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# Relationship of Hepatic Peroxisome Proliferation and Replicative DNA Synthesis to the Hepatocarcinogenicity of the Peroxisome Proliferators Di(2-ethylhexyl)phthalate and [4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic Acid (Wy-14,643) in Rats

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## ABSTRACT

**The mechanism of hepatocarcinogenesis caused by peroxisome proliferators (PP) is poorly understood, making it difficult to predict the carcinogenicity of PP to rodents or other species. It has been suggested that the carcinogenic potential of individual PP in rodents is correlated with the degree of PP-induced hepatic peroxisome proliferation. To evaluate this possible correlation, di(2-ethylhexyl)phthalate (DEHP) at 1.2% and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) at 0.1% were fed to male F-344 rats for up to 365 days and hepatocytic peroxisome proliferation and DNA replication were measured. All rats fed Wy-14,643 for 365 days had numerous grossly visible nodules in comparison to none in the livers of DEHP-fed or control rats. Despite this difference in the induction of tumors, both DEHP and Wy-14,643 increased the peroxisomal volume density 4- to 6-fold from 8 to 365 days of treatment. Peroxisomal β-oxidation enzyme activities were increased 8-fold by both DEHP and Wy-14,643 after 18 days. At later time points (77 to 365 days), these enzyme activities were about 25% higher in livers of Wy-14,643- than DEHP-fed rats. DEHP or Wy-14,643 increased absolute liver weights 50 to 75% above controls after 18 to 365 days of feeding. Labeling of hepatocyte nuclei with a single injection of tritiated thymidine revealed a rapid burst in replicative DNA synthesis in both DEHP and Wy-14,643-fed rats, with a return to control levels by 4 days. Additional rats were implanted with 7-day osmotic pumps containing tritiated thymidine. With this more extended method of labeling a 5- to 10-fold increase in replicative DNA synthesis was observed in rats receiving Wy-14,643 for 39 to 365 days as compared to DEHP-fed rats or controls. In conclusion, when performed under conditions similar to the tumorigenicity studies, the degree of peroxisome proliferation correlated poorly with the relative hepatocarcinogenicity of DEHP and Wy-14,643. However, a strong correlation was observed between the relative hepatocarcinogenicity of DEHP and Wy-14,643 and the ability to induce a persistent increase in replicative DNA synthesis. These data emphasize the possible importance of cell replication in the mechanism of PP-induced hepatocarcinogenesis.**

## INTRODUCTION

A wide variety of structurally unrelated chemicals cause proliferation of peroxisomes in rodent liver (1–9). Chronic administration of PP[4] results in the induction of hepatocellular carcinomas in rats and mice (10, 11). The mechanism of PP-induced carcinogenesis is poorly understood because classical genotoxicity tests have been uniformly negative (12–21) and promotion studies have shown variable results (22). In general, PP cause large increases in the activity of the peroxisomal $H_2O_2$-producing enzyme fatty acyl-CoA oxidase (23), but minimal increases in the activity of peroxisomal catalase (24–26), the enzyme responsible for $H_2O_2$ degradation. These data led Reddy *et al.* (27) to hypothesize that the carcinogenic mechanism of the PP may involve leakage of $H_2O_2$ from peroxisomes and subsequent oxidative damage to macromolecules (28). Recent data support the leakage of hydrogen peroxide out of peroxisomes during high rates of peroxisomal β-oxidation in livers of PP-treated rats (29).

Although PP by definition cause proliferation of peroxisomes in hepatocytes and the associated increases in the activities of peroxisomal β-oxidation enzymes, the carcinogenicities of individual PP vary widely (30–35). For example, DEHP fed at 1.2% in the diet produced a 10% incidence of male rats with hepatocellular carcinomas after 2 years (30, 36), whereas Wy-14,643 at 0.1% in the diet resulted in a 100% incidence of male rats with hepatocellular carcinomas after 60 weeks (37–39). Because of the possible importance of peroxisome proliferation as a quantitative indicator of the carcinogenic risk of individual PP, we measured peroxisome proliferation following the feeding of DEHP or Wy-14,643 under conditions similar to the above carcinogenicity studies.

