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The Hepatocarcinogen Methapyrilene but Not the Analog Pyrilamine Induces Sustained Hepatocellular Replication and Protein Alterations in F344 Rats in a 13-Week Feed Study

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Methapyrilene (MPH) was a widely used antihistamine until it was found to produce hepatocellular carcinoma and cholangiocarcinoma in Fischer 344 rats. The structurally similar antihistamine pyrilamine (PYR) was marginally or noncarcinogenic in a similar study. The peroxisome proliferator Wy-14,643 was included in this study as a positive control. As part of a program to investigate the mechanisms whereby structurally similar chemicals produce different toxicities, we studied these three chemicals for the induction of cell proliferation in the liver of F344 rats. Male rats were treated for up to 13 weeks with feed dosed with MPH (HCl salt) at 0, 50, 100, 250, or 1000 ppm or PYR (maleate salt) at 1000 ppm to duplicate the route of administration and high-dose groups used in the carcinogenesis assay. In addition, the nongenotoxic hepatocarcinogen peroxisome proliferator Wy-14,643 was included as a positive cell-proliferating chemical. Cell proliferation was quantitated by measuring the incorporation of bromodeoxyuridine (BrDU) administered by osmotic minipump for 7 days and the appearance of proliferating cell nuclear antigen (PCNA) immunohistochemically. The BrDU-labeling index showed a large and sustained increase in rats treated with MPH at 250 and 1000 ppm, sustaining greater than 50% labeling in the higher dose group of 4-, 6-, and 13-week treatment groups. PYR at 1000 ppm demonstrated no significant increase in labeling above control levels at any time point. PCNA-labeling indexes showed similar but reduced increases for MPH and were comparable to control for the PYR dose groups. Two-dimensional gel electrophoresis was used for the detection of quantitative changes in gene expression and qualitative changes in the charges of specific mitochondrial and cytosolic proteins. Quantitative changes in 32 proteins induced by MPH and 39 changes induced by Wy-14,643 were detected throughout the 13-week study. Specific mitochondrial protein charge shifts were associated with high-dose MPH treatment that were not observed in animals treated with Wy-14,643. PYR induced no significant qualitative or quantitative protein alterations. Hepatocellular proliferation of the large magnitude observed following dietary administration of MPH, and not PYR may contribute to the mechanism of carcinogenesis of MPH. © 1995 Academic Press, Inc.

Methapyrilene (MPH[2]; Fig. 1) is an H_1 receptor antagonist that was widely used in over-the-counter sedatives and allergy medications. As a result of the induction of hepatocellular and cholangiocellular carcinomas by MPH (Lijinsky *et al.*, 1980), it was removed from these preparations and often replaced with the structurally similar antihistamine pyrilamine (PYR; Fig. 1). MPH demonstrates an almost total lack of activity in short-term genotoxicity assays, including *Salmonella* (Andrews *et al.*, 1980), DNA binding studies (Casciano *et al.*, 1988; Lijinsky *et al.*, 1988), and unscheduled DNA synthesis *in vitro* (Steinmetz *et al.*, 1988), although MPH was positive in some cell transformation assays and the L5178Y/TK$^{+/-}$ mouse lymphoma assay (Turner *et al.*, 1987). A comprehensive review of the genotoxicity of MPH has been published (Mirsalis, 1987). Structure–mutagenicity analyses recently conducted on MPH have produced equally conflicting results (Rosenkranz and Klopman, 1990; Ashby, 1992). MPH produces severe hepatotoxicity following a single gavage treatment of 225 mg/kg (Steinmetz *et al.*, 1988). This acute administration produced hepatic necrosis, increased mitotic figures, and elevated serum enzyme levels characteristics of hepatotoxicity over the next 96 hr. MPH failed to produce unscheduled DNA synthesis following *in vivo* exposure, consistent with other data that indicate it has little or no genotoxicity

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[2] Abbreviations used: MPH, methapyrilene hydrochloride; PYR, pyrilamine maleate; Wy-14,642, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid; BrDU, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen.

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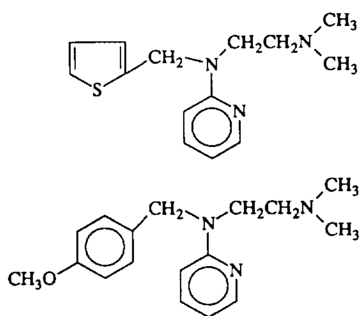

FIG. 1. Structures of the methapyrilene (top) and pyrilamine (bottom) showing their structural similarity.

