Biochemical Pharmacology, Vol. 34, No. 2, pp. 249–255, 1985.
Printed in Great Britain.

0006–2952/85 $3.00 + 0.00
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CHLOROFORM-INDUCED ALTERATION OF GLUTATHIONE *S*-TRANSFERASE ACTIVITY*

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(*Received* 23 *February* 1984; *accepted* 16 *May* 1984)

Abstract—The effect of chloroform treatment on the hepatic glutathione *S*-transferases was studied in phenobarbital-treated rats. The apparent isozymic composition of glutathione *S*-transferases in hepatic cytosol was changed after chloroform treatment. Glutathione *S*-transferases AA, A, B, C, and D + E were observed in hepatic cytosol from untreated rats; in contrast, the catalytic activity associated with basic glutathione *S*-transferases, such as AA, A, B, and C, decreased with time after chloroform treatment. Glutathione *S*-transferase B was not detectable 2 hr after chloroform treatment, and glutathione *S*-transferases AA and C were scarcely detectable after 5 hr. Twenty-four hours after chloroform treatment, glutathione *S*-transferases A and C were clearly detectable as was D + E and a small amount of B. Hepatic cytosolic glutathione *S*-transferase activity was decreased by chloroform treatment, and reached a minimum at 5 hr after treatment. Corresponding to the decrease of hepatic cytosol glutathione *S*-transferase activity, serum glutathione *S*-transferase activity was elevated maximally 5 hr after chloroform treatment and returned to almost normal by 24 hr. Treatment of rats with SKF 525-A or cysteine inhibited the chloroform-induced elevation of serum glutathione *S*-transferase activity. The chromatographic properties of the gluathione *S*-transferases present in serum were similar to glutathione *S*-transferase D + E. Furthermore, after incubation of partially purified cytosolic glutathione *S*-transferases with chloroform in the presence of hepatic microsomes and NADPH, only transferase D + E was detected. The addition of bilirubin to partially purified cytosolic glutathione *S*-transferase decreased the basic character of glutathione *S*-transferases B and C. In conclusion, chloroform caused a release of hepatic cytosolic glutathione *S*-transferases into serum. Both the active metabolite of chloroform, which was produced by the microsomal cytochrome P-450 system, and bilirubin, which was increased by chloroform treatment, played roles in altering the properties of the glutathione *S*-transferases.

Chloroform is a known hepatotoxic chemical, whose toxicity is increased by phenobarbital treatment [1]. Chloroform toxicity is attributed to its biotransformation to phosgene by microsomal cytochrome P-450-dependent mixed-function oxidases [2]. The glutathione *S*-transferases (EC 2.5.1.18) are a group of multifunctional proteins involved in the biotransformation of xenobiotics [3, 4]. These enzymes catalyze the conjugation of electrophilic compounds with glutathione and also play a role in the binding and storage of toxic compounds, such as bilirubin and azo-dye carcinogens [5–7]. In rat liver cytosol, several glutathione *S*-transferases have been identified and designated as AA, A, B, C, and D + E [8].

Previous studies (J. L. Stevens and M. W. Anders, unpublished observations) showed that the chromatographic profiles of hepatic cytosolic glutathione *S*-transferases were altered after chloroform treatment; only glutathione *S*-transferase D + E was observed. Moreover, evidence for the covalent binding of chloroform metabolites to the glutathione *S*-transferases was presented, but it was not clear that the alteration of the physical behaviour and catalytic activity of the glutathione *S*-transferases was due to covalent modification by chloroform metabolites.

The objective of the present study was to clarify the mechanism of alteration of glutathione *S*-transferases by chloroform treatment. It was found that chloroform-induced bilirubin formation and an active metabolite of chloroform played roles and that glutathione *S*-transferases were released from liver cytosol into serum after chloroform treatment.

MATERIALS AND METHODS

Sephadex G-100 (superfine) was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). DE52§ and CM52 were obtained from Whatman, Inc. (Clifton, NJ); CDNB was purchased from the Eastman Chemical Co. (Rochester, NY). Bilirubin was obtained from the Sigma Chemical Co. (St, Louis, MO).

Male Long–Evans rats (200–300 g) were given phenobarbital sodium (75 mg/kg) intraperitoneally once daily for 3 days. Chloroform (0.2 ml/kg as a 30% solution in corn oil) was injected intraperitoneally 24 hr after the last dose of phenobarbital. SKF 525-A hydrochloride (75 mg/kg) was

* A preliminary report of this work has been published in *Fedn Proc.* **26**, 125 (1984).