In addition to peroxisome proliferation, PP commonly cause hepatomegaly associated with a short burst in DNA replication (10, 40). Since DNA replication plays an important role in the induction of chemical carcinogenesis by other classes of chemicals (37, 41), replicative DNA synthesis was also quantitated in DEHP- and Wy-14,643-fed rats. Osmotic pumps were used to deliver tritiated thymidine to label the replicating DNA over 7 days. Compared to the traditional pulse labeling of animals with tritiated thymidine, the use of osmotic pumps increased the ability to measure low levels of DNA synthesis in liver. In the present study a strong correlation between increases in replicative DNA synthesis and hepatocarcinogenicity was observed while there was no quantitative correlation between peroxisome proliferation and hepatocarcinogenicity.

## MATERIALS AND METHODS

**Animal Treatment.** Male Fischer 344 rats were obtained from Charles River Breeding Laboratories, Inc. (Raleigh, NC), at 6–7 weeks of age (121.3 ± 6.8 g) and were housed 5 rats per cage. Upon arrival, the animals were quarantined for 2 weeks, after which time they were found to be virus-free as determined by the standard murine antibody determination tests (Microbiological Associates, Bethesda, MD). Rats were maintained on NIH-07 chow and purified water *ad libitum* and housed in biologically clean rooms with filtered air and a 12-h night/day cycle. Temperature and relative humidity were held at 22 ± 2°C and 50 ± 5% (SD), respectively. Body weights and clinical observations were recorded weekly, while food consumption was recorded biweekly throughout the study. Treated animals were given the same rodent chow blended with either DEHP (Eastman Chemical Products, Kingsport, TN) or Wy-14,643 (ChemSyn Science Laboratories, Lenexa, KS) at target

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[4] The abbreviations used are: PP, peroxisome proliferators; DEHP, di(2-ethylhexyl)phthalate; Wy-14,643, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]-acetic acid.

concentrations of 1.2 and 0.1%, respectively. The concentration of DEHP or Wy-14,643 in the feed was assayed after each blending with measured values of 1.208 ± 0.027 and 0.093 ± 0.003%, respectively.

Seven days prior to sacrifice on days 8, 18, 39, 77, 151, and 365, Alzet osmsotic pumps (Palo Alto, CA; flow rate, 10 μl/h; 7-day delivery) containing 2 mCi of 40 Ci/mmol tritiated thymidine (Amersham, Arlington Heights, IL) were implanted s.c. in five rats per group. Two h prior to sacrifice on the mornings of days 1, 2, 4, 8, 18, and 39, five additional rats per group were given 1 μCi/g body weight tritiated thymidine (New England Nuclear, Boston, MA; specific activity, 6.7 Ci/mmol) ip.

**Histopathology and Autoradiography.** On the mornings of days 1, 2, 4, 8, 18, 39, 77, 151, and 365 five or ten animals per group were killed by exsanguination while under methoxyflurane anesthesia. At the time of necropsy terminal body weights and wet liver weights were recorded.

Liver sections were made for hematoxylin and eosin staining and autoradiography. Autoradiographic sections were dipped in emulsion (Kodak NTB-2) and then allowed to develop in darkness at −20°C. Sections from the left lobe of pulse-labeled rats were developed for 8 weeks while those from pump-implanted rats were developed for 13 weeks. A section of ileum was included from each rat as a positive control of nuclear labeling. Labeled and unlabeled hepatocyte nuclei were counted in randomly generated fields of nonlesional tissue. At least 2000 hepatocyte nuclei were counted per rat to calculate the percentage of labeled nuclei (labeling index).

At the 365-day time point, livers were sectioned at 1–2-mm intervals for quantification of grossly observable lesions. Frozen and formalin-fixed sections of lesions were taken for histological evaluation.

**Peroxisome Morphometry.** Additional sections of nonnodular liver were taken from the left lobe for electron microscopy and stained using a diaminobenzidine technique (42). Twelve electron micrographs were taken randomly from each of 3 rats/group at days 8, 39, 151, and 365. Peroxisomal volume density was determined from the area density of peroxisomes in cross-sections of nonlesional hepatocyte cytoplasm at a final magnification of ×20,000 (43). Peroxisomal mean volume and numerical density determinations were calculated using a minor modification of a standard stereological technique (44). Peroxisomes were assumed to be spherical, with a minimal identifiable radius of 0.1 μm.