(Steinmetz *et al.*, 1988). However, MPH induced increases in S-phase synthesis and mitotic figures after a single dose, both indicators of cell division. PYR induced unscheduled DNA synthesis in rat hepatocyte culture following *in vivo* treatment (McQueen and Williams, 1981) and was mutagenic in the L5178Y/TK$^{+/-}$ mouse lymphoma assay requiring S9 activation (Turner *et al.*, 1987).

PYR (as the maleate salt) was tested for chronic toxicity and carcinogenicity at 2000 ppm in the diet in male and female F344 rats in parallel with rats treated with MPH at 125 or 250 ppm in the diet. After 110 weeks, animals were placed on normal diet until they died naturally. At 2000 ppm PYR, the only statistical difference between controls and treated was the number of hepatic neoplastic nodules plus carcinomas in female rats, 10/20, compared to 3/20 in the untreated control group.

Rats given MPH at 250 ppm had hepatic carcinoma or neoplastic nodules (Lijinsky, 1984) in 18/20 (males) and 20/20 (females). The author concluded that PYR produced an equivocal increase in liver neoplasms at 2000 ppm in the diet for 2 years, and that if PYR was carcinogenic, it was very much less potent than MPH (Lijinsky, 1984). A larger and more comprehensive study of PYR would be necessary to assess the possible carcinogenicity of PYR, but within the limits of this study, the relative carcinogenic potency of PYR and MPH is clear.

The present study was conducted in order to test the hypothesis that the carcinogenicity of MPH is associated with the production of chronic hepatotoxicity when administered to F344 rats at the same dose rate and route of administration as was conducted in the carcinogenesis study. It was also intended to compare the results for MPH with a similar study on the marginal or noncarcinogenic antihistamine analog pyrilamine as a negative control (Lijinsky, 1984).

Mechanistic studies have been conducted in our laboratories to elucidate the biochemical differences between structurally similar chemicals which produce widely dissimilar effects upon chronic treatment in rodents (Cunningham *et al.*, 1991, 1993, 1994a,b; Cunningham and Matthews, 1991). Our studies have demonstrated that carcinogenic compounds increase cell proliferation in the target tissue for carcinogenesis and that noncarcinogens fail to do so. The present study used two endpoints of cell proliferation, BrDU incorporation and proliferating cell nuclear antigen (PCNA) as indexes of cell proliferation to determine their respective value in the performance of such investigations. The nongenotoxic peroxisome proliferator hepatocarcinogen Wy-14,643 was included in this study as a positive control for increased hepatocellular cell proliferation (Marsman *et al.*, 1988).

Recent reports have demonstrated that MPH treatment results in the covalent modification of mitochondrial proteins resulting in altered protein migration when analyzed by two-dimensional polyacrylamide gel electrophoresis (PAGE) (Richardson *et al.*, 1992, 1993). We confirmed these results and extended these data to demonstrate that the nonhepatocarcinogenic analog pyrilamine does not induce similar protein alterations. Evaluation of mitochondrial as well as cytosolic proteins were included in the present study since mitochondria have been identified as a unique target organelle for the toxic effects of MPH (Reznik-Schüller and Lijinsky, 1982). The results in the present report provide evidence that events early in the course of chemical treatment may have predictive and mechanistic significance in the evaluation of chemical carcinogenesis in rodents. Preliminary results of these studies have been presented previously (Pippin *et al.*, 1993).

MATERIALS AND METHODS

Chemical treatment. Methapyrilene hydrochloride (crystalline; purity >99%) and pyrilamine maleate (crystalline; purity >99%) were purchased from Sigma Chemical Co. (St. Louis, MO). Wy-14,643 ([4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetate) (purity ≥98%) was purchased from Chemsyn Science Laboratories (Lenexa, KS). Feed was prepared in 7-kg batches by adding ground NIH-07 feed and chemical and mixing in a Patterson–Kelly blender for 15 min with the intensifier bar in operation for the first 10 min. Control animals received an identical chow mixture without addition of chemical.

Animal treatment. Male F344 rats (Harlan Sprague Dawley, Indianapolis, IN) approximately 7 weeks of age were maintained on a standard NIH 07 diet *ad libitum* and a daily cycle of alternating 12-hr periods of light and darkness. The animals were acclimated to this environment for 2 weeks prior to the beginning of the experiment and were maintained at 21–23°C at 35–65% relative humidity. Rats were randomly assigned by body weight to treatment groups of 40 animals per group and allowed 7 days to adapt to a new cage environment. Groups of 10 rats were euthanized following 2, 4, 6, or 13 weeks on treated diets. All animals were identified by tail tattoos with indelible ink that lasted the duration of the study. Experiments were performed according to the guidelines established in the NIH Guide for the Care and Use of Laboratory Animals. Statistical analysis was performed by ANOVA and Dunnett's *t* test.

