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Delineation of the Role of Metabolism in the Hepatotoxicity of Trichloroethylene and Perchloroethylene: A Dose–Effect Study

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Delineation of the Role of Metabolism in the Hepatotoxicity of Trichloroethylene and Perchloroethylene: A Dose–Effect Study. BUBEN, J. A., AND O'FLAHERTY, E. J. (1985). *Toxicol. Appl. Pharmacol.* 78, 105–122. The relationships among dose, metabolism and hepatotoxicity in mice which resulted from subchronic exposure to the chlorinated solvents trichloroethylene (TRI) and perchloroethylene (PER) were examined. Male Swiss-Cox mice received either TRI (0 to 3200 mg/kg/day) or PER (0 to 2000 mg/kg/day) in corn oil by gavage for 6 weeks. Urinary metabolites from individual mice were quantified to estimate the extent to which each compound was metabolized. Four parameters of hepatotoxicity were assessed: liver weight, triglycerides, glucose-6-phosphatase (G6P) activity, and SGPT activity. TRI significantly affected liver weight and G6P activity; PER affected all four parameters. The metabolism of TRI was linearly related to dose through 1600 mg/kg, but then became saturated. The metabolism of PER was saturable. The dose–effect curves of the affected hepatotoxicity parameters of both compounds were nonlinear and resembled the dose–metabolism graph of the corresponding solvent. Plots of the hepatotoxicity data of each compound against total urinary metabolites were linear in all cases, suggesting that the hepatotoxicity of both PER and TRI in mice is directly related to the extent of their metabolism. This pattern is consistent with formation of the toxic intermediate in the primary metabolic pathway of each compound. © 1985 Academic Press, Inc.

Studies by the National Cancer Institute (NCI) have demonstrated that mice treated chronically with high doses of the widely used industrial solvents trichloroethylene (TRI) and perchloroethylene (PER) had high incidences of hepatocellular carcinoma (National Cancer Institute, 1976, 1977). Attempts to assess the carcinogenic risk to man of exposure to these and other compounds found to cause tumors in animals are being made. However, such assessments have been challenged because of lack of information concerning two important relationships: the relationship between disposition of the compound at high and low doses, and the relationship between dose-related pharmacokinetic factors and toxic response. Insights into both of these relationships can be gained from an examination of the pharmacokinetic properties of such compounds. The importance of pharmacokinetic analysis in interpreting toxic response has been reviewed by Andersen (1981).

In most carcinogenicity bioassays only a few very high doses are used. The percentage of animals developing tumors from chronic treatment at these doses is determined. These data form the basis for assessment of the carcinogenic risk of the compound to man. An extrapolation is made from the animal response data at high doses to the low doses more characteristic of human exposure. Such

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an extrapolation may utilize any of a number of mathematical models (Fishbein, 1980), but each assumes that the pharmacokinetics of the compound in question obeys first-order kinetics over the range of the extrapolation. Such an assumption is rarely valid.

A number of processes involved in the disposition of a compound, particularly the metabolic processes, are often dose dependent. This dose dependency has particular importance since it is now well established that many chemicals known to be carcinogens are metabolized to reactive intermediates which can interact with cellular components to initiate the tumorigenic process (Miller, 1978; Weisburger and Williams, 1980). At high doses, the metabolism of many chemicals is likely to be saturated, a result which can lead to shifts in the relative importance of metabolic pathways and to relative increases or decreases in the toxic response. For this reason, the need to examine the pharmacokinetics of a compound when risk assessment or other evaluation of dose–response relationships is to be undertaken has been stressed (Munro, 1977; Watanabe *et al.*, 1977; Andersen, 1981).

Despite the problems associated with high-dose data, little has been done to examine the effect of high doses on both metabolism and toxic response. TRI and PER are good models for such a study. Not only have both been shown to cause hepatic tumors in mice in NCI carcinogenicity bioassays, but both are believed to be metabolized through an epoxide intermediate, an intermediate often involved in initiation of carcinogenesis by other chemicals (Henschler, 1977; Van Duuren, 1975; Weisburger and Williams, 1980). Both compounds can also be eliminated unchanged via the lungs, a nontoxic pathway (Daniel, 1963). However, despite these similarities, there is evidence that the kinetics of TRI and PER metabolism differ significantly. The metabolism of TRI has been reported to be linearly related to dose, while that of PER is saturable (Ikeda *et al.*, 1972). This difference, together with their similar metabolic pathways and their common site of action, makes them ideal candidates for studying the effect of metabolism on toxicity.

The purpose of this study was to investigate the relationships among TRI and PER dose, metabolism, and hepatotoxicity in mice after subchronic exposure to these solvents, with particular interest in how these relationships are affected at high doses. A dosing format similar to those used in the NCI carcinogenicity bioassays was followed. Studies by Schumann *et al.* (1980) on PER and by Stott *et al.* (1982) on TRI have suggested that the carcinogenicity of these solvents to mice occurs via an epigenetic mechanism, involving repeated toxic insult to the liver as a prerequisite to tumor formation. An understanding of the above relationships, therefore, could have significant bearing on an assessment of the carcinogenic potential of these solvents.

METHODS

Chemicals. Trichloroethylene and perchloroethylene (tetrachloroethylene) were obtained from the Aldrich Chemical Company. Trichloroethylene was distilled before use. Perchloroethylene had a purity greater than 99% and was used without further purification.

Animals. Male Swiss-Cox mice (outbred) were obtained by breeding from a colony at the Kettering Laboratory. When the mice were between 3 and 5 months of age, males from each litter were randomly assigned to each dose group and were tail marked. Three to five mice were generally housed together in stainless-steel cages with wire-grid bottoms. The mice had access to Purina Rodent Laboratory Chow at all times. Water was available *ad libitum* through an automatic watering system. The room was maintained on a 12-hr light/dark cycle at 21 ± 2°C.

Dosing. Dose mixtures were prepared fresh two or three times weekly by dissolving calculated amounts of the solvents in corn oil. The mice were weighed three times each week; the weight of each mouse determined the amount of dose mixture it received. Mouse weights ranged from 34 to 45 g; dose volumes ranged from 0.18 to 0.24 ml. The mice were dosed by gavage five times each week for 6 weeks, a total of either 29 or 30 doses. Doses for PER were 0, 20, 100, 200, 500, 1000, 1500, and 2000 mg/kg/day. Those for TRI were 0, 100, 200, 400, 800, 1600, 2400, and 3200 mg/kg/day. Controls received corn oil. Twelve to fifteen mice were used in most dose groups; the 100- and 1200-mg TRI/kg/day groups and the 1500- and 2000-mg PER/kg/day groups

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contained 4 to 6 mice. The two control groups consisted of 24 and 26 mice.