**Enzyme Assays.** Liver from the left lobe was used to prepare 20% homogenates in 50 mM Tris-HCl-154 mM KCl, pH 7.2. Samples were kept on ice until frozen at −20°C. The postnuclear supernatant of the thawed liver homogenate was prepared on the day of enzyme assays by centrifugation at 2500 × *g* for 10 min.

Fatty acyl-CoA oxidase activity was assayed in the postnuclear supernatant of 5 or 10 rats/group by a measurement of hydrogen peroxide production in the presence of 25 μM palmitoyl-CoA (45). Cyanide-insensitive $NAD^+$ reduction activity was measured using the same supernatants (46). Enzyme activity was normalized per g of protein using a commercial kit (Abbott Labs, Irving, TX). Since no difference in enzyme activity was noted between liver homogenates from pulse-labeled and pump-implanted animals, the data from these two groups were combined for statistical analysis.

**Statistical Analysis.** Data from the groups were analyzed for homogeneity of variance. Statistical significance was assessed with the *t* test, using a Bonferroni correction factor for multiple comparisons. All data points are reported as means ± SEM for samples of size $n = 5$ or 10.

## RESULTS

**Effects on Feed Consumption, Body Weight, Liver Weight, and Hepatocarcinogenesis.** Feed consumption (w/w) fell by about 10% in Wy-14,643-fed rats initially but returned to control levels by day 18 (data not shown). Body weight gains of both DEHP- and Wy-14,643-fed rats were lower than controls, with terminal body weights of Wy-14,643-fed rats being statistically significantly lower than those of rats fed DEHP for 18 to 365 days (Fig. 1). Both compounds increased absolute liver weights between 18 and 365 days, with liver weights of 15 to 17 g, compared to control weights of 10 to 12 g (data not shown). The relative liver weight of Wy-14,643-fed rats was higher than DEHP-fed rats or controls from 18 to 365 days (Fig. 2), reflecting the lower body weights of the Wy-14,643-fed rats.

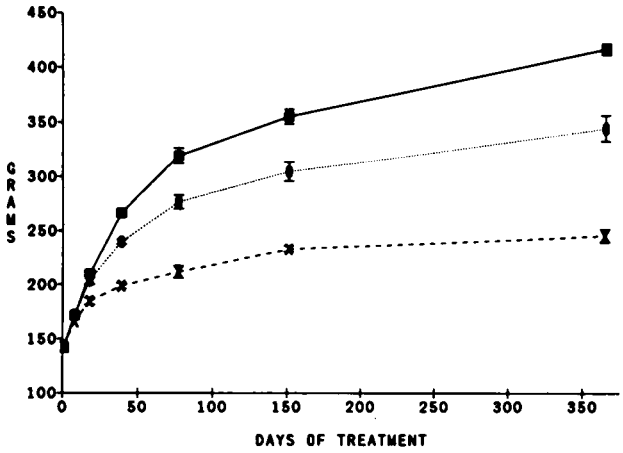


Fig. 1. Terminal body weights of rats given control, DEHP, or Wy-14,643 diet for up to 1 year. ■, control; O, DEHP diet; ×, Wy-14,643 diet. *Bars*, SEM.

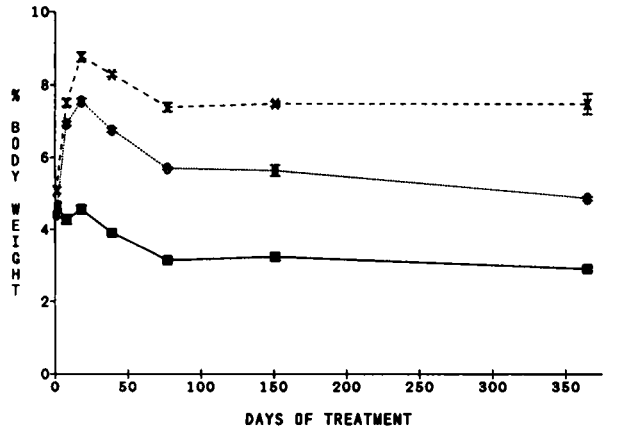


Fig. 2. Relative liver weights as a percentage of body weights for rats given control, DEHP, or Wy-14,643 diet for up to 1 year. ■, control; O, DEHP diet; ×, Wy-14,643 diet. *Bars*, SEM.

