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CUMULATIVE EFFECTS OF REPEATED DOSES OF COMPOUNDS TRANSFORMED INTO REACTIVE METABOLITES*

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Abstract—Several drugs and chemicals are transformed in the liver by cytochrome P-450 into reactive metabolites that may (a) destroy hepatic cytochrome P-450, (b) deplete hepatic glutathione, and (c) covalently bind to hepatic proteins. The possibility was investigated that daily administration of the parent compounds may have cumulative effects on some of these phenomena. Rats received 1–3 i.p. doses of bromobenzene, 0.25 mmole/kg daily, trichloroethylene, 1 mmole/kg daily, or vinyl chloride, 0.5 mmole/kg daily. (a) Cytochrome P-450 concentration was not significantly decreased after a single dose, but was progressively decreased after repeated doses of the three compounds. (b) Although hepatic glutathione concentration was decreased 4 hr after the administration of a single dose of bromobenzene, it was not decreased 24 hr after a single or repeated doses of bromobenzene. (c) The amount of metabolite irreversibly bound to proteins progressively increased in the liver during repeated administration of the three compounds. It is concluded that daily administration of compounds transformed into reactive metabolites may lead to a progressive decrease in hepatic cytochrome P-450 concentration and to the accumulation of metabolites irreversibly bound to proteins in the liver.

Several drugs and chemicals [1, 2], including bromobenzene [3–8], trichloroethylene [9–18] and vinyl chloride [19–28] are transformed in the liver by cytochrome P-450 into reactive metabolites that may (a) destroy hepatic cytochrome P-450 and thus limit further metabolism, (b) be inactivated by binding to hepatic glutathione whose hepatic concentrations may be, in the process, decreased, or (c) covalently bind to hepatic proteins. The above mentioned effects have been well studied after the administration of a single dose of the parent compounds but not after several daily doses.

The possibility was investigated that daily administration of the parent compounds may have cumulative effects on some of these phenomena. In this communication we report the effects of a single and of repeated daily doses of bromobenzene, trichloroethylene and vinyl chloride on hepatic cytochrome P-450 and glutathione concentrations and on the amounts of metabolites irreversibly bound to proteins in the liver and in various tissues.

MATERIALS AND METHODS

Animals were male Sprague–Dawley rats weighing 180–220 g. Rats were given food (Autoclave 113, UAR) and water *ad lib.*

[^{14}C]Bromobenzene (uniformly labeled, sp. act. 57 mCi/mmole) was purchased from New England Nuclear, Boston, MA. [^{14}C] Trichloroethylene (uniformly labeled, sp. act. 1 mCi/mmole) and [^{14}C] vinyl chloride (uniformly labeled, sp. act. 1 mCi/mmole) were purchased from Commissariat à l'Energie Atomique, Saclay, France. The radiochemical purity of the 3 compounds were checked by gas–liquid chromatography to be higher than 99 per cent.

Rats received one of the three following treatments: [^{14}C] bromobenzene, 0.25 mmole/kg (25 μCi/kg) in 0.5 ml/kg of methanol i.p. daily for 1–6 days, or [^{14}C] tricholoroethylene, 1 mmole/kg (25 μCi/kg) in 0.5 ml/kg of methanol i.p. daily for 1–3 days, or [^{14}C] vinyl chloride, 0.5 mmole/kg (500 μCi/kg) in 0.5 ml/kg of methanol i.p. daily for 1–6 days. Some rats were treated with $CoCl_2$, 6 H_2O, 35 mg/kg s.c. twice daily for 4 days and received either a single dose of the labeled compound on day 4 of the $CoCl_2$-treatment or 3 daily doses of the labeled compound on days 2, 3, and 4 of the $CoCl_2$-treatment. Other rats received 1, 3 or 6 doses of methanol, 0.5 ml/kg daily.