Procedure. Four parameters were chosen to assess hepatic toxicity: increases in liver weight, decreases in liver glucose-6-phosphatase (G6P) activity, increases in liver triglycerides, and increases in serum glutamate-pyruvate transaminase (SGPT) activity. Mice were killed by cervical fracture the day following their last dose. A blood sample was obtained by cardiac puncture. The blood was allowed to clot and the serum, obtained after centrifugation, was frozen until its assay for SGPT. The inferior vena cava on the underside of the liver was cut, and the liver was perfused with 5 ml of ice-cold saline via the left ventricle of the heart. The liver was removed, washed in cold saline, blotted dry, and weighed. It was homogenized in 9 vol of 0.1 M Tris–maleate buffer (pH 6.2). Aliquots were frozen until assay. Triglyceride concentrations were determined on 1-ml aliquots of hepatic homogenate by the method of Van Handel and Zilversmit (1957) as modified by Butler *et al.* (1961). Glucose-6-phosphatase activity was determined by measuring the amount of phosphate released after incubation of 0.1 ml homogenate with 25 μmol of glucose 6-phosphate in 0.1 M Tris–maleate buffer (pH 6.2, total volume = 1 ml) for 20 min at 37°C. Four milliliters of 10% trichloroacetic acid was used to stop the reaction. Phosphate was measured by the method of Fiske and Subbarow (1925) and is expressed as micrograms of phosphate per milligram of protein. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. SGPT activity was determined on 0.1 ml of serum by the method of Reitman and Frankel (1957). DNA was measured by the method of Burton (1968).

Portions of freshly removed liver were fixed in 10% neutral Formalin, embedded in paraffin, and sectioned. The sections were processed by standard procedures, stained with hematoxylin and eosin, and submitted for histological evaluation. Weighed portions of some livers were placed in an oven at 75°C until a constant dry weight was obtained.

Hepatotoxicity data were analyzed by analysis of variance, and comparisons between each dose and its respective control were done by the Student–Newman–Keuls procedure (Sokal and Rohlf, 1969).

Urinary metabolites. During the dosing period, individual mice from each of the dose groups were placed into mouse metabolism cages supplied by Wahmann Manufacturing Company. Feed and water were always available. After an acclimation day, a 24-hr urine collection was taken. Two to four urine samples were collected per mouse throughout the 6-week dosing period.

Preliminary studies to investigate day-to-day and week-to-week variations in the pattern of urinary metabolites were also carried out. For these studies, mice were housed in metabolism cages throughout the dosing period and daily urine collections were made.

Quantification of the urinary metabolites trichloroethanol (TCE) and trichloroacetic acid (TCA) was by the method of Humbert and Fernandez (1976). A Hewlett–Packard Model 7620A gas chromatograph equipped with a tritium electron capture detector was used for the analysis. The column, containing 5% OV-17, was heated at 92°C.

Oxalic acid in urine was determined by the method of Hodgkinson and Williams (1972). A method reported by Rajagopal and Ramakrishnan (1975) was adapted to determine whether ethylene glycol was a urinary metabolite of PER.

Data analysis. The hepatotoxicity data were plotted against both dose and total urinary metabolites. The data points were fit using the Nonlin computer program provided by the Upjohn Company (Metzler *et al.*, 1974), or by linear regression analysis, as appropriate.

RESULTS

Hepatotoxicity

Mice tolerated the 6-week po dosing with either compound. Few deaths occurred except at the highest dose of each compound. These deaths appeared to be the result of central nervous system depression. Mice in all dose groups continued to gain weight throughout the 6-week dosing period.

Both solvents caused dose-related increases in the liver weight to body weight ratio (Table 1). Since the body weights of the mice were generally unaffected by any of the treatments, these increases represent true liver weight increases. Doses as low as 100 mg/kg/day of each compound were sufficient to cause statistically significant increases in the liver weight/body weight ratio. The increases in liver size were attributable to hypertrophy of the liver cells as revealed by histological examination and by a decrease in the DNA concentration of the livers (Table 2). Mice in the highest dose group of each solvent displayed liver weight/body weight ratios which were about 75% greater than those of controls.

PER caused a marked dose-related accumulation of triglycerides in the liver (Table 1). Mice in the high-dose groups had six times as much triglyceride per gram of liver

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TABLE 1

EFFECT OF TRICHLOROETHYLENE AND PERCHLOROETHYLENE ON THE RELATIVE LIVER WEIGHT, HEPATIC TRIGLYCERIDE CONCENTRATION, GLUCOSE-6-PHOSPHATASE (G6P) ACTIVITY, AND SERUM GLUTAMATE PYRUVATE TRANSAMINASE (SGPT) ACTIVITY OF MICE AFTER A 6-WEEKS EXPOSURE[a]

Dose (mg/kg/day)	Liver weight/body weight (%)	Liver triglycerides (mg/g liver)	G6P (μg phosphate/mg protein/20 min)	SGPT (units/0.1 ml serum)
Trichloroethylene				
0	5.22 ± 0.09 (24)	3.08 ± 0.29 (24)	125.5 ± 3.2 (12)	11.8 ± 1.3 (15)
100	5.84 ± 0.20 (5)**	3.12 ± 0.49 (5)	117.8 ± 6.0 (5)	—
200	5.99 ± 0.13 (12)***	4.41 ± 0.76 (12)	116.4 ± 2.8 (9)	11.3 ± 2.3 (7)
400	6.51 ± 0.12 (12)***	4.53 ± 1.05 (12)	117.3 ± 4.6 (9)	11.7 ± 2.5 (6)
800	7.12 ± 0.12 (12)***	5.76 ± 0.85 (12)	111.7 ± 3.3 (9)**	11.1 ± 1.3 (7)
1600	8.51 ± 0.20 (12)***	5.82 ± 0.93 (12)	89.9 ± 1.7 (9)***	14.1 ± 2.1 (7)
2400	8.82 ± 0.15 (12)***	6.89 ± 1.40 (12)**	83.8 ± 2.1 (8)***	31.5 ± 6.8 (11)**
3200	9.12 ± 0.15 (4)***	7.02 ± 0.69 (4)	83.0 ± 7.0 (3)***	26.8 ± 3.1 (4)*
Perchloroethylene				
0	5.21 ± 0.09 (26)	3.23 ± 0.29 (26)	136.5 ± 3.1 (15)	11.8 ± 1.0 (18)
20	5.51 ± 0.11 (13)	2.72 ± 0.27 (13)	132.0 ± 7.6 (6)	11.3 ± 0.7 (10)
100	5.97 ± 0.11 (13)***	7.66 ± 1.78 (13)*	120.4 ± 7.7 (5)	11.8 ± 1.1 (10)
200	6.45 ± 0.12 (15)***	13.21 ± 2.28 (15)***	118.4 ± 12.4 (5)	15.3 ± 1.9 (9)
500	7.35 ± 0.16 (15)***	22.69 ± 1.61 (15)***	110.4 ± 7.5 (5)**	28.4 ± 2.7 (10)***
1000	7.89 ± 0.16 (19)***	25.68 ± 1.54 (19)***	101.5 ± 4.1 (8)***	44.6 ± 5.3 (12)***
1500	8.10 ± 0.27 (6)***	24.52 ± 2.59 (5)***	94.5 ± 2.1 (4)***	44.1 ± 5.3 (6)***
2000	9.00 ± 0.11 (6)***	20.10 ± 2.08 (6)***	99.2 ± 4.0 (4)***	46.4 ± 5.6 (4)***

[a] Values are $\bar{x} \pm SE$ determined in (N) mice.
* $p < 0.05$ from control group.
** $p < 0.01$ from control group.
*** $p < 0.001$ from control group.