Table 1 *Grossly visible nodules and hepatocellular carcinomas in the livers of rats fed control, DEHP, or Wy-14,643 diet for 365 days*

Rats were treated and livers were examined as in "Materials and Methods." Data are expressed as the mean frequency of lesions ± SEM for each size category.

| | Mean diameter of lesions (mm) in treatment group | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | ≥5 |
| Control | ND[a] | ND | ND | ND | ND |
| DEHP | ND | ND | 0.2 ± 0.2 | ND | ND |
| Wy-14,643[b] | 53.0 ± 6.7 | 5.0 ± 0.9 | 2.0 ± 0.7 | 0.5 ± 0.3 | 1.0 ± 0.7 |

[a] ND, no lesions detected.
[b] Only Wy-14,643 induced significant hepatic lesions over controls, $P < 0.05$ for all lesion size classes.

Macroscopic lesions were not observed in rats fed the control or DEHP-containing diet for 365 days (Table 1). In contrast, livers of rats fed Wy-14,643 for 365 days contained numerous macroscopic (Table 1) and microscopic lesions (data not shown) not present in DEHP or control animals. In hematoxylin and eosin-stained sections the Wy-14,643-induced lesions were observed only at 365 days and were randomly distributed throughout the liver lobule of all lobes examined. The cells of these lesions characteristically had large, irregularly staining nuclei with multiple nucleoli and basophilic cytoplasm. A majority of even the smallest lesions identified had an autonomous orga-

nization with distinct borders and were compressing adjacent tissue.

**Peroxisome Proliferation.** Peroxisomal fatty acyl-CoA oxidase activity increased after 1 or 2 days of treatment with Wy-14,643 or DEHP, respectively (Fig. 3*A*). Peak activity was observed in both groups by 18 days with values 8-fold higher than controls. From 39 to 365 days of treatment the activity in livers from DEHP-fed rats was 70 to 80% of that in Wy-14,643-fed rats.

Peroxisomal $\beta$-oxidation activity as measured by cyanide-insensitive $NAD^+$ reduction was increased after 2 or 4 days of Wy-14,643 or DEHP feeding, respectively (Fig. 3*B*). Again, peak activity was observed in both treatment groups at 18 days with values 8-fold higher than those of controls. Only at later time points were differences noted between Wy-14,643- and DEHP-fed rats. The activity in livers from DEHP-fed rats was about 35% lower than from Wy-14,643-fed rats after 1 year.

Quantitative evaluation of electron micrographs indicated that both Wy-14,643 and DEHP induced the proliferation of numerous catalase-positive peroxisomes (Table 2). Increases in the peroxisomal volume density was similar in magnitude to the observed increases in the activity of the peroxisomal $\beta$-oxidation system with maximal values about 6-fold higher than controls at all time points studied. The peroxisomal mean volume from livers of treated and control rats increased over time, with a concomitant decrease in the numerical density. The increase in peroxisomal volume density associated with DEHP and Wy-14,643 treatment was due to an increase in both the numerical density and the mean volume of the peroxisomes.