Cell proliferation measurements. Seven days prior to termination, osmotic minipumps (Alza Corporation, Palo Alto, CA, Model 2002) were implanted subcutaneously into the backs of the rats under Ketamine/Rompun anesthesia. The minipumps delivered bromodeoxyuridine (BrDU; 30 mg/ml in 0.01 N NaOH) (Sigma Chemical Co.) which is incorporated into the DNA of newly replicating cells. Seven days later, the animals were euthanized by Metofane anesthesia and exsanguination and liv-

ers were blotted, weighed, and fixed in neutral-buffered formalin. A midlobe radial section of the right anterior lobe was fixed in neutral-buffered formalin for 24 hr. A cross section of small intestine was also fixed as a positive control for the proper operation of the minipump and the staining technique because these cells are constantly in S phase. Tissues were embedded in paraffin and serial sections mounted onto Superfrost Plus slides. Following deparaffination and rehydration, one set of slides was stained with hematoxylin and eosin for histological analysis and another set was stained immunohistochemically for BrDU incorporation by a variation on the method of Sugihara *et al.* (1986), as described previously (Cunningham *et al.*, 1991; Cunningham and Matthews, 1991). PCNA analysis was performed on serial sections used for BrDU analysis and was stained as described previously (Foley *et al.*, 1991). Random areas of the slides were chosen for counting stained and unstained hepatocyte nuclei (>1000 hepatocytes/animal). Statistics were performed using nonparametric Kruskal–Wallis test and the Mann–Whitney *U* test for pairwise comparisons when a significant *F* ratio was detected.

Two-dimensional gel electrophoresis for hepatic protein analysis. Proteins from liver samples were solubilized with 8 vol (w/v) of 9 M urea, 2% NP-40 detergent, 0.5% dithiothreitol, and 2% pH 9–11 ampholytes. Acidic and neutral soluble proteins were resolved based on molecular weight and isoelectric point using sodium dodecyl sulfate-denaturing polyacrylamide gels as previously described (Anderson *et al.*, 1992). Proteins were separated using 20 × 25 cm ISODALT system, fixed, and stained, and the protein spots were digitized. Proteins showing significant changes were defined as those that met the following four criteria: *t* test *p* value less than 0.001 versus controls; average within-group coefficient of variation (CV; standard deviation/mean) less than 20%; group average value at least 30% different from control value for at least one treatment group; and the protein spot was detected in at least all but five gels of every experimental group. These criteria together select protein spots showing meaningful treatment-related changes. The charge modification index was developed to describe the level of charge modification of individual proteins (Anderson *et al.*, 1992). This index is the sum of abundances of various charge modified forms weighted by the number of charges added, divided by the unweighted sum of abundances. The index yields the overall average number of charges added per protein molecule and gives a useful measure of the level of covalent chemical modification of a target protein by a reactive drug metabolite or other reactive species. Proteins used include three major mitochondrial proteins (Mitcon:1, :2, and :3). Mitcon:1 is the β subunit of the mitochondrial F_1 ATPase located on the inner mitochondrial membrane. Mitcon:2 is a mitochondrial matrix protein which is inducible by heat shock (also called hsp 58) and appears to be analogous to the *Escherichia coli* groEL gene product. The latter protein is involved in the assembly of large multimeric protein structures. Mitcon:3 is another mitochondrial matrix polypeptide which belongs to the family of mammalian stress-inducible proteins (under the name grp 75, for "glucose-regulated protein"), binds ATP, and appears to be analogous to both the *E. coli* dnaK and yeast SSC1 proteins. This family of proteins appears to be involved in the ATP-dependent stabilization of proteins being incorporated into larger structures. For parallel measurement of the level of drug-induced modification of cytosolic proteins, two major, well-resolved cytosolic proteins were chosen (MSN 8 and MSN 55). The 2D map location of the three mitochondrial and two cytosolic protein markers used to compute the charge modification index are shown on Fig. 4 (labeled M:1,2,3 and C: 1,2). Covalent modification as used here is defined as a modification of the protein's physical properties in such a way that net charge (and thus isoelectric point) are changed under the strongly denaturing conditions (9 M urea, 2% NP-40, 0.5% dithiothreitol) present in the gel. This suggests but does not prove that an adduct is present on the protein.