TABLE 2

DNA CONTENT, HISTOPATHOLOGICAL EVALUATION, AND WET WEIGHT/DRY WEIGHT RATIOS OF LIVERS FROM TREATED MICE

Group	DNA[a] (mg/g liver)	Degeneration	Karyorrhexis	Necrosis	Polyploidy	Wet wt/dry wt ratio[b]
Control	2.83 ± 0.17	(±)[c]	−	−	−	3.45 ± 0.12
400 mg TRI/kg	2.57 ± 0.14*	++	+	−	+	3.49 ± 0.07
1600 mg TRI/kg	2.15 ± 0.08***	+++	++	+	+	3.48 ± 0.15
200 mg PER/kg	2.52 ± 0.27	+++	+	(±)	(±)	3.23 ± 0.13*
1000 mg PER/kg	2.36 ± 0.21**	++++	++	+	+	3.18 ± 0.20*

[a] $\bar{x} \pm SD$ ($n = 4$ or 5).
[b] $\bar{x} \pm SD$ ($n = 5$).
[c] Histological evaluations are graded: −, negative; + to ++++, increasing severity of observed pathology.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

as control mice. In contrast, TRI had very little effect on hepatic triglycerides. Although mice in the highest dose groups had mean triglyceride concentrations twice the control value, the triglyceride concentrations in some of these mice were not elevated over those of controls. The gross appearances of livers from PER- and TRI-treated mice were consistent with these results. The livers of almost all mice receiving 200 mg/kg/day or more of PER were pale and mottled in appearance, a pattern characteristic of fatty liver. The livers of TRI mice, although enlarged, appeared normal.

Both PER and TRI caused dose-related decreases in glucose-6-phosphatase activity (Table 1). These decreases were small, only about 10%, until the dose reached 800 mg TRI/kg or 500 mg PER/kg. Mice receiving the highest doses of TRI and PER had G6P activities which were 66 to 70% of the control value.

SGPT activity was not increased in TRI-treated mice except at the two highest doses (Table 1). Even at the 2400-mg/kg/day dose, half of the mice had normal values. PER caused significant increases in SGPT activity at 500 mg/kg/day. A threshold appeared to exist at 100 mg/kg/day. At the highest PER doses, SGPT activity averaged about 45 transaminase units per 100 μl serum, four times the control value. This increase reflects mild to moderate hepatic damage. As a comparison, several mice were treated with two or three doses of $CHCl_3$ (400 mg/kg) or CCl_4 (1600 mg/kg), enough to cause severe hepatotoxicity; their SGPT activities ranged up to several hundred units per 100 μl. With either TRI or PER, SGPT activity rarely exceeded 70 units per 100 μl.

Histopathology

The livers of mice from several dose groups were histopathologically examined. A summary of the observations is shown in Table 2. Liver degeneration, manifested by swollen hepatocytes, was common in all four treatment groups. Cells had indistinct borders; their cytoplasm was clumped and a vesicular pattern was apparent. The swelling was not simply due to edema, as wet weight/dry weight ratios did not increase (Table 2). Evidence of karyorrhexis, the disintegration of the nucleus, was present in nearly all specimens and suggested impending cell death. Central lobular necrosis was present in some specimens. Polyploidy was also characteristic in the central lobular region. Hepatic cells had two or more nuclei or had enlarged nuclei containing increased amounts of chromatin, suggesting that a regenerative process was ongoing. Both solvents caused similar pathology, but it was generally more severe in the PER-treated mice. Also, fine droplets of lipid were commonly present in the cytoplasm of the hepatocytes of PER-treated, but not TRI-treated, mice.

Preliminary Metabolism Studies

Trichloroethanol and trichloroacetic acid were found in the urine of mice treated with TRI. Verification of the metabolites was accomplished by comparisons with known standards. TCE was the predominant metabolite, and much of it was conjugated with glucuronic acid. TCA generally accounted for between 15 and 30% of the total urinary metabolite. Urine from several mice was collected daily throughout the 6-week dosing period. The amount of urinary metabolite did not show significant day-to-day variability or week-to-week trends.

Gas chromatographic analysis of urine from PER-treated mice identified TCA as the only metabolite. The possible presence of dechlorinated metabolites such as oxalic acid or ethylene glycol, reported as PER metabolites by other investigators (Pegg *et al.*, 1979; Daniel, 1963; Dmitrieva, 1967), was investigated. Six control mice excreted 0.327 ± 0.085 mg of urinary oxalic acid/day. Nine mice receiving PER doses ranging from 200 to 2000 mg/kg/day had 0.351 ± 0.081 mg of oxalic acid/day in their urine. Therefore

oxalic acid was eliminated as a kinetically significant metabolite. Ethylene glycol also was not found.

There were day-to-day trends in the amount of TCA excreted by mice dosed with PER. The amount of TCA excreted each day tended to increase, before leveling off toward the end of the week. This pattern occurred because the metabolism and/or excretion of PER was slower than that of TRI, and 10 to 15% of the metabolized PER was not excreted within 24 hr, but was carried over to the following day. No week-to-week trends in TCA urinary excretion were observed. The influence of the day-to-day trend in urinary excretion of TCA was minimized by consistently collecting urine from the mice on specific days late in the week.

The contribution of fecal elimination of metabolites in mice dosed with either TRI or PER was examined. The amount of metabolites in the feces of mice treated with either compound was less than 5% of the amount found in urine, and its contribution was neglected.

TRI Metabolism

A biphasic relationship between amount of urinary metabolite and TRI dose was observed (Fig. 1). The initial portion of this curve was linear through 1600 mg TRI/kg, before an abrupt plateau was reached. The linearity of this initial portion of the curve was verified by the fractional metabolism occurring at each dose. With the exception of the 200-mg/kg dose, 27.5% of each dose in the range 100 to 1600 mg/kg/day was converted into urinary metabolite. Above 1600 mg/kg the fraction of each dose metabolized decreased, suggesting that TRI metabolism approached saturation beyond this dose.

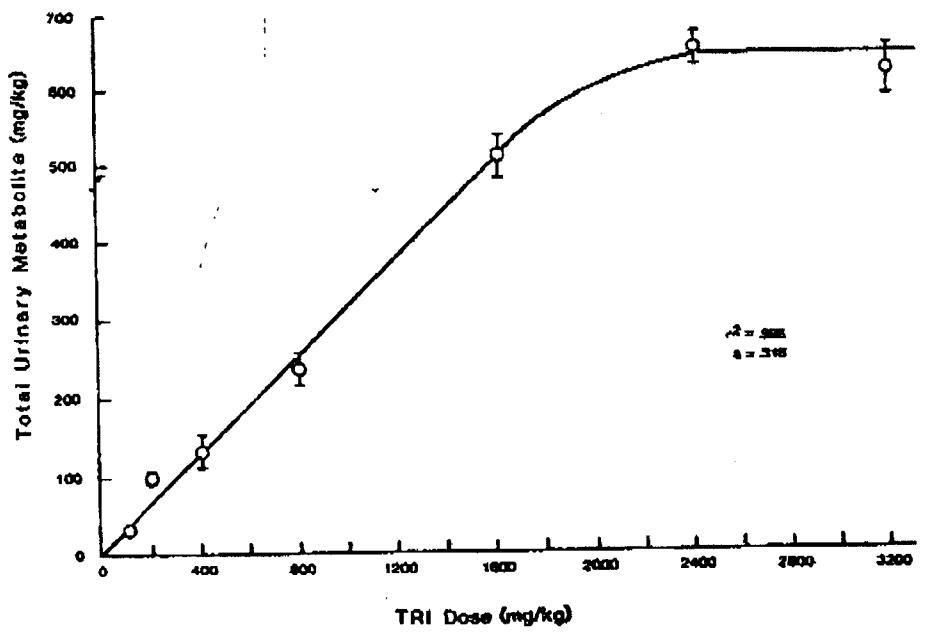

FIG. 1. Relationship between the TRI dose and the amount of total urinary metabolite excreted per day by mice in each group. Values represent $\bar{x} \pm$ SE. N = 7 to 9 mice per group except for the 100- and 3200-mg TRI/kg groups where N = 4 and 3, respectively. The slope a and r^2 value for the linear regression fit of the 100- to 1600-mg/kg data points are given.