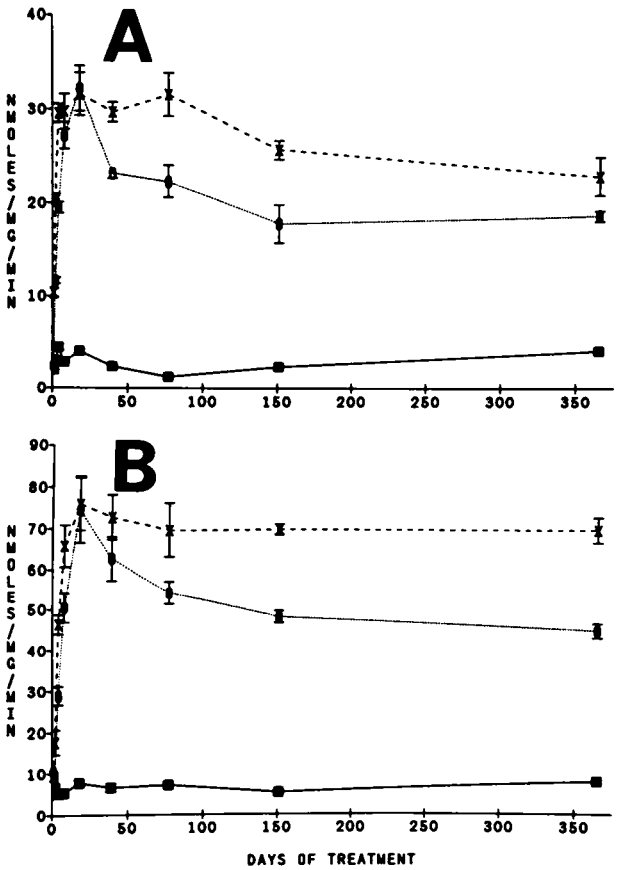


Fig. 3. Effect of control, DEHP, or Wy-14,643 diets on peroxisomal enzyme activities in the postnuclear supernatant of liver homogenates. *A*, palmitoyl-CoA oxidase activity; *B*, cyanide-insensitive $NAD^+$ reduction activity. ■, control; ○, DEHP diet; ×, Wy-14,643 diet.

Table 2 *Morphometry of hepatic peroxisomes from rats fed control, DEHP, or Wy-14,643 diets*

Rats were treated and liver sections were prepared as in "Materials and Methods." Peroxisome evaluation were performed for 3 rats/group by image analysis from electron micrograph prints at a final magnification of ×20,000.

| Treatment | Days of treatment | Vv[a] | Nv | Mean volume |
|---|---|---|---|---|
| Control | 8 | 1.7 ± 0.1[b] | 0.20 ± 0.04 | 0.09 ± 0.02 |
| | 39 | 2.9 ± 0.4 | 0.33 ± 0.06 | 0.09 ± 0.01 |
| | 151 | 2.8 ± 0.3 | 0.20 ± 0.02 | 0.14 ± 0.01 |
| | 365 | 1.8 ± 0.4 | 0.07 ± 0.02 | 0.28 ± 0.09 |
| DEHP | 8 | 8.9 ± 1.6[c] | 0.64 ± 0.04[c] | 0.14 ± 0.03 |
| | 39 | 13.0 ± 0.9[c] | 0.69 ± 0.05[c] | 0.19 ± 0.03[d] |
| | 151 | 7.9 ± 0.5[c] | 0.40 ± 0.04[d] | 0.20 ± 0.04 |
| | 365 | 12.0 ± 5.7[d] | 0.17 ± 0.02[d] | 0.71 ± 0.31 |
| Wy-14,643 | 8 | 9.0 ± 1.2[d] | 0.40 ± 0.07[d] | 0.23 ± 0.02[d] |
| | 39 | 17.4 ± 2.6[c] | 0.75 ± 0.03[c] | 0.23 ± 0.03[d] |
| | 151 | 12.7 ± 0.6[c] | 0.33 ± 0.05 | 0.41 ± 0.07[d] |
| | 365 | 7.7 ± 1.6[d] | 0.13 ± 0.02 | 0.63 ± 0.17 |

[a] Vv, volume density (percentage of cytoplasm occupied by peroxisomes); Nv, numerical density (number of peroxisomes/$\mu m^3$ of cytoplasm; mean volume, average volume of a peroxisome in $\mu m$.
[b] Mean ± SEM.
[c] Significantly higher than control values, $P < 0.01$.
[d] Significantly higher than control values, $P < 0.05$.

**Effects on DNA Synthesis.** Increases in hepatocytic DNA synthesis as identified by autoradiograms using the 2-h pulse-labeling technique (Table 3) corresponded well with increases in liver weights. In the control rats, higher labeling indices were observed at early as compared to late time points. The labeling index peaked in Wy-14,643- and DEHP-fed rats at 1 or 2 days, respectively, and then returned to control levels by 4 days in both treatment groups.

With the osmotic pump labeling technique a large increase in the nuclear labeling index was noted at 8 days in livers of both DEHP- and Wy-14,643-fed rats (Table 3) similar to the burst of DNA synthesis noted after pulse administration of tritiated thymidine. In agreement with the pulse-labeling technique, at Day 8 the control labeling index was elevated. However, in contrast to the results after pulse-labeling, with the osmotic pump technique a 5- to 10-fold increase in nuclear labeling was observed between 77 and 365 days in Wy-14,643-fed compared to DEHP-fed rats and controls. At all time points examined, the majority of the nuclear labeling in the livers of Wy-14,643-fed rats was periportal, with occasional labeled nuclei observed in other regions of the liver lobule. A small but statistically significant increase in the labeling index was noted in the livers of DEHP-fed rats at 365 days.