RESULTS

Toxicity. No compound-related deaths were observed in any dose group over the course of the study. Few clinical signs of toxicity were noted, except for decreased rate of body weight gain in the 1000 ppm MPH dose group at all time points compared to control. This was supported by a concomitant decrease in food consumption (data not shown). Rats treated with Wy-14,643 demonstrated significantly less body weight gain compared to untreated control animals, although they consumed significantly more feed. No significantly different body weights or food consumption occurred in the 50, 100, and 250 ppm MPH dose groups or the 1000 ppm PYR negative control groups compared to untreated controls.

A significant decrease in absolute liver weight was observed in rats treated with 1000 ppm MPH at 6 and 13 weeks. However, these animals exhibited an increased liver/body weight ratio due to their decreased body weight (Table 1). Rats treated with Wy-14,643 exhibited a significant increase in liver weights and liver/body weight ratio compared to untreated controls at all time points. Liver weights and liver/body weight ratios were not affected in any other treatment group at any time point.

Hepatotoxicity as observed on histopathologic evaluation of H&E-stained sections appeared only in livers of rats treated with MPH at 250 and 1000 ppm. This toxicity, characterized by marked hyperplasia of the bile ducts, individual hepatocellular necrosis, and prominent hypertrophy of hepatocytes, was observed mainly in the periportal areas and was present at all time points. No significant lesions were observed in any other MPH dose group, or the PYR or Wy-14,643 treatment groups.

Hepatocellular replication analysis by BrDU incorporation and PCNA. Animals treated with MPH at 1000 ppm showed a significant increase in the number of cells staining positive for BrDU incorporation compared to untreated controls (Fig. 2). Maximum cell turnover was observed in this group by 4 weeks and was sustained over the 13-week treatment period. The cell replication induced by the hepatocarcinogen Wy-14,643 was also increased above control levels, but of reduced magnitude compared to the 1000 ppm MPH dose group. Cell replication induced by MPH at 250 ppm was not different from untreated controls at 4 weeks but was significantly elevated at 6 and 13 weeks of exposure. Interestingly, cell replication by MPH at 50 and 100 ppm was slightly but statistically lower than that of untreated controls at 4 weeks, but not at 6 weeks or 13 weeks (Fig. 2). The weak or nonhepatocarcinogen PYR at 1000 ppm produced no increase in BrDU incorporation at any time examined, and produced a slight decrease in labeling index at 4 weeks, similar to the 50 and 100 ppm doses of MPH, and was not considered to be compound related. Due to technical difficulties, BrDU LI at 2 weeks could not be evaluated.

The results of cell replication analysis performed by PCNA were similar to those of the BrDU incorporation for the 1000 ppm MPH dose groups. PCNA-labeling indexes

TABLE 1
Compound Consumption and Liver/Body Weight Ratios of F344 Rats Treated with Methapyrilene, Wy-14,643, or Pyrilamine Maleate in the Feed

Treatment	2 Weeks		4 Weeks		6 Weeks		13 Weeks	
	Cumulative[a] dose (mg/kg)	Liver/body weight ratio	Cumulative dose (mg/kg)	Liver/body weight ratio	Cumulative dose (mg/kg)	Liver/body weight ratio	Cumulative dose (mg/kg)	Liver/body weight ratio
Untreated	0	4.6 ± 0.4	0	4.3 ± 0.1	0	4.1 ± 0.1	0	3.6 ± 0.1
Methapyrilene, 50 ppm	55	4.5 ± 0.3	99	4.4 ± 0.2	122	4.0 ± 0.2	171	3.4 ± 0.1
Methapyrilene, 100 ppm	109	4.7 ± 0.4	201	4.2 ± 0.1	248	3.9 ± 0.2	396	3.6 ± 0.1
Methapyrilene, 250 ppm	276	4.6 ± 0.3	504	4.3 ± 0.1	648	4.3 ± 0.2	900	3.6 ± 0.1
Methapyrilene, 1000 ppm	1156	5.3 ± 0.5*	2103	4.7 ± 0.2	2615	4.5 ± 0.3*	4095	4.2 ± 0.1*
Wyeth 14,643, 50 ppm	59	10.0 ± 0.6*	111	9.3 ± 0.2*	144	8.5 ± 0.5*	252	8.7 ± 0.3*
Pyrilamine, 1000 ppm	1056	4.5 ± 0.2	1983	4.3 ± 0.3	2520	4.0 ± 0.1	3618	3.6 ± 0.2

[a] (Grams of feed consumed per animal per day) (concentration in feed) (number of days on test) ÷ body weight.
* $p < 0.05$ compared to untreated control group by ANOVA and Dunnett's t test; $n = 5$–10 animals per group.