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PER Metabolism

The relationship between PER metabolism and dose is shown in Fig. 2. The data points were fit with an equation analogous to the Michaelis–Menten expression, $Y = (M_{max}X)/(K_m + X)$, which describes capacity-limited kinetics. X is the dose. Y is the amount of metabolite resulting, M_{max} is the maximum amount of metabolite formed and excreted in 24 hr, and K_m is, in this case, the dose at which the amount of metabolite excreted in a 24-hr period is half the apparent maximum amount. The data fit the expression very well; r^2 was 0.996. M_{max} was 136 mg/kg/day and K_m was 660 mg PER/kg.

The fraction of each dose metabolized decreased with increasing dose, consistent with capacity-limited metabolism. About 25% of a very low PER dose was metabolized, but only 5% of a very high dose.

Effect of TRI Metabolism on Hepatotoxicity

TRI significantly affected only two of the four hepatotoxicity parameters, liver weight and G6P activity. When dose was plotted on a linear scale against the percentage increase in liver weight, or against the percentage inhibition of G6P activity, biphasic curves exactly analogous to the TRI metabolism curve resulted (Figs. 3A and B). In each case, the relationship between dose and the toxic effect was linear up through 1600 mg TRI/kg/day, reaching a plateau at high doses.

However, when these same hepatotoxicity data points were plotted against the amount of metabolism (i.e., total urinary metabolites) at each respective dose, linear relationships were observed throughout the entire dose range (Figs. 4A and B). The points corresponding to the plateau region of the dose–effect curves fall around the regression line

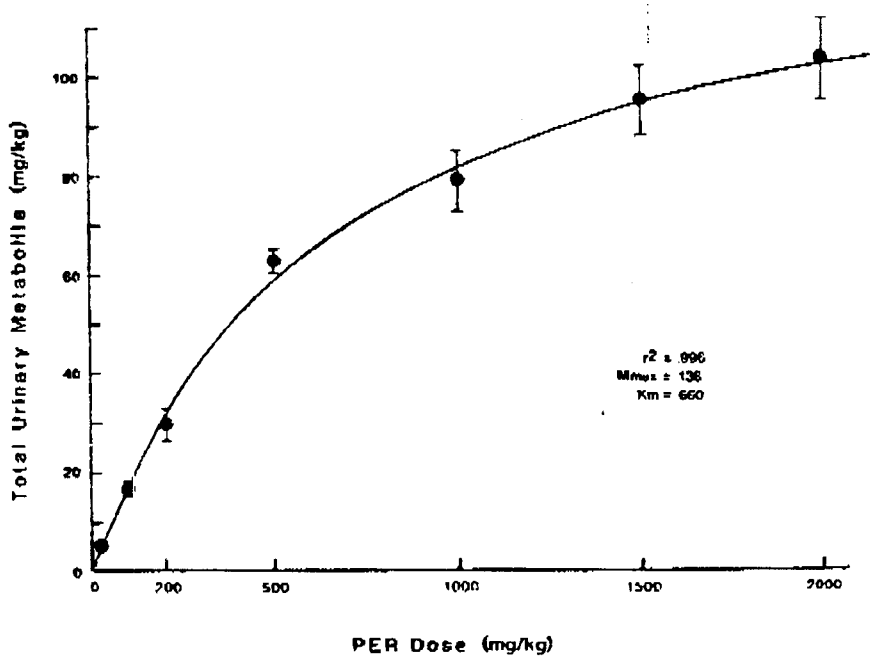

FIG. 2. Relationship between the PER dose and the amount of total urinary metabolite excreted per day by mice in each group. Values represent $\bar{x} \pm$ SE. N = 9 to 11 mice per group except for the 1500- and 2000-mg/kg doses where N = 4 or 5. The points were fit by the Michaelis–Menten equation, and the values for M_{max} and K_m are given, together with the r^2 value.

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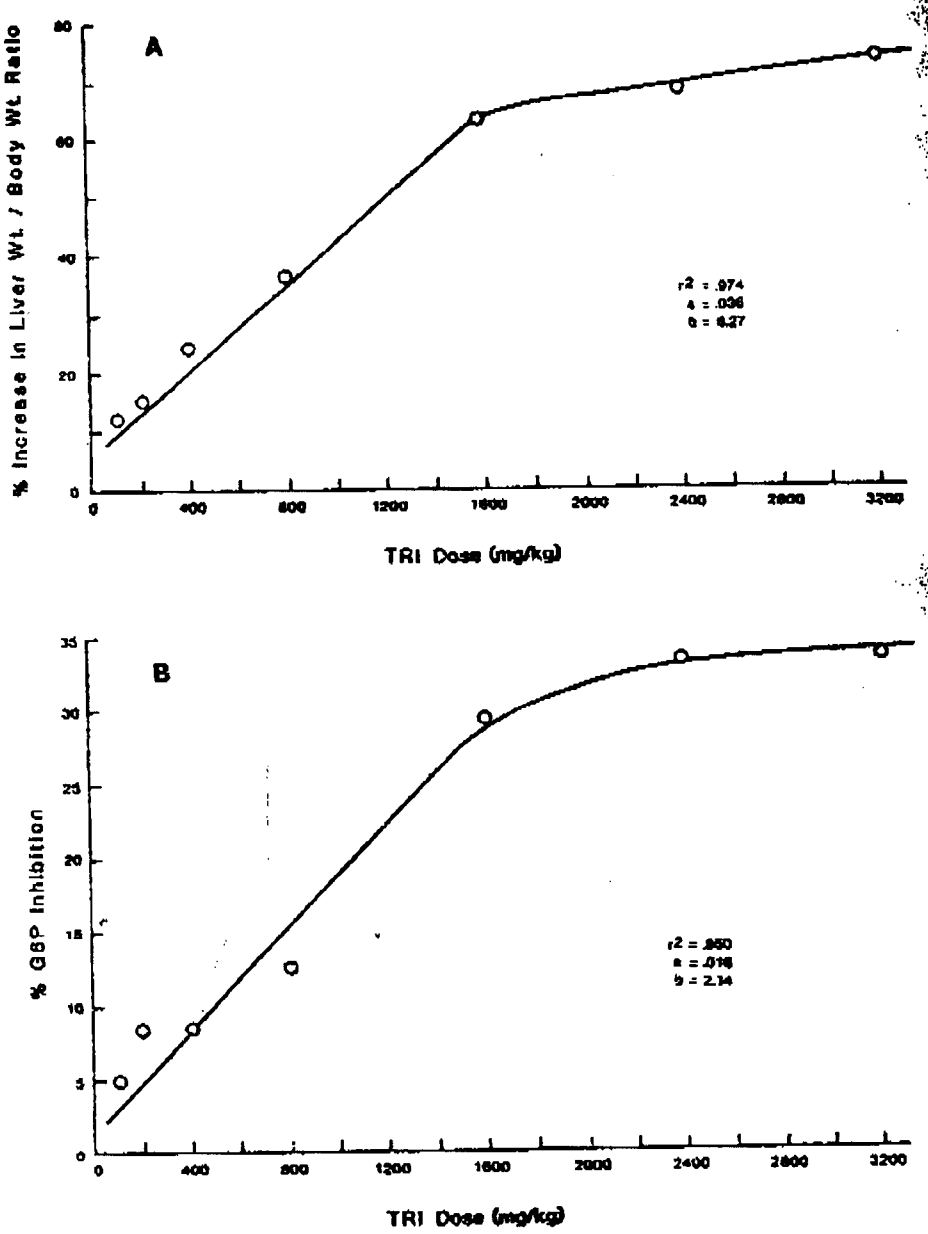

FIG. 3. Dose-effect relationships between TRI dose and the hepatotoxicity parameters of (A) liver weight increases and (B) G6P inhibition. Indicated values are the slope a, intercept b, and r^2 value for the linear regression of the 100- to 1600-mg/kg data points.

in the metabolism–effect graphs. This indicates that the nonlinearity seen in the dose–effect relationships can be explained by a change in the kinetics of TRI metabolism.