## DISCUSSION

Proliferation of peroxisomes occurs in rats and mice after the administration of hypolipidemic agents, plasticizers, halogenated solvents, and herbicides (47). Following the observation that many of the chemicals that induce hepatic peroxisomes are also hepatocarcinogenic, Reddy *et al.* (27) hypothesized a causal relationship between peroxisome proliferation and hepatocarcinogenesis. Despite the data supporting an association between peroxisome proliferation and hepatocarcinogenicity, a causal relationship has not been established. Comparing PP with widely different carcinogenic activities could help define the relationship between peroxisomal induction and the subsequent formation of hepatic tumors. The current feeding study was designed to examine the possible correlations between peroxisome proliferation, replicative DNA synthesis, and the hepatocarcinogenicity of DEHP and Wy-14,643 under conditions of the original feeding studies (30, 36, 39). The informa-

Table 3 *Hepatic nuclear labeling indices in rats fed control, DEHP, or Wy-14,643 diets*

Five male rats were fed the diets shown for up to 365 days. Pulse-labeled animals received 1 μCi/g body weight i.p. of 6.7 Ci/mmol tritiated thymidine 2 h prior to sacrifice. Pump-infused animals were implanted s.c. with an osmotic pump (10 μCi/h) containing 40 Ci/mmol tritiated thymidine 7 days prior to sacrifice. Autoradiograms were prepared and labeling index was quantitated as described in "Materials and Methods."

| Days of treatment | Labeling index for pulse-labeled rats | | | Labeling index for pump-infused rats | | |
|---|---|---|---|---|---|---|
| | Control | DEHP | Wy-14,643 | Control | DEHP | Wy-14,643 |
| 1 | 2.0 ± 0.3[a] | 2.6 ± 0.6 | 8.8 ± 1.2[d] | [b] | [b] | [b] |
| 2 | 2.0 ± 0.5 | 5.6 ± 0.9[c] | 5.3 ± 0.9[c] | [b] | [b] | [b] |
| 4 | 1.3 ± 0.2 | 1.2 ± 0.2 | 1.6 ± 0.1 | [b] | [b] | [b] |
| 8 | 2.0 ± 0.6 | 1.0 ± 0.1 | 1.3 ± 0.2 | 8.5 ± 0.7 | 39.8 ± 5.2[d] | 50.0 ± 3.2[d] |
| 18 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.9 ± 0.1[c] | 2.5 ± 0.4 | 5.1 ± 1.2 | 9.6 ± 1.2[c] |
| 39 | 0.3 ± 0.0 | 0.4 ± 0.1 | 0.4 ± 0.0 | 1.2 ± 0.3 | 3.2 ± 0.5 | 5.9 ± 1.3[d] |
| 77 | [b] | [b] | [b] | 0.9 ± 0.3 | 0.7 ± 0.2 | 10.4 ± 1.7[d] |
| 151 | [b] | [b] | [b] | 2.2 ± 1.2 | 1.5 ± 0.1 | 9.6 ± 0.7[d] |
| 365 | [b] | [b] | [b] | 1.0 ± 0.1 | 1.5 ± 0.3[c] | 9.5 ± 1.1[d] |

[a] Mean ± SEM.
[b] Nuclear labeling not measured.
[c] Significantly higher than control values, $P < 0.05$.
[d] Significantly higher than control values, $P < 0.01$.

tion from the present comparative study is important in determining the usefulness of peroxisome proliferation and DNA replication as early predictors of subsequent PP-induced carcinogenicity. In addition, this information may give direction to further studies of the mechanism of PP-induced cancer.