Rats were killed at 9:00–11:00 a.m., 24 hr after any given dose of the labeled compounds. Blood was drawn from the inferior vena cava. The liver, the kidneys and the sartorius muscle were removed. A liver fragment was placed in Bouin's fluid. Serum was prepared from a blood sample. Blood sample and tissue fragments were homogenized in 3 vol. of ice-cold 1.15 M KCl, 0.01 M Na^+K^+ phosphate buffer, pH 7.4 with a glass–Teflon Potter–Elvehjem homogenizer. The remaining liver fragment was perfused with ice-cold 0.154 M NaCl through the portal vein, homogenized in 3 vol. of 5% TCA and used for the determination of hepatic glutathione by the

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method of Ellman [29]. Hepatic microsomes were prepared with the liver homogenate and used for the determination of hepatic cytochrome P-450 by the method of Omura and Sato [30]. Serum glutamic pyruvic transaminase (SGPT) activity was measured according to Reitman and Frankel [31]. The liver fragment placed in Bouin's fluid was embedded in paraffin 24 hr later, cut and stained with hematoxylin and eosin.

The amount of [^{14}C] material irreversibly bound to tissue proteins in the tissue homogenates was measured as previously described [8, 17, 28]. Samples (250 μl) of the tissue homogenates were applied to filter paper disks (Durieux 111, France) that had been dipped into 5% trichloroacetic acid (TCA) and had been dried at 40°. After application of the samples, the paper disks were dried at 40°. The dried paper disks were then successively placed in the following 200-ml baths: 10% TCA for 60 min, 5% TCA for 30 min, 5% TCA for 15 min, methanol for 15 min, methanol for 15 min, heptane for 10 min and ethyl ether for 10 min. No further radioactivity could be removed by introducing additional solvent baths. The paper disks were then dried and placed on the bottom of scintillation vials; 10 ml of Dimilume 30 (Packard) scintillation fluid were added and radioactivity was counted in an Intertechnique, ABAC SL 40 scintillation counter with automatic background and quench (external standardization) correction.

The Student's *t*-test for independent data was used to assess the significance of differences between means.

RESULTS

After administration of 1 or 3 doses of the labeled parent compound, a labeled material was irreversibly bound to proteins in various tissues (Tables 1–3). Treatment of rats with $CoCl_2$ for 4 days decreased

Table 1. Effect of treatment with $CoCl_2$ on the amount of [^{14}C] material irreversibly bound to tissue proteins after administration of [^{14}C] bromobenzene*

	[^{14}C] material irreversibly bound to proteins (nmoles/g tissue)			
	Liver	Kidney	Blood	Muscle
One dose of bromobenzene in control rats	19.8 ± 1.6	5.3 ± 1.6	2.8 ± 0.6	1.4 ± 1.0
One dose of bromobenzene in $CoCl_2$-treated rats	1.6 ± 0.5†	1.9 ± 0.2†	0.4 ± 0.2†	0.4 ± 0.2
Three doses of bromobenzene in control rats	29.8 ± 1.8‡	15.4 ± 2.6‡	5.1 ± 0.8‡	4.4 ± 1.2‡
Three doses of bromobenzene in $CoCl_2$-treated rats	3.4 ± 0.4†	2.5 ± 0.2†	1.6 ± 0.3†	1.2 ± 0.5†

* The amount of bound material (mean ± S.D. for at least 3 rats) was measured 24 hr after a single, or the last of 3 doses of [^{14}C] bromobenzene, 0.25 mmole/kg i.p. daily. Some rats received $CoCl_2$, $6H_2O$, 35 mg/kg s.c. twice daily for 4 days and either a single dose of [^{14}C] bromobenzene on day 4 of the $CoCl_2$-treatment or 3 doses, given respectively on day 2, 3 and 4 of the $CoCl_2$-treatment.

† Significantly different from that in control rats, $P < 0.05$.

‡ Significantly different from that after one dose of [^{14}C] bromobenzene, $P < 0.01$.