Effect of PER Metabolism on Hepatotoxicity

Graphs relating PER dose to each of the four hepatotoxicity parameters resulted in

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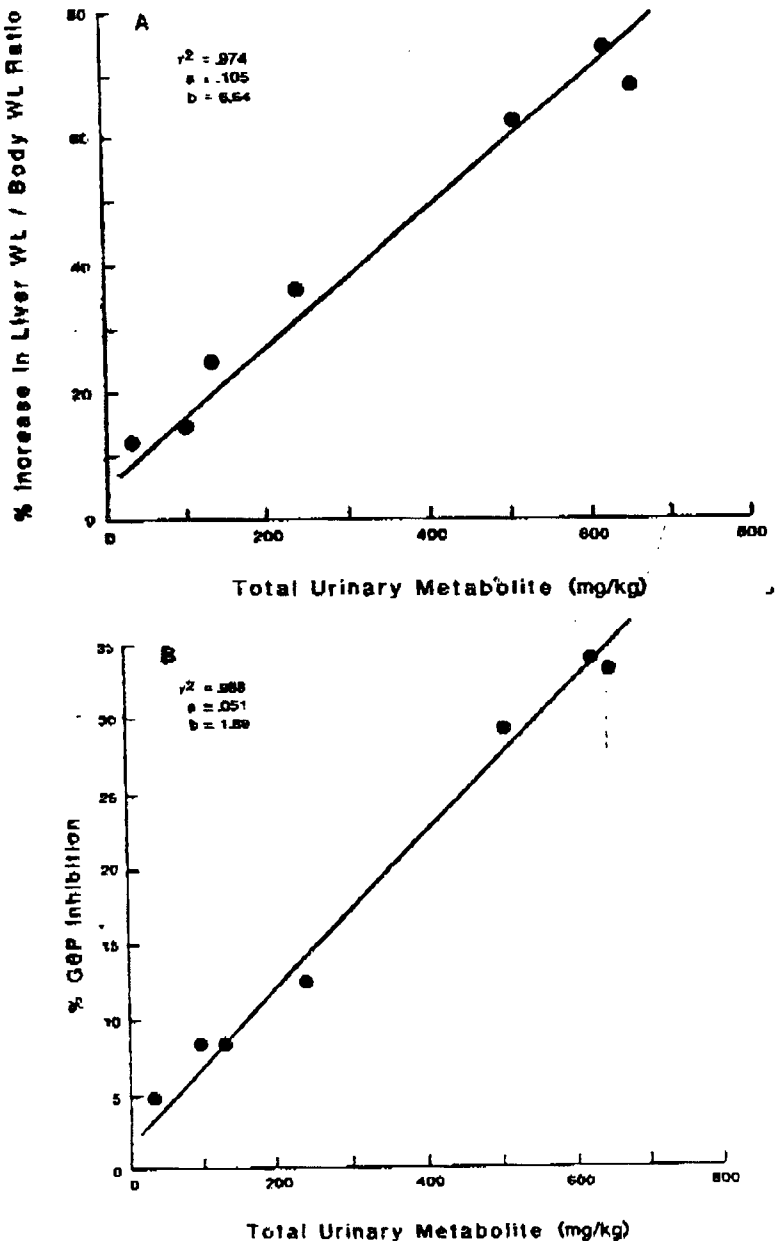

FIG. 4. Relationships between the hepatotoxicity parameters of (A) liver weight increases and (B) G6P inhibition and total urinary metabolite excreted per day by mice from the various TRI dose groups. Indicated values are the slope a, intercept b, and r^2 value of the linear regression of all points.

hyperbolic curves analogous to the PER metabolism graph in Fig. 2 (Figs. 5A–D). The data points in each case were fit by the expression $Y = (E_{max}X)/(K_m + X)$, where E_{max} is the maximum effect, and the resulting E_{max} and K_m values, as well as the r^2 values,

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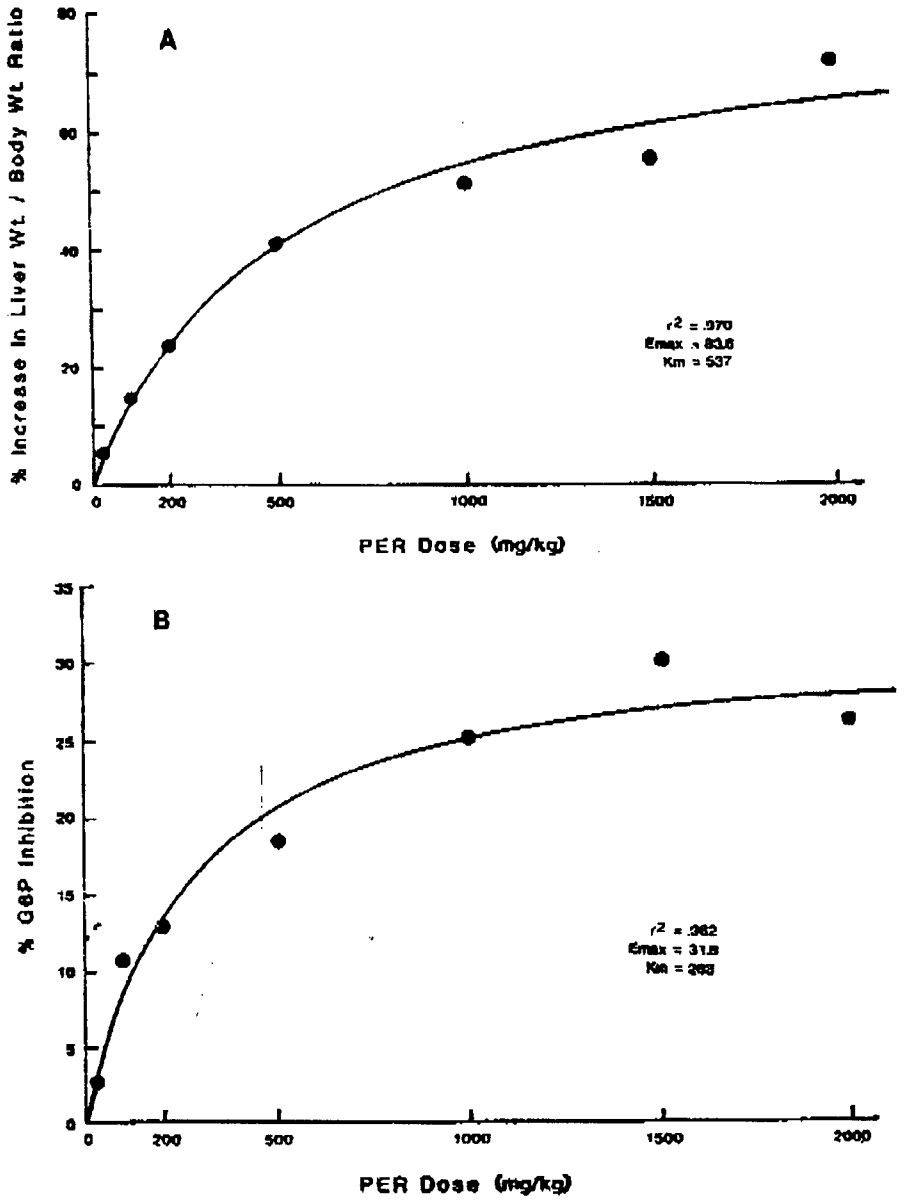