Several previous studies have addressed the possible quantitative relationship between the degree of peroxisome proliferation and hepatocarcinogenesis. Reddy *et al.* (34) and Tomaszewski *et al.* (33) compared peroxisome proliferation induced by several PP to historical hepatocarcinogenicity bioassay data. Both groups (33, 34) concluded that the degree of peroxisome proliferation was correlated to the incidence of hepatocellular cancer. However, there are several potential problems in the above comparative studies. Some of the doses of PP used by Reddy *et al.* (34) were not the same as that used in the carcinogenicity bioassays. In the studies by Tomaszewski *et al.* (33), the animals were dosed by gavage, making it difficult to quantitatively compare peroxisome proliferation to the tumor incidence in previous bioassays where the chemical was blended in the diet. Finally, the evaluation of only a single early time point in both studies makes it difficult to determine what role peroxisome proliferation may have on the progression of hepatic tumors. Interestingly, in contrast to the results from the above studies, fatty acyl-CoA oxidase and peroxisomal β-oxidation activities (Fig. 3) were identical in rats receiving DEHP or Wy-14,643 for 18 days at dietary concentrations used in the original bioassays. While comparative studies are an important tool for investigating the mechanism of carcinogenicity of PP, duplication of sex and strain of rat, dose and route of administration of the PP, and examination of multiple time points is necessary to confidently compare treatment-induced changes with historical carcinogenicity bioassay data.

**Peroxisome Proliferation.** Differences in peroxisomal enzyme activities in rats receiving DEHP and Wy-14,643 were small and observed only at later time points. Measurements of peroxisome proliferation in nonnodular tissue by morphometry agreed closely with the measurements of enzyme activities. While minor differences in peroxisomal enzyme activities at later time points may qualitatively correspond with the carcinogenicity of the two compounds, the degree of peroxisome proliferation does not quantitatively correlate to the cancer incidences shown here or reported elsewhere (30, 36, 39). It is plausible that a threshold may exist, above which the induction of peroxisomes or peroxisomal specific β-oxidation may be sufficient to result in leakage of hydrogen peroxide from peroxisomes and subsequent macromolecular damage. In addition, these measurements do not test the "leakiness" of peroxisomes or the intracellular detoxification of the active oxygen species (29). However, the marginal quantitative differences noted in peroxisome proliferation suggest that other factors are important in modulating the ultimate carcinogenic response. The lack of a quantitative correlation between the degree of peroxisome proliferation to hepatocarcinogenicity in the present study suggests that measurements of PP-induced peroxisome proliferation are not reliable predictors of the carcinogenic potential of these chemicals.

**Replicative DNA Synthesis.** As expected (47), persistant hepatomegaly was observed following the start of DEHP- or Wy-14,643-feeding. The burst of replicative DNA synthesis noted during the first several days of DEHP- or Wy-14,643 treatment could facilitate the fixation of carcinogen-induced genetic alterations. Enhanced cell replication profoundly enhances the tumorigenicity of an initiator (37, 48), and is believed to be important at multiple stages in chemically induced hepatocarcinogenesis (41). An elevation in replicative DNA synthesis was detected on Day 1 in Wy-14,643-treated compared to Day 2 for DEHP-treated rats, corresponding to a 1-day-earlier induction of hepatic peroxisomal enzymes in Wy-14,643-treated rats. The earlier induction of both parameters suggests a different rate of delivery for two chemically divergent hypolipidemic agents. One interesting observation was the higher replicative DNA synthesis in the control rats during the first 8 days of the study compared to later time points, even though the age and weights of the rats used in the present study were selected so that they were at least as old as those in the original reports for DEHP (36) and Wy-14,643 (39). What effect the early elevation in background cell proliferation due to normal growth may have had on the eventual carcinogenic response in these studies is unknown but it has been shown in numerous systems that there are sometimes striking differences in the response to xenobiotics in the immature animal (49).

In contrast to the early effects of PP on hepatic cell replication, both the present study and past studies using pulse labeling with tritiated thymidine have not detected increases in replicative DNA synthesis in rat liver at later time points. Limited data in mice (50) have suggested a minor enhancement of cell replication with chronic PP treatment. Because of the low rate of replicative DNA synthesis in hepatocytes of normal adult rats, we decided to quantitate replicative DNA synthesis using osmotic pump infusion for 7 continuous days.