Table 2. Effect of treatment with $CoCl_2$ on the amount of [^{14}C] material irreversibly bound to tissue proteins after administration of [^{14}C] trichloroethylene*

	[^{14}C] material irreversibly bound to proteins (nmoles/g tissue)			
	Liver	Kidney	Blood	Muscle
One dose of trichloroethylene in control rats	427 ± 51	151 ± 12	45 ± 5	29 ± 2
One dose of trichloroethylene in $CoCl_2$-treated rats	58 ± 5†	35 ± 10†	17 ± 3†	6 ± 1†
Three doses of trichloroethylene in control rats	633 ± 81‡	248 ± 32‡	121 ± 18‡	51 ± 4‡
Three doses of trichlorethylene in $CoCl_2$-treated rats	112 ± 8†	91 ± 5†	35 ± 8†	30 ± 7

* The amount of bound material (mean ± S.D. for at least 3 rats) was measured 24 hr after a single, or the last of 3 doses of [^{14}C] trichloroethylene, 1 mmole/kg i.p. daily. Some rats received $CoCl_2$, $6H_2O$, 35 mg/kg s.c. twice daily for 4 days and either a single dose of [^{14}C] trichloroethylene on day 4 of the $CoCl_2$-treatment or 3 doses given respectively on day 2, 3 and 4 of the $CoCl_2$-treatment.

† Significantly different from that in control rats, $P < 0.05$.

‡ Significantly different from that after one dose of [^{14}C] trichloroethylene, $P < 0.05$.

Table 3. Effect of treatment with $CoCl_2$ on the amount of [^{14}C] material irreversibly bound to tissue proteins after administration of [^{14}C] vinyl chloride*

	[^{14}C] material irreversibly bound to proteins (nmoles/g tissue)			
	Liver	Kidney	Blood	Muscle
One dose of vinyl chloride in control rats	38 ± 7	13 ± 4	7 ± 1	3 ± 1
One dose of vinyl chloride in $CoCl_2$-treated rats	4.5 ± 0.2†	3.0 ± 1.4†	1.7 ± 0.4†	0.7 ± 0.3†
Three doses of vinyl chloride in control rats	116 ± 20‡	52 ± 6‡	28 ± 3‡	9 ± 3‡
Three doses of vinyl chloride in $CoCl_2$-treated rats	7.1 ± 0.7†	2.4 ± 0.7†	1.6 ± 0.3†	0.9 ± 0.9†

* The amount of bound material (mean ± S.D. for at least 3 rats) was measured 24 hr after a single, or the last of 3 doses of [^{14}C] vinyl chloride, 0.5 mmole/kg i.p. daily. Some rats received $CoCl_2$, $6H_2O$, 35 mg/kg s.c. daily for 4 days and either a single dose of [^{14}C] vinyl chloride on day 4 of the $CoCl_2$-treatment or 3 doses given respectively on day 2, 3 and 4 of the $CoCl_2$-treatment.

† Significantly different from that in control rats, $P < 0.01$.

‡ Significantly different from that after one dose of [^{14}C] vinyl chloride, $P < 0.01$.

cytochrome P-450 by 33, 46, 61 and 73 per cent at 24, 48, 72 and 96 hr, respectively. After administration of 1 or 3 doses of the labeled parent compound (Tables 1–3), the amount of bound material was lower in rats treated with $CoCl_2$ than in control rats. After administration of 1, 3 or 6 doses of methanol, 0.5 ml/kg daily, hepatic cytochrome P-450, hepatic glutathione, SGPT activity and liver histology were unchanged (not shown).

After administration of bromobenzene, 0.25 mmole/kg daily, hepatic cytochrome P-450 concentration was not significantly decreased after the first dose but progressively decreased as further doses were administered (Fig. 1); hepatic glutathione concentration was decreased 4 hr after the first dose (Table 4) but was not modified 24 hr after one or several doses (Fig. 1); SGPT activity was unchanged (Fig. 1) and histologic examination showed no liver necrosis after 1, 3 or 6 doses (not shown); the amount of [^{14}C] material irreversibly bound to proteins progressively increased in various tissues during administration of the first 3 doses but then decreased in the liver and in the kidney (Fig. 1).