were significantly elevated by 2 weeks of 1000 ppm MPH treatment and remained elevated at 4, 6, and 13 weeks (Fig. 3). MPH at 250 ppm demonstrated a significant increase in hepatic labeling index above the untreated control group after 6 and 13 weeks of treatment. The positive control, Wy-14,643, produced elevated PCNA after 6 weeks of treatment only. MPH at 50 and 100 ppm showed decreased PCNA only at the 4-week time point, similar to results observed for BrDU incorporation. PYR at 1000 ppm showed a decrease in PCNA-labeling index at 4 weeks, similar to the results from the BrDU analysis, and was not different from untreated control for the rest of the study (Fig. 3). Both BrDU- and PCNA-labeling indexes exhibited a periportal distribution and the labeling at the 1000 ppm doses

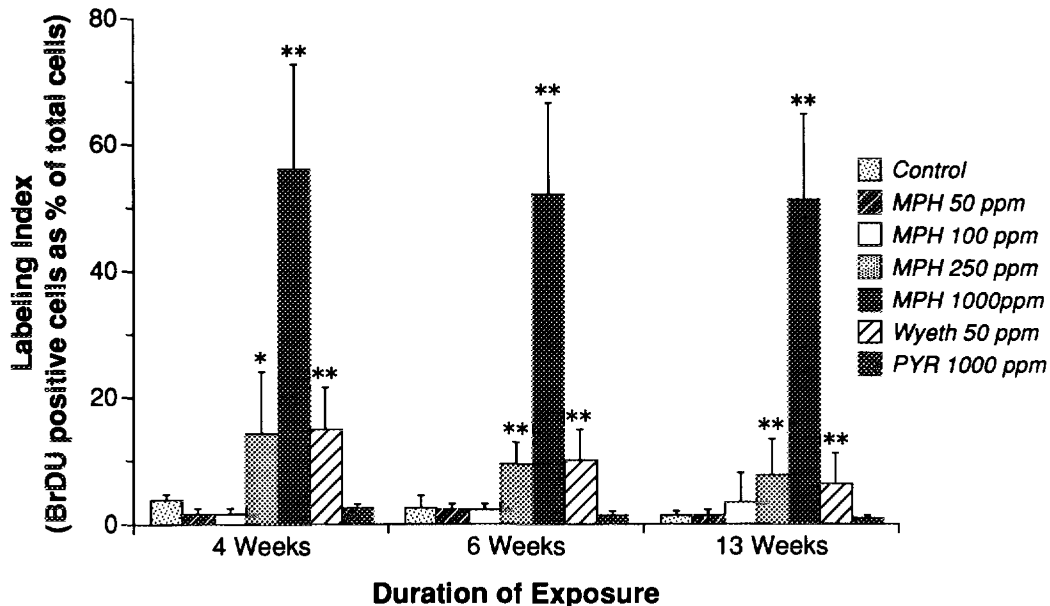

FIG. 2. Labeling indexes in livers of male F344 rats treated with methapyrilene, pyrilamine, or Wy-14,643. Labeling of newly replicated cells was performed by exposure to BrDU via osmotic minipump for the last 7 days prior to termination. *$p < 0.05$; **$p < 0.01$.