FIG. 5. Dose–effect relationships between PER dose and the hepatotoxicity parameters: (A) liver weight increases; (B) G6P inhibition; (C) triglyceride increases; (D) SGPT activity increases. The points were fit with the Michaelis–Menten equation, and the values for E_{max} and K_m and the r^2 value are given. The fit for C was based only on the 0- to 1000-mg/kg data points.

are presented on each graph. The K_m values of three of the four hepatotoxicity parameters are similar to the K_m of PER metabolism, a result which is expected if a relationship exists between metabolism and hepatotoxicity.

Graphs of the four hepatotoxicity parameters against metabolism result in linear fits

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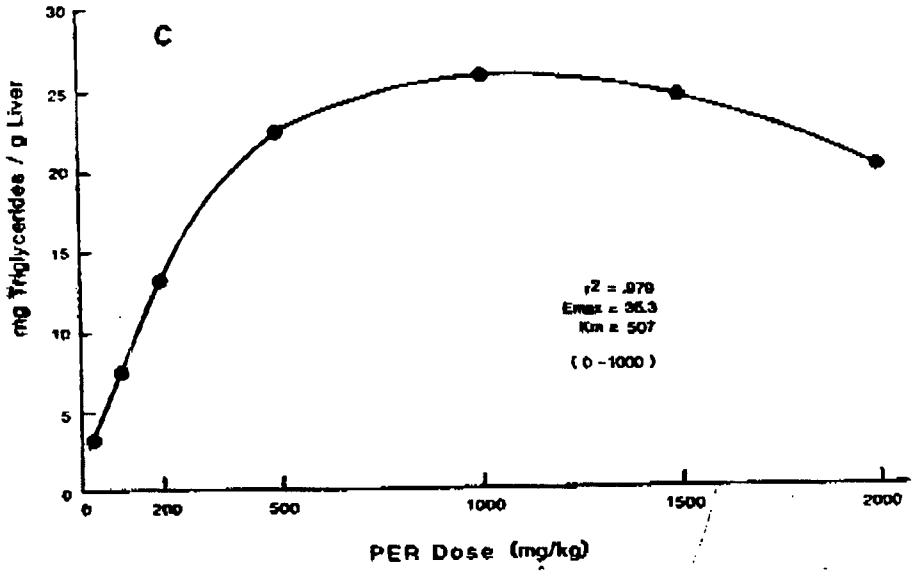

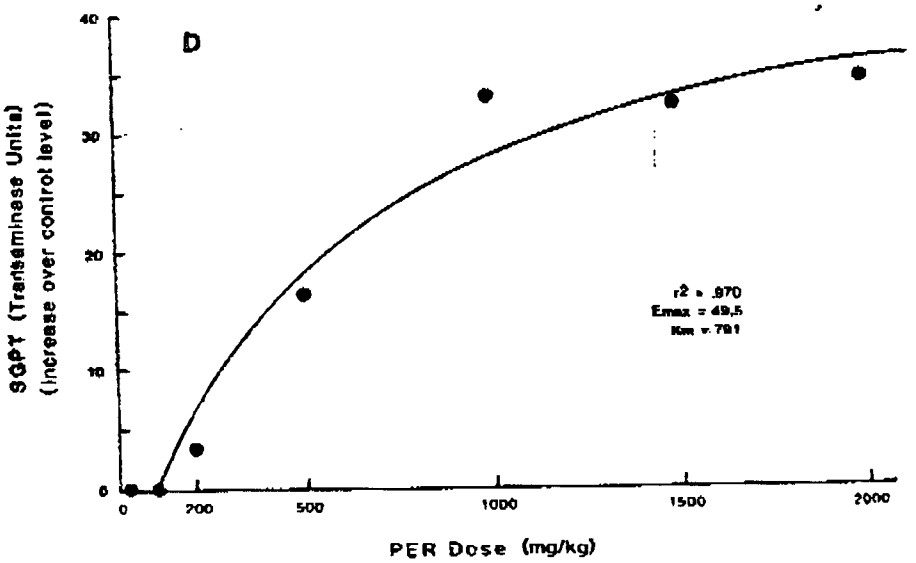

FIG. 5—*Continued.*

(Figs. 6A–D). These results strongly suggest that the toxicity of PER is also related to the amount of metabolism. The metabolism–effect graph of hepatic triglycerides (Fig. 6C) deviates from linearity above the 1000-mg/kg/day dose point. This deviation may be the result of achievement of a maximum effect. Triglyceride concentrations increase with PER dose up to 1000 mg/kg/day, but decrease at higher dose rates (Table 1). If a maximum in the total amount of hepatic triglycerides had been reached by the 1000-mg/kg/day dose, the larger liver weights of the higher dose groups would result in a relative decrease in triglyceride concentration per gram of liver at these higher doses.

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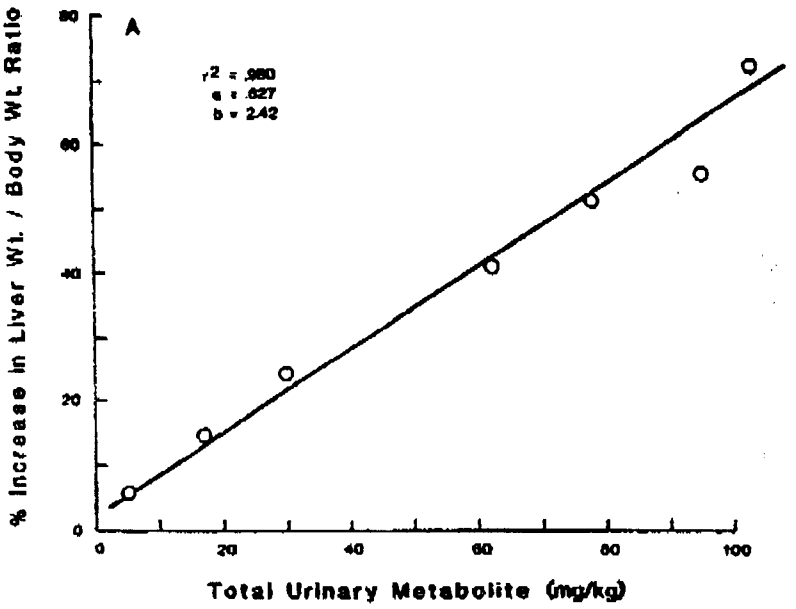