After administration of trichlorethylene, 1 mmole/kg daily, hepatic cytochrome P-450 concentration was not significantly decreased after the

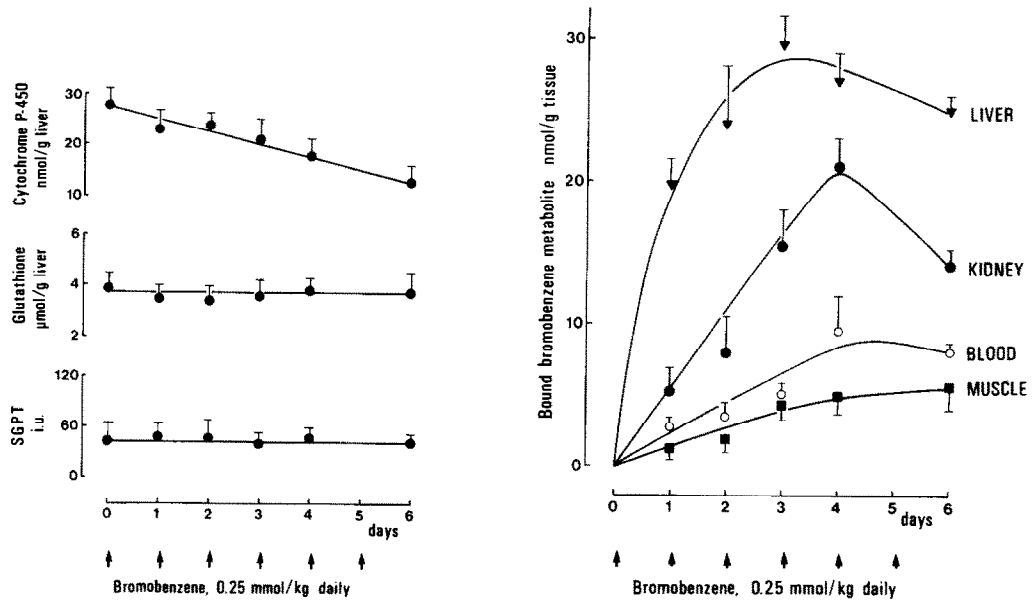

Fig. 1. Effects of repeated doses of bromobenzene, 0.25 mmole/kg daily. Hepatic microsomal cytochrome P-450 concentration, hepatic glutathione concentration, SGPT activity, and the amount of metabolite irreversibly bound to proteins in various tissues were measured 24 hr after a single dose, or the last of repeated doses, of bromobenzene. Points and bars represent mean and S.D. in 10 rats for hepatic cytochrome P-450 concentration, hepatic glutathione concentration and SGPT activity and in at least 3 rats for the amount of metabolite irreversibly bound to proteins.

