabromobiphenyl, isolated as a minor component of the commercial polybrominated biphenyl mixture, Firemaster BP-6. It was proposed that the single isomer possessed the structural requirements for a cytochrome P-448 inducer (3,3′4,4′,5-substitution) and the addition of the single ortho bromo substituent did not eliminate this activity and was also sufficient to impart the capability of microsomal cytochrome P-450 induction. A test for this mixed induction