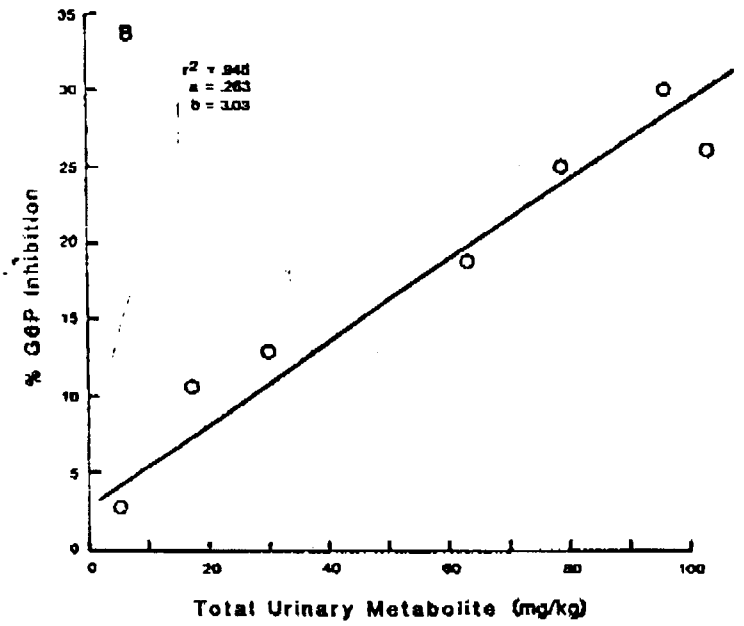

FIG. 6. Relationships between the hepatotoxicity parameters: (A) liver weight increases, (B) G6P inhibition, (C) triglyceride increases, and (D) SGPT activity increases, and total urinary metabolite excreted per day by mice from the various PER dose groups. Data points were fit by linear regression (only the 0 to 1000 data points were used for C), and the slopes a, intercepts b, and r^2 values are indicated.

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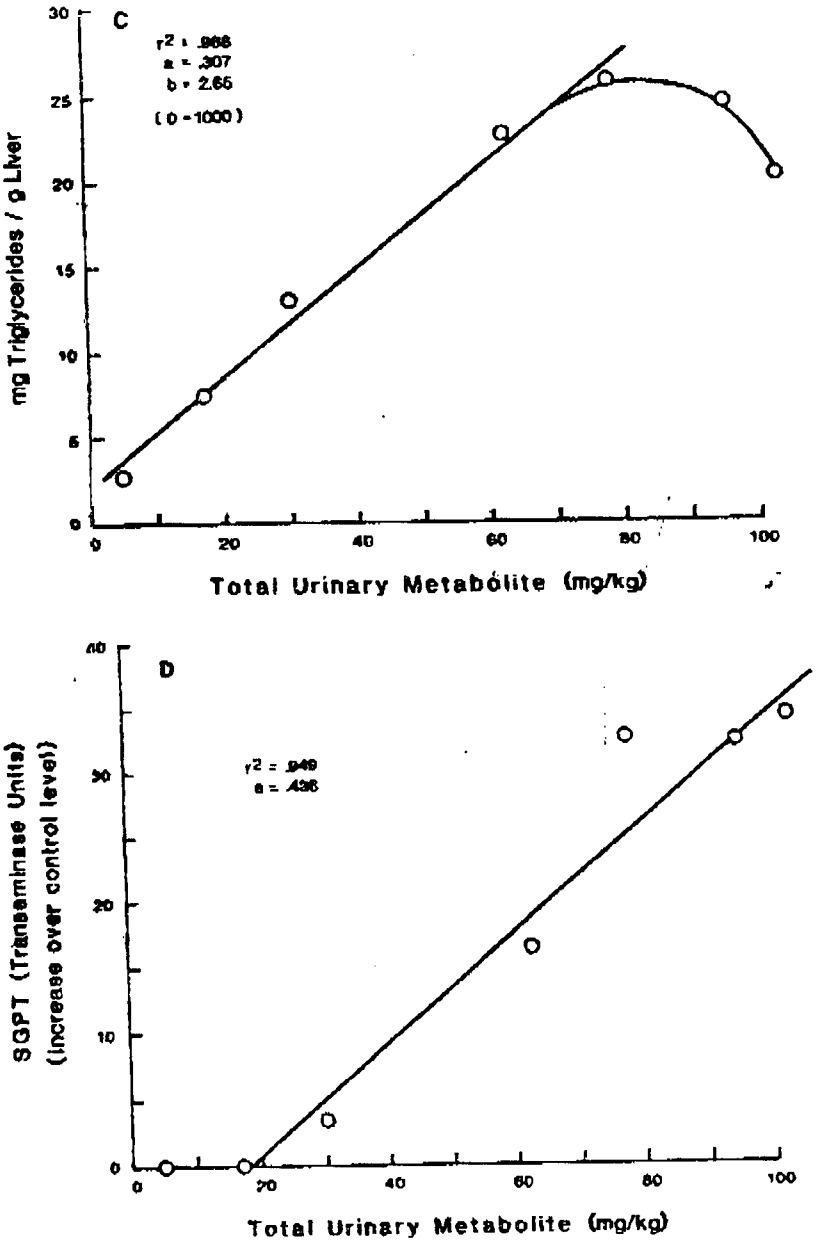

FIG. 6—*Continued.*

Two observations support the concept of a triglyceride maximum: (1) total hepatic lipids (triglyceride concentrations × liver weight) did not greatly differ among the three highest PER groups; (2) mice receiving two or three doses of CCl_4 or $CHCl_3$ (1600 and 400 mg/kg/day, respectively), hepatotoxicants known to induce fatty liver, had hepatic triglyceride concentrations of approximately 24 ± 1 mg/g liver, virtually identical to those

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seen in the mice receiving 1000 and 1500 mg PER/kg/day, suggesting that this value may be the upper limit of triglyceride accumulation in this strain of mice.

DISCUSSION

The objective of this study was to examine the relationships among dose, metabolism, and hepatotoxicity in mice after exposure to either TRI or PER for 6 weeks to determine the part that metabolism plays in the toxicity of these solvents. The linearity seen in all six metabolism–effect graphs, which relate the various hepatotoxicity parameters to the amount of metabolism occurring at each dose of TRI and PER, indicates that the hepatotoxicity of both these compounds is directly proportional to the extent to which the solvents are metabolized.

Such a result is consistent with the proposed metabolic scheme of both compounds. Both TRI and PER are believed to be metabolized by the mixed-function oxidase system to epoxide intermediates, and then to stable terminal metabolites excreted mainly in the urine (Daniel, 1963; Henschler, 1977; Leibman and Ortiz, 1977; Yllner, 1961). TRI epoxide is converted into chloral hydrate, which is further metabolized to TCE and TCA by alcohol dehydrogenase and chloral hydrate dehydrogenase, respectively. PER epoxide is believed to convert spontaneously to trichloroacetyl chloride, which is rapidly hydrolyzed to TCA. The epoxide is believed to be the toxic intermediate in each case, reactive enough to bind to cellular macromolecules (Henschler, 1977; Van Duuren, 1975; Leibman and Ortiz, 1977; Allemand *et al.*, 1978). Covalent binding of both compounds has been demonstrated (Van Duuren and Banerjee, 1976; Banerjee and Van Duuren, 1978; Bolt *et al.*, 1977; Uehleke and Poplawski-Tabarelli, 1977; Bolt and Filser, 1977; Allemand *et al.*, 1978; Pegg *et al.*, 1979).