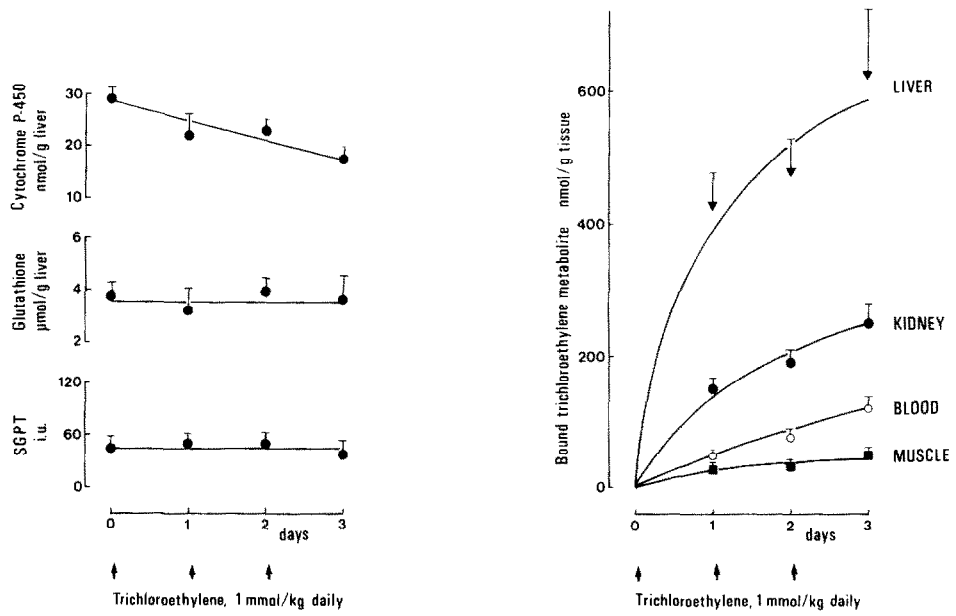

Fig. 2. Effects of repeated doses of trichloroethylene, 1 mmole/kg daily. Hepatic microsomal cytochrome P-450 concentration, hepatic glutathione concentration, SGPT activity, and the amount of metabolite irreversibly bound to proteins in various tissues were measured 24 hr after a single dose, or the last of repeated doses, of trichloroethylene. Points and bars represent mean and S.D. in 10 rats for hepatic cytochrome P-450 concentration, hepatic glutathione concentration and SGPT activity and in at least 3 rats for the amount of metabolite irreversibly bound to proteins.

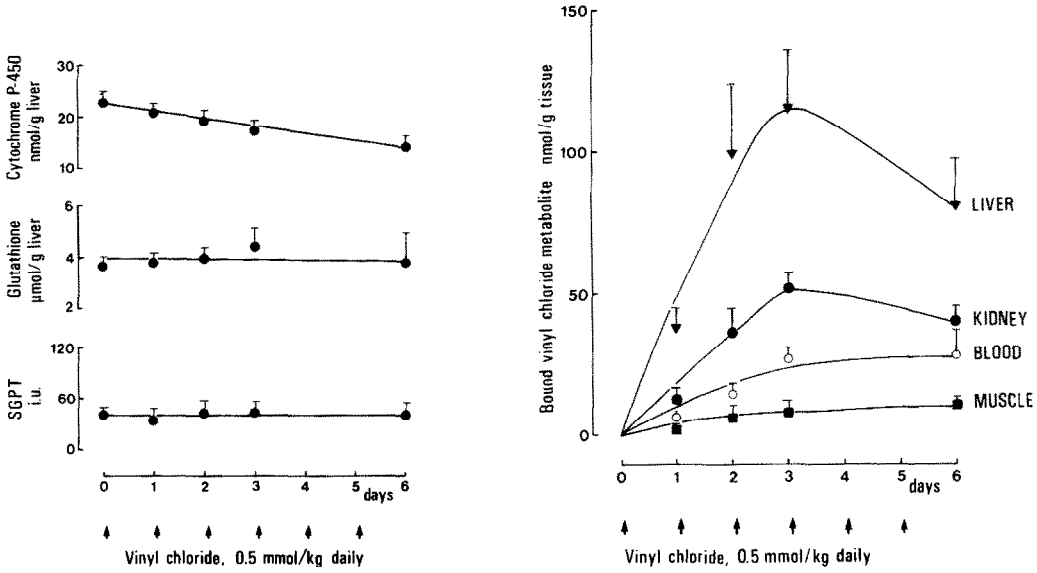

Fig. 3. Effects of repeated doses of vinyl chloride, 0.5 mmole/kg daily. Hepatic microsomal cytochrome P-450 concentration, hepatic glutathione concentration, SGPT activity, and the amount of metabolite irreversibly bound to proteins in various tissues were measured 24 hr after a single dose, or the last of repeated doses, of vinyl chloride. Points and bars represent mean and S.D. in 10 rats for hepatic cytochrome P-450 concentration, hepatic glutathione concentration and SGPT activity and in at least 3 rats for the amount of metabolite irreversibly bound to proteins.