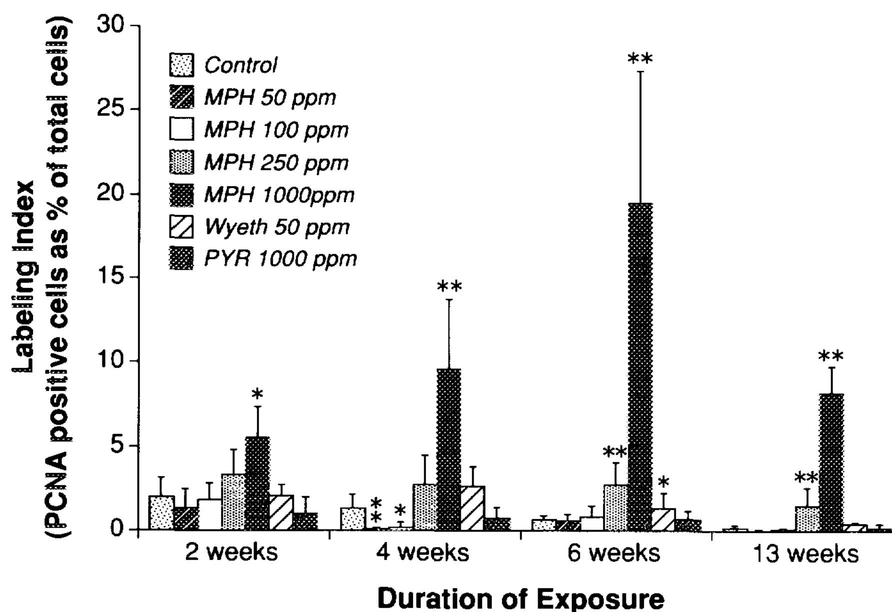

FIG. 3. Labeling indexes in livers of male F344 rats treated with methapyrilene, pyrilamine, or Wy-14,643. Labeling of cells was performed by staining for PCNA as described under Materials and Methods. $*p < 0.05$; $**p < 0.01$.

was so intense that the labeling became diffuse. Differences in the two methods for quantitating cell proliferation, BrDU and PCNA, produced similar though not identical results which reflect the bases for their identification of replicating cells.

Protein abundance changes and charge modifications measured by 2D electrophoresis. The results of the protein mapping indicated that several significant alterations in abundance changes occurred following MPH treatment (Fig. 4). At least 32 proteins underwent significant abundance changes at the high dose of MPH and 39 protein changes were observed in the Wy-14,643 treatment group evaluated after 6 weeks of treatment (Table 2). Similar changes occurred at 2, 4, and 13 weeks (data not shown). Many, but not all, of the protein changes in the MPH-dosed animals were common in the Wy-14,643 group. Treatment with PYR produced no significant quantitative protein changes, as judged by the same criteria used for MPH and Wy-14,643. MPH produced significant covalent modification of mitochondrial, but not cytosolic, proteins as measured by a charge modification index (Table 2). The effect is maximal at the 1000 ppm dose, measurable at 250 ppm, but becomes indistinguishable from control at 100 and 50 ppm. Neither PYR nor Wy-14,643 caused covalent modification of the mitochondrial or cytosolic proteins examined. The mitochondrial protein charge modification produced by MPH occurred at all four time points although the charge shift peaked at 4 weeks and declined thereafter. Quantitative abundance changes in at least 32 proteins were also observed and in most cases showed a maximum level of change at 29 days. Wy-14,643 did not produce protein charge shifts at any time point but did produce a consistent set of abundance changes in at least 39 proteins at all time points in this study (Table 2).

DISCUSSION

The early effects of carcinogenic chemicals that exert little or no genotoxicity in *in vitro* assays have been a subject of much interest in our laboratory (Yarbrough *et al.*, 1991; Thottassery *et al.*, 1992; Cunningham *et al.*, 1994a,b). The fact that a nongenotoxic compound such as methapyrilene produces such a dramatic carcinogenic effect in rats seems to contradict the somatic cell mutation hypothesis. Based on the data presented in this paper, we conclude that the ability of MPH to produce almost a 100% rate of hepatic carcinoma is related to its ability to produce a massive, sustained increase in cell replication when animals are exposed at the same dose and route of exposure as was performed in a carcinogenesis assay. This conclusion is supported by the inability of pyrilamine to produce an increase in cell proliferation at the same dose that methapyrilene induced a 20- to 40-fold increase (Figs. 2 and 3). Measurement of cell proliferation by BrDU incorporation or PCNA analysis produced similar qualitative results. BrDU produced higher LI due to its action to identify all labeled cells incorporating BrDU during the time that the pump is in place, whereas PCNA measures cells replicating at the time of euthanasia only.