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§ Abbreviations: DE52, diethylaminoethyl-cellulose; CM52, carboxymethyl-cellulose; SKF 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride; and CDNB, 1-chloro-2,4-dinitrobenzene.

given 60 min before chloroform administration, and cysteine (150 mg/kg) was given both 15 min before and 60 min after chloroform treatment. Control rats were given corn oil intraperitoneally. Rats were killed by decapitation, and blood was collected. The liver was removed after perfusion *in situ* with ice-cold 1.15% potassium chloride solution and was homogenized with a Teflon–glass homogenizer in 2 vol. of ice-cold deionized water. The homogenate was centrifuged at 9,000 *g* for 30 min, and the supernatant fraction was centrifuged at 105,000 *g* for 60 min. The resulting supernatant fraction was used as the cytosolic fraction.

Glutathione *S*-transferase activity in hepatic cytosol and in serum was assayed by the method of Habig *et al.* [8] with CDNB as the substrate. Serum bilirubin concentrations were determined by the method of Jendrassik and Grof [9] with Sigma Kit No. 605 (Sigma Chemical Co.). Protein concentrations were measured by the method of Lowry *et al.* [10].

Glutathione *S*-transferases were isolated according to the procedure of Habig *et al.* [8] with minor modifications. Briefly, the DE52 fractions with glutathione *S*-transferase activity were concentrated by ultrafiltration (Amicon, Danvers, MA) and applied to a Sephadex G-100 column. The fractions with transferase activity that eluted from the Sephadex G-100 column were dialyzed against 0.01 M phosphate buffer (pH 6.7) and chromatographed on a CM52 column. Serum glutathione *S*-transferases were isolated from blood samples taken 5 hr after chloroform treatment. The pooled serum samples were diluted with 2 vol. of 0.01 M Tris–HCl buffer (pH 8.0) and chromatographed on a DE52 column, as described for hepatic cytosol.

In the *in vitro* experiments, the Sephadex G-100 fraction was used as a source of partially purified hepatic cytosolic glutathione *S*-transferases. The effect of chloroform *in vitro* was studied in a reaction mixture that contained 2 ml of Sephadex G-100 fraction (5.6 mg protein in 0.01 M phosphate buffer, pH 7.4), 1 ml of hepatic microsomes (22.1 mg protein in 0.01 M phosphate buffer, pH 7.4), NADPH (2.5 μmoles), and 5 μl chloroform in a total volume of 4 ml. After incubation at 37° for 10 min, the mixture was diluted with 2 ml of 0.01 M phosphate buffer, pH 6.7, and centrifuged at 105,000 *g* for 30 min. The supernatant fraction was dialyzed against 0.01 M phosphate buffer, pH 6.7, for 2 hr and chromatographed on a CM52 column with the same conditions as employed for hepatic cytosolic glutathione *S*-transferase. An incubation mixture without chloroform was used as control. When the effect of bilirubin was studied, 2 ml of Sephadex G-100 fraction (5.6 mg protein), dialyzed against 0.01 M phosphate buffer, pH 6.7, was incubated with 0.025 ml of bilirubin (final concentration 5 μM) in 0.1 N sodium hydroxide in a total volume of 5 ml at room temperature for 10 min. Then the reaction mixture was chromatographed on a CM52 column. Control reaction mixtures contained 0.025 ml of 0.1 N sodium hydroxide instead of bilirubin.