Table 4. Early effects of a single dose on hepatic glutathione concentration*

	Hepatic glutathione (μmoles/g liver)	
	Control	Treated
Bromobenzene, 0.25 mmole/kg	4.1 ± 0.5	2.9 ± 0.6†
Trichloroethylene, 1mmole/kg	3.8 ± 0.6	3.7 ± 0.7
Vinyl chloride, 0.5 mmole/kg	4.3 ± 0.5	3.5 ± 0.7

* Hepatic glutathione concentration (mean ± S.D. for 8 rats) was measured in control rats and in rats that had received bromobenzene, trichloroethylene or vinyl chloride 4 hr before sacrifice.

† Significantly different from that in control rats, $P < 0.05$.

first dose but was decreased ($P < 0.05$) after 3 doses (Fig. 2); hepatic glutathione concentration (Table 4, Fig. 2) and SGPT activity (Fig. 2) were unchanged and there was no liver necrosis after 1 or 3 doses (not shown); the amount of [^{14}C] material irreversibly bound to proteins increased in various tissues during administration of the first 3 doses (Fig. 2). Administration of a fourth dose led to the death of most animals; there was severe peritonitis with intestinal perforations but neither liver nor kidney necrosis. These effects may be related to a local toxic effect of the trichloroethylene–methanol mixture: they were not observed after repeated administration of much higher doses of trichloroethylene dissolved in liquid paraffin [18].

After administration of vinyl chloride, 0.5 mmole/kg daily, hepatic cytochrome P-450 was not significantly decreased after 1 dose, but progressively decreased after repeated doses (Fig. 3); hepatic glutathione (Table 4, Fig. 3) and SGPT activity (Fig. 3) were unchanged and histologic examination of liver specimens obtained after 1, 3 or 6 doses showed no liver necrosis (not shown). The amount of [^{14}C] material irreversibly bound to proteins increased in various tissues during administration of the first 3 doses but then tended to decrease in the liver and in the kidney (Fig. 3).

DISCUSSION

Acute administration of bromobenzene, trichloroethylene and vinyl chloride has been previously shown to destroy hepatic cytochrome P-450, deplete hepatic glutathione, and result in the covalent binding of metabolites to tissue proteins [3–28]. In this study, the possibility was investigated that repeated daily doses may have cumulative effects on some of these phenomena.

Cytochrome P-450. With the three compounds (Figs. 1–3), cytochrome P-450 concentration was not significantly decreased 24 hr after the first dose but progressively decreased during repeated administration of the parent compound. The mechanism responsible for this progressive decrease is inkown. It is not related to hepatic necrosis which was found neither after one or nor after several doses. The half-life of cytochrome P-450 heme is about 1 day in the rat [32]. The progressive decrease in hepatic cytochrome P-450 concentration may be due either to a sustained decrease in cytochrome P-450 synthesis or to a cumulated destruction of cytochrome P-450 by the repeated doses: a slight decrease in hepatic cytochrome P-450 concentration produced by the first dose may persist when the second dose is administered; this second dose may further destroy cytochrome P-450 so that repeated doses might lead, through this mechanism, to a progressive decrease in hepatic cytochrome P-450 concentration.

Glutathione. None of the treatments had any significant effect on hepatic glutathione concentration measured 24 hr after one or several daily doses (Figs. 1–3), although administration of bromobenzene did decrease hepatic glutathione 4 hr after the first dose (Table 4). These findings are consistent with the view that hepatic glutathione, whose half-life is only 4 hr in the rat [33], was restored to normal levels 24 hr after any given dose of bromobenzene, so that repeated doses of bromobenzene had no cumulative effects on hepatic glutathione concentration.