Toxicity should parallel metabolism in any compound for which the reactive intermediate responsible for the toxic effect is part of the primary metabolic pathway, as is the case with TRI and PER. Although the data are consistent with the view that the metabolic pathways of both compounds involve the epoxide as the toxic intermediate, they are not restricted by it. Recently, Miller and Guengerich (1982, 1983) have proposed that the epoxide is not an obligate intermediate in TRI metabolism. The results of the present study demonstrate that formation of the reactive intermediate, whatever its structure, must be proportional to the overall amount of metabolism, since when metabolism reaches a constant maximum value the toxicity also does not increase further.

It should be pointed out that use of total urinary metabolites as the index of metabolism is an approximation. Most of the TRI or PER which is metabolized through reactive intermediates is converted to either TCA or TCE and excreted in the urine. Very little is excreted in the feces. Some metabolized compound (both TRI and PER) is bound to macromolecules, and some is metabolized to carbon dioxide (Daniel, 1963; Pegg *et al.*, 1979; Schumann *et al.*, 1980; Stott *et al.*, 1982; Parchman and Magee, 1982). To the extent to which these factors are involved, total urinary metabolite is an underestimate of the actual amount of metabolism. However, at the doses used, urinary excretion is by far the major route of disposition of metabolized compound. Therefore, total urinary metabolites should be a reasonable approximation of the amount of metabolism.

The relationship between the metabolism of these compounds and their toxicity is not an unexpected finding. It has been shown that the toxicity of numerous compounds is a result of their metabolism to reactive intermediates (Mitchell *et al.*, 1976; Gillette *et al.*, 1974), and Moslen *et al.* (1977) have demonstrated an association between TRI metabolism and hepatotoxicity. However, the implications of such a relationship, particularly the quantitative association demonstrated here, are important.

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First, it is noteworthy that the relationship between toxicity and metabolism is demonstrated by both TRI and PER despite differences in the kinetics and rates of their metabolic processes. The metabolism of TRI is linear with dose until high doses are reached. Then it abruptly reaches saturation and levels off (Fig. 1). This type of behavior has recently been described in inhalation uptake studies of a number of small halogenated compounds, including TRI (Filser and Bolt, 1979; Andersen *et al.*, 1980; Andersen, 1981), and has been termed "flip-flop" kinetics. It occurs when the hepatic enzymes are able to metabolize the substrate readily (i.e., the K_m value is small). At low and intermediate doses, the major factor limiting the rate of metabolism is the blood flow to the liver (Andersen, 1981). If the concentration of the compound in the blood is proportional to the dose, the amount of metabolism would also be proportional to dose. As long as perfusion remains rate limiting, first-order kinetics will be obeyed. However, as dose is increased, at some point the capacity of the metabolizing enzymes is reached and they become saturated. At that point the kinetics shift from first order to zero order, and a plateau in the dose–metabolism relationship occurs. This kinetic behavior seen with TRI indicates that metabolism of TRI by the liver is a high affinity, high capacity process.

In contrast, the metabolism of PER (Fig. 2) displays classical saturable kinetics. This pattern suggests, especially with its high K_m value of 660 mg PER/kg, that extraction and/or metabolism of PER by the hepatic enzymes is not efficient. The extent of PER metabolism is dose dependent, even at low concentrations. In addition, the liver has a low capacity for metabolizing PER; the M_{max} of 136 mg TCA/kg/day is less than one-fifth (20%) of the extent to which TRI can be metabolized. This limited capacity for PER metabolism has been observed in numerous species (Ikeda *et al.*, 1972; Daniel, 1963; Monster, 1979; Pegg *et al.*, 1979). Despite the contrasts in the kinetics of TRI and PER metabolism, the toxicity of each compound is proportional to the extent of its metabolism over the entire dose range studied.

A second inference, regarding the relative toxicities of the reactive metabolites, can be drawn from the results. A comparison of the hepatotoxicity elicited by TRI and PER shows that there were both quantitative and qualitative differences. Both compounds caused increases in liver weight, decreases in glucose-6-phosphatase activity, and similar histopathology. Comparison of the doses which result in equivalent effect suggest that PER is at least twice as potent as TRI on a molar basis with respect to these toxic indices. The difference in potency is considerably greater with regard to SGPT and triglyceride increases. Since at any given dose from two to five times more TRI than PER is metabolized, it follows that the toxic metabolite of PER is considerably more potent than that of TRI.

Third, the dose–effect graphs (Figs. 3 and 5) illustrate that a maximum occurs in each effect as dose is increased. With the exception of the effect of PER on hepatic triglycerides, each maximum results from a change in the kinetics of metabolism. This result illustrates one way by which a nonlinearity between dose and effect can arise.

Finally, the findings shed light on several factors involved in risk assessment. First, they establish that the amount of metabolism of both TRI and PER is directly related to their hepatotoxicity. It is the internal parameter upon which assessment of their toxicity should be based. Since there is strong evidence supporting the proposal that TRI and PER cause hepatocellular tumors in mice as a result of an epigenetic mechanism involving repeated liver damage (Schumann *et al.*, 1980; Stott *et al.*, 1982), these results involving the effect of metabolism of TRI and PER on hepatic toxicity have implications with respect to their carcinogenicity as well.

Risk extrapolation estimates to date have related dose to response. It would be much more fruitful to base the extrapolation of risk on metabolism rather than on dose for those

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compounds whose toxicity or carcinogenicity has been demonstrated to be dependent on metabolic activation. Other authors have discussed the merits of using internal parameters to explain toxic response or estimate carcinogenic potential (Gillette, 1974a,b; Gehring and Blau, 1977; Andersen, 1981). For risk assessment purposes, preliminary pharmacokinetic studies should be undertaken to relate the administered dose to the significant internal parameter of metabolism. This principle may apply to many genetic carcinogens as well. Many of these agents are first metabolized to toxic intermediates which react with cellular nucleic acids. The extent of their metabolism would likely be more directly related to their carcinogenic activity than would dose.

Second, the results of this study demonstrate that use of high doses in studies such as the NCI carcinogenicity bioassays often lead to saturation of metabolism. The metabolism of PER deviated from linearity at doses above 100 mg/kg, considerably lower than the doses utilized in the NCI study (National Cancer Institute, 1977). Even the metabolism of TRI, a compound which is readily metabolized, approached saturation at 2400 mg/kg, a dose equivalent to the maximum tolerated dose used in the NCI bioassay (National Cancer Institute, 1976). As pointed out earlier, such saturated metabolism was associated with the occurrence of maxima in measured effects. This saturated metabolism may explain the absence of a dose-related response in the hepatic tumorigenicity data of mice in the NCI study of PER, as well as the rather small increase observed among male mice despite a doubling of the TRI dose (National Cancer Institute, 1976, 1977). Extrapolation for purposes of risk assessment of such high-dose data without correction for the pharmacokinetics of the metabolic process may lead to erroneous results (Watanabe *et al.*, 1977) and may greatly overestimate the actual risk. It is unlikely that the pharmacokinetics of TRI and PER metabolism are unusual. The metabolism of many compounds will likely resemble the kinetics of one or the other. Thus, implications, drawn from the results of this study, concerning the use of high doses in risk assessment have general applicability. The nonlinearities of the dose–effect curves which result from saturation of metabolic processes demonstrate the need to include at least one dose significantly lower than the maximum tolerated dose in carcinogenicity bioassays.

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