Nuclear labeling of hepatocytes in rats implanted with the 7-day osmotic pumps clearly showed that Wy-14,643 but not

DEHP treatment enhanced hepatocellular DNA replication at later time points (*i.e.*, 39 to 365 days). Unpublished work from this laboratory suggests that foci and nodules of Wy-14,643-fed rats can be reliably identified by both hematoxylin and eosin, as well as the accepted ATPase histochemistry. By eliminating all visible preneoplastic lesions identified by hematoxylin and eosin staining prior to counting, the elevated level of cell replication in the Wy-14,643-fed animals indicates a general phenomenon of hepatocytes. The predominantly periportal distribution of the nuclear labeling with random distribution of the preneoplastic lesions in this study offers additional evidence that the increased rate of DNA synthesis is a generalized hepatocytic response and not merely a reflection of enhanced replication within foci or nodules. The rates of replicative DNA synthesis induced by Wy-14,643 as well as DEHP at 365 days correlate well with their carcinogenic activities. This increase in DNA synthesis noted in the Wy-14,643-fed rats may be a direct mitogenic effect on hepatocytes, starting initially with the massive burst in DNA synthesis in the first days of treatment and continuing at a low level over time. However, since the liver weights of rats fed either DEHP or Wy-14,643 were remarkably similar at all time points, it seems likely that Wy-14,643-fed rats lost hepatocytes through toxicity. Only marginal elevations in serum enzymes as a measure of hepatotoxicity have been demonstrated, suggesting that hepatocellular toxicity, if present, may be at or below the current capabilities of detection (51). Although an increase of hepatocellular ploidy cannot be excluded (52), it is difficult to explain the magnitude of the rate without concomitant cell death. The persistent increase in replicative DNA synthesis observed in Wy-14,643-fed rats may help explain the high incidence of cancer with this PP.

Regardless of the underlying mechanism, the difference in levels of persistent replicative DNA synthesis between Wy-14,643- and DEHP-fed rats identifies this response as potentially important in understanding the divergent carcinogenicity of these two agents. A chronic level of hepatocellular toxicity from some effect of the Wy-14,643 such as oxidative stress (53) or some unidentified factor could cause cell death and the subsequent need for cellular replacement. It was interesting to note that a small but significant increase in DNA replication was occurring in the DEHP-fed animals at 1 year. What relationship this may have to the induction of tumors by DEHP is not clear. However, the persistent replicative DNA synthesis induced by Wy-14,643 could by itself be necessary for promotion of altered hepatocytes or be important in the expression of the promotional event(s) (54).

Cell replication has been suggested to be important in the initiation, promotion, and progression of cancer (54). It is important to realize that in many tissues, such as the epidermal layer of the skin, the intestinal epithelium, and the hematopoietic system, high levels of cell replication are normal and do not typically cause these tissues to become neoplastic. However, many chemical carcinogens do induce forced or regenerative cell replication. Schulte-Hermann (55) lists numerous compounds and treatments that induce a transient burst in cell replication in rodents, only some of which have been proved to be carcinogenic in the tissues shown to have enhanced replication. It has historically been presumed for these "inducers" of cell replication that the proliferation rate returns to control levels within a few days, even when the administration of the inducing substance continues (55). However, it is now evident from this study that the factor which makes the livers of Wy-14,643-fed rats quantitatively different from those of DEHP-fed rats is not the early, short burst of cell replication but the elevated, persistent level of cell replication that has been described here at all later time points. These results further suggest that the dependency in numerous studies upon pulse administration of labeled thymidine for detection of cell proliferation may have failed to address potential effects of chemicals on forced cell replication. While it is clear that the cumulative magnitude of the Wy-14,643-induced hepatocellular replication is great, what role it may play in chemically induced carcinogenesis is unknown. Additional studies are necessary to determine the effect of this elevated replication upon spontaneous mutations, exogenous initiating events, promotion, and possible alterations in the regulation of hepatocyte proliferation.

In conclusion, the lack of a quantitative correlation between the degree of peroxisome proliferation and carcinogenicity of DEHP and Wy-14,643 suggests that peroxisome proliferation alone is insufficient to explain the carcinogenicity induced by PP. The difference in replicative DNA synthesis after chronic administration of DEHP or Wy-14,643 suggests that induction of cell replication may be important in the carcinogenic mechanism of PP. The persistent elevation of DNA replication in Wy-14,643- as opposed to DEHP-fed rats represents the identification of a biological end point that is both qualitatively and quantitatively correlated with carcinogenesis.

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