Protein-bound material. After administration of the labeled compound, a labeled material became bound to tissue proteins (Tables 1–3); treatment of the animals with $CoCl_2$ decreased hepatic cytochrome P-450 and decreased the amount of bound material present in various tissues after 1 or 3 doses (Tables 1–3). The findings are consistent with the view that the bound material is a metabolite formed by cytochrome P-450. The binding of the metabolites to proteins had not been disrupted by repeated washings and repeated extractions with various solvents of various polarities. This apparently irreversible binding suggests that the metabolites were attached to proteins by a covalent bond. A covalent bond between a metabolite and a protein may occur through 2 main reactions: (a) the covalent binding of a chemically reactive metabolite to an already formed protein; or (b) the utilization of a small stable metabolite for the synthesis of normal amino acids and their subsequent incorporation into a newly synthetized protein. Incorporation is unlikely to occur with bromobenzene whose benzene ring is unlikely to be split; although incorporation might occur to some extent with trichloroethylene [17] and with vinyl chloride [22], it has been estimated that it could only represent a negligible fraction of the amounts of metabolites bound to protein after administration of [^{14}C] vinyl chloride [22]. Rather, it is assumed that the labeled materials irreversibly attached to tissue proteins after administration of [^{14}C] bromo-

benzene [3–8], [^{14}C] trichloroethylene [15, 17] and [^{14}C] vinyl chloride [22, 25–28] mainly represent chemically reactive metabolites which have reacted with, and have bound to, proteins.

After one or several doses of the parent compound, the amount of bound metabolite was highest in the liver, intermediate in the kidney, and lowest in blood and muscle (Figs. 1–3). This sequence in the extent of binding may be consistent with a similar sequence in the activity of cytochrome P-450 in these various organs [34]. However, it is not yet clear whether metabolites bound in extrahepatic tissues are mainly formed *in situ* or mainly exported from the liver.

Trichloroethylene [13] and vinyl chloride [35] have half-lives of 1 hr or less in rats; only 2 per cent of the initial concentration of unchanged bromobenzene was found in the liver 24 hr after the administration of bromobenzene, 4.85 mmole/kg, in rats [6]. These observations suggest that after each daily dose, metabolism was essentially complete when the next dose was administered. Although metabolism was essentially complete 24 hr after the first dose, significant amounts of protein-bound metabolites persisted in the tissues at that time (Tables 1–3). This finding is consistent with previous observations showing late persistance of protein-bound metabolites in the liver after administration of a single dose of vinyl chloride [22] or of bromobenzene [6]; with the latter compound, about 40 and 20 per cent of the metabolite initially bound to proteins were still present in the liver 2 and 4 days, respectively, after administration of 0.41 mmole/kg [^{14}C] bromobenzene in mice [6]. Late persistance of protein-bound metabolites in the liver is not unexpected, since (a) metabolites bound to proteins may be cleared away only as the proteins to which they are attached are themselves split or excreted, and (b) the overall half-life of hepatic proteins is 2–4 days in rats [36].

Theoretically, persistence of high amounts of bound metabolites at the time the second dose of the parent compound is administered suggests that protein-bound metabolites may accumulate in the liver upon daily administration of the parent compounds. If the daily formation rate of the reactive metabolites remains constant over the days, then the amount of protein-bound metabolites should reach a plateau when the amount of metabolite bound daily equals that removed daily by protein excretion and/or breakdown. If, on the contrary, the progressive decrease in hepatic cytochrome P-450 concentration (Figs. 1–3) is associated with a progressive decrease in the daily binding rate of the metabolite to proteins, then the amount of protein-bound metabolites may first increase after the first doses and then decrease as the daily binding rate of the metabolites progressively decreases. Figures 1–3 show that the amount of reactive metabolites irreversibly bound to proteins progressively increased in the liver after the first three doses but then tended to decrease when further doses were administered.

It is concluded that daily administration of compounds transformed into reactive metabolites may lead to the accumulation of protein-bound metabolites in the liver and in various tissues. Accumulation may, however, be limited by a progressive decrease in hepatic cytochrome P-450 concentrations.

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