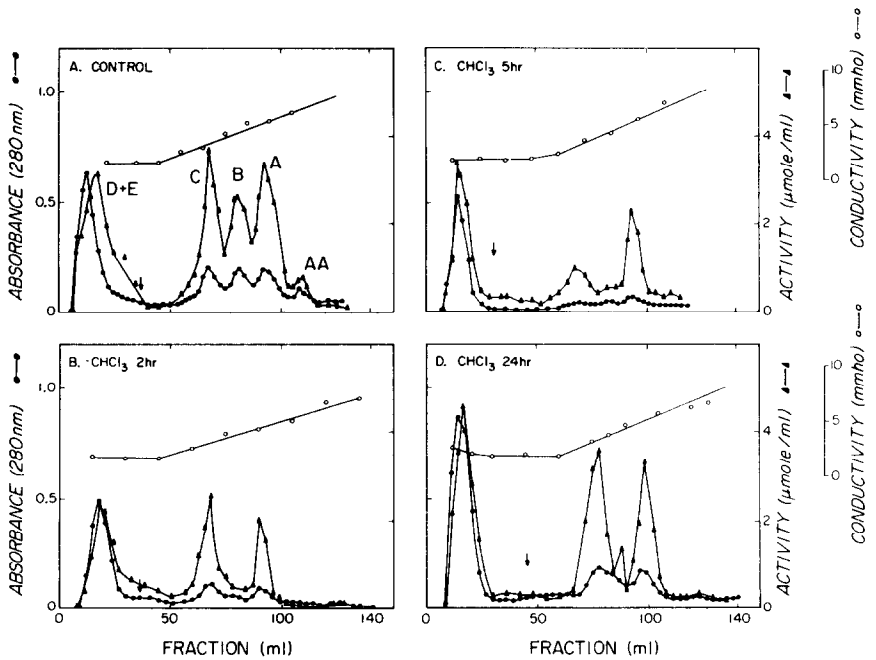

Fig. 1. CM52 column chromatography of hepatic cytosol glutathione *S*-transferases. Hepatic cytosolic glutathione *S*-transferases from control and chloroform-treated rats were isolated as described in Materials and Methods. After concentration and dialysis, the Sephadex G-100 fractions with glutathione *S*-transferase activity were applied to the CM52 column (0.9 × 22 cm) and were eluted with a linear gradient of potassium chloride (0–100 mM) in 0.01 M potassium phosphate buffer (pH 6.7). The amounts of protein applied to the column were: A (15 mg), B (13 mg), C (14 mg), and D (18 mg). A = control; B = 2 hr after chloroform treatment; C = 5 hr after chloroform treatment; and D = 24 hr after chloroform treatment.

Table 1. Protein recovery and catalytic activity of glutathione S-transferases in liver cytosol after chloroform treatment*

Treatment	Glutathione S-transferase									
	Cytosol		D + E		A		B		C	
	mg protein (%)	μmoles (%)	mg protein (%)	μmoles (%)	mg protein (%)	μmoles (%)	mg protein (%)	μmoles (%)	mg protein (%)	μmoles (%)
Control	193.0	313.0	5.7	46.6	1.5	24.5	1.1	17.5	1.2	22.3
	(100)	(100)	(2.9)	(14.7)	(0.7)	(7.8)	(0.6)	(5.6)	(0.6)	(7.1)
$CHCl_3$ 2 hr	150.0	236.7	6.3	31.4	0.8	14.1			1.2	23.3
	(100)	(100)	(4.1)	(13.2)	(0.5)	(5.9)	ND†	ND	(0.7)	(9.8)
5 hr	150.8	123.2	6.7	35.8	1.0	20.9			0.5	9.7
	(100)	(100)	(4.4)	(29.0)	(0.6)	(16.9)	ND	ND	(0.3)	(7.8)
24 hr	226.5	292.0	7.9	37.2	1.4	25.9			1.3	30.3
	(100)	(100)	(3.4)	(12.7)	(0.6)	(8.8)	ND	ND	(0.5)	(10.3)

* Rats were given chloroform (0.2 ml/kg, i.p.) and were killed at the times shown. Hepatic cytosolic fractions were isolated and chromatographed on a CM52 column, as described in Materials and Methods. Results are shown as mg protein (percent) and as μmoles product formed per fraction with CDNB as the substrate.

† ND = not detectable (activity < 5 μmoles).

RESULTS

Time course of chloroform-induced alterations in glutathione S-transferase activity. The CM52 column chromatographic profiles of the liver cytosolic glutathione *S*-transferases isolated at 2, 5, and 24 hr after chloroform treatment are shown in Fig. 1. Glutathione *S*-transferases AA, A, B, C, and D + E were observed in untreated rats, and the conductivity (mmho) at the peak of each fraction was about 5.8, 5.0, 4.0, and 3.1 for transferases AA, A, B, and C respectively. After chloroform treatment, a decrease in the activity of peaks corresponding to glutathione *S*-transferases AA, A, B, C, and D + E was observed. Glutathione *S*-transferase B, which eluted at 3.9 to 4.0 mmho in control rats, was not detected 2 hr after chloroform treatment. Similarly, the activity associated with glutathione *S*-transferase C was almost lost 5 hr after the treatment. Twenty-four hours after chloroform treatment, glutathione *S*-transferases A and C were clearly detectable as was D + E and a small amount of B. As shown in Table 1, protein recovery of basic glutathione *S*-transferases A, B, and C in cytosol from chloroform-treated rats was decreased 2 and 5 hr after treatment. These results suggested that glutathione *S*-transferases are altered or lost from liver as a consequence of chloroform treatment.