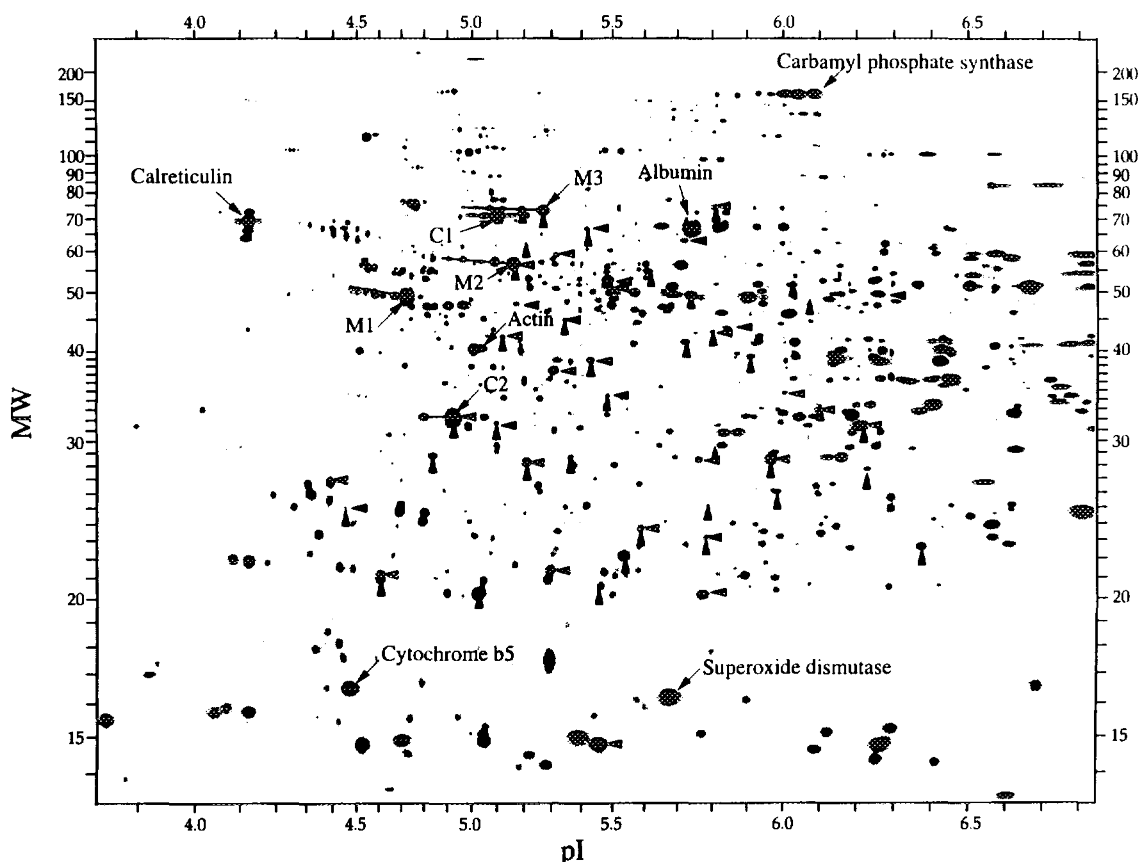

FIG. 4. Reference master 2D pattern of F344 rat liver proteins, with several landmark spots identified. M1, M2, and M3 are the three mitochondrial proteins and C1 and C2 are the two cytosolic proteins used in the calculation of charge modification indexes. C1 is hsc70, cytosolic stress protein believed to be a clathrin-uncoating ATPase. C2 is the 30-kDa senescence marker protein. A horizontal line indicates the set of charge shift spots used in the calculation of each of the five charge modification values. Upward pointing arrowheads indicate the 39 spots showing a significant effect of Wy-14,643, while the horizontal arrowheads point to the 32 protein spots significantly affected by MPH. Isoelectric point and molecular weight (kilodaltons) scales were calculated from best fits to the positions of a series of proteins of known sequence.

Pyrilamine was determined to be an equivocal or noncarcinogen in rats (Lijinsky, 1984). The nonmutagenic but potent hepatocarcinogen Wy-14,643 was also able to induce hepatic cell proliferation in this model, further supporting the link between induced cell proliferation and carcinogenesis. Data in support of this conclusion were also presented in a study published during preparation of this manuscript. Richardson *et al.* (1992) demonstrated that MPH at 1000 ppm produced hepatocellular labeling indexes of up to 67.8% in rats treated for 12 weeks. This strong degree of cell proliferation was not observed at 12 weeks of exposure to 1000 ppm MPH in the mouse, a species resistant to the hepatocarcinogenicity of MPH. They concluded that chronic toxicity was related to the carcinogenicity of MPH in the rat and that both toxicity and carcinogenicity were absent in the mouse.

Based on histopathological examination of liver sections, methapyrilene produces extensive hepatotoxicity after dietary exposure including hepatocellular necrosis and pigment deposition. The increased cell proliferation observed in the present study may be considered compensatory hyperplasia to replace necrotic cells. This is in contrast to the cell proliferation induced by Wy-14,643 that is not associ-

TABLE 2

Protein Abundance Changes and Charge Modification Indexes of Three Mitochondrial Proteins in Livers of Male Fischer 344 Rats Treated with Methapyrilene, Wyeth-14,643, or Pyrilamine for 6 Weeks

Treatment	Number of protein abundance changes	Charge modification index		
		Mitcon:1	Mitcon:2	Mitcon:3
Untreated control	—	−0.30	−0.34	−0.36
Methapyrilene				
50 ppm	0	−0.34	−0.34	−0.35
100 ppm	2	−0.37	−0.39	−0.37
250 ppm	6	−0.59	−0.61	−0.66
1000 ppm	32	−1.45	−1.45	−1.32
Wyeth-14,643, 50 ppm	39	−0.39	−0.40	−0.33
Pyrilamine, 1000 ppm	0	−0.24	−0.31	−0.31

ated with toxicity and may be considered to be due to a mitogen-type stimulation. It is interesting that both types of induced cell proliferation are associated with high levels of hepatocarcinogenesis. It has been speculated that increased cell proliferation per se may result in DNA lesions as a consequence of unfaithful DNA replication, mitotic recombination, or gene conversion (Ames *et al.*, 1993). Mitogenesis increasing mutagenesis is an appealing theory to explain the effects of nongenotoxic carcinogens that are negative in *in vitro* assays for mutagenesis but are positive carcinogens *in vivo*. Mutagenic by-products of cytotoxicity and cell division such as active oxygen species and lipid peroxidation metabolites may be produced that induce mutagenesis *in vivo* and yet remain undetected in the commonly used mutagenesis assays (Ames *et al.*, 1993). Rapidly proliferating cells may not have sufficient time to repair DNA damage from whatever source. Enhanced cell replication may also result in oncogene activation produced by hypomethylation of chromatin (Vorce and Goodman, 1989; Ray *et al.*, 1994). Chronic chemically induced cell proliferation may also promote the clonal expansion of preneoplastic cells during the promotion and progression stages in multistage carcinogenesis.

A similar conclusion was reached concerning the basis for the observed lack of carcinogenicity of a series of mutagenic chemicals. Although testing positive as mutagens in short-term assays, 2,6-diaminotoluene, 1-nitropropane, and several organophosphate compounds such as dimethoate and dioxathion were found not to induce tumors in 2-year NTP rodent bioassays. The structurally similar mutagenic compounds 2,4-diaminotoluene, 2-nitropropane, and tris(2,3-dibromopropyl) phosphate were able to induce liver or kidney tumors under bioassay conditions similar to those used for the noncarcinogens. Research in our laboratory demonstrated that one difference between the mutagenic carcinogens and the mutagenic noncarcinogens was in the ability of the carcinogens but not the noncarcinogens to induce cell proliferation in the target organ for carcinogenesis (Cunningham *et al.*, 1991, 1993, 1994a,b; Cunningham and Matthews, 1991). These results provide support to the hypothesis that chemically induced cell proliferation is associated with carcinogenesis. Similar conclusions have been made by other investigators (Cohen and Ellwein, 1990; Preston-Martin *et al.*, 1990). Recent reports (Tennant *et al.*, 1987; Zeiger, 1987) have demonstrated that *in vitro* mutagenicity data for a chemical are not sufficient to predict its carcinogenicity in 2-year bioassays as conducted by the National Toxicology Program. Results presented here represent a continued effort to determine the sources of these apparently discordant results and are of value in understanding the differences between *in vitro* and *in vivo* systems for the evaluation of results of animal bioassays for chemical carcinogenesis.

Protein analysis by two-dimensional electrophoresis has been demonstrated to be a useful tool for investigational toxicology studies. In the present study, protein changes that were different from control were identified in MPH dose groups as low as 100 ppm (Fig. 3). These changes occurred in a dose-dependent manner, increasing with increasing MPH exposure. These results confirm the protein changes observed by other groups (Anderson *et al.*, 1992; Richardson *et al.*, 1993). The hepatocarcinogen Wy-14,643 also produced a set of protein changes concomitant with its effects as a cell proliferator and a peroxisome proliferator (Table 2). Of immense interest was the observation that the nonhepatocarcinogen pyrilamine at 1000 ppm produced no detectable protein changes in the liver by this technique that showed major protein changes by the hepatocarcinogenic compounds. Also of interest is the identity of the proteins changed by chemical treatment. Of the 32 proteins changed in livers of the 1000 ppm MPH animals, 20 are common in livers of the Wy-14,643 group. These 20 may represent proteins expressed in the mechanics of cell replication, since both MPH and Wy-14,643 produce this effect. The remaining protein changes are less well characterized and may represent the unique biochemical toxic effects of these chemicals. The significance of the protein alterations is not definite, but may be of predictive value for assessing the carcinogenicity and toxicity of chemicals in preclinical assays. A chemical which does not produce a large number of protein changes would be considered safer than a similar chemical that produces many changes. This analysis may also be useful as a biomarker of exposure in human populations. It is possible that low-level and/or chronic exposures to chemicals may be detectable in human populations using this approach.

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