Figure 2 shows the relationship among serum glutathione *S*-transferase activities, serum bilirubin concentrations, and hepatic cytosolic glutathione *S*-transferase activities after chloroform treatment. Hepatic cytosolic glutathione *S*-transferase activity was decreased after chloroform treatment; the activity was 41% of the control value 2 hr after treatment, 27% at 5 hr, and 61% at 24 hr. In control rats, serum glutathione *S*-transferase activity was not detected, but, after chloroform treatment, transferase activity appeared in the serum, and a marked elevation was observed at 5 hr. Low glutathione *S*-transferase activities were still detectable in serum at 8 and 24 hr. On the other hand, serum bilirubin concentrations increased gradually after chloroform treatment and reached a plateau after 5 hr. Thus, the decrease in hepatic glutathione *S*-transferase activity was accompanied by an increase in serum glutathione *S*-transferase activity.

To determine the identity of the glutathione *S*-transferases present in serum, the protein was purified by the same procedure as that employed for the liver. As shown in Fig. 3, only activity corresponding to glutathione *S*-transferase D + E was detected in serum after chloroform treatment.

Treatment with cysteine and SKF 525-A. The effects of cysteine and SKF 525-A on chloroform-induced toxicity were studied (Table 2). The serum bilirubin concentrations increased to 210% of the control after chloroform treatment, and, when chloroform-treated rats were given cysteine or SKF 525-A, the bilirubin concentrations were decreased, relative to rats given chloroform alone, to 170 or 140% of control values respectively. The chloroform-induced increase in serum glutathione *S*-transferase activity was decreased by SKF 525-A or cysteine treatment, and the decrease in hepatic glutathione

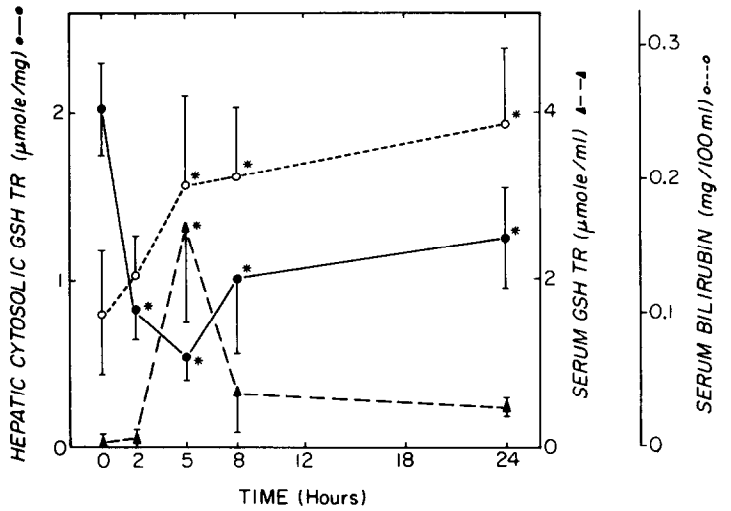

Fig. 2. Effect of chloroform treatment on serum and hepatic glutathione *S*-transferase activities and on serum bilirubin concentrations. Phenobarbital sodium (75 mg/kg) was injected intraperitoneally once daily for 3 days. Chloroform (0.2 ml/kg) was given intraperitoneally 24 hr after the last dose of phenobarbital. The glutathione *S*-transferase activities in serum and in hepatic cytosol as well as the serum bilirubin concentrations were measured as described in Materials and Methods. Asterisks (*) indicate significantly different from zero time, $P < 0.05$.

S-transferase activity was also partially blocked by SKF 525-A or cysteine treatment.

Treatment of hepatic cytosolic glutathione S-*transferase with chloroform or bilirubin.* Incubation of cytosolic glutathione *S*-transferases with chloroform in the presence of microsomes and NADPH decreased the activity to 17% of the control, and the remaining activity corresponded to glutathione *S*-transferase D + E (Fig. 4). The recovery of protein corresponding to transferase D + E was 4.6 and 4.0 mg after chloroform treatment and in control incubations respectively. Total protein recovery, including glutathione *S*-transferases AA, A, B, and C, in control incubations was 4.7 mg. Thus, glutathione *S*-transferases were altered by chloroform treatment and were recovered as transferase D + E.

In the case of bilirubin treatment, glutathione *S*-transferase activity was decreased to 66% of control values by bilirubin. Glutathione *S*-transferases AA and A were observed, but no glutathione *S*-transferase C and low glutathione *S*-transferase B activities were detected, as shown in Fig. 5. Protein recovery corresponding to glutathione *S*-transferases D + E, B, and C was 2.0 mg in control incubations and 2.2 mg after bilirubin treatment. Thus, glutathione *S*-transferases B and C were recovered as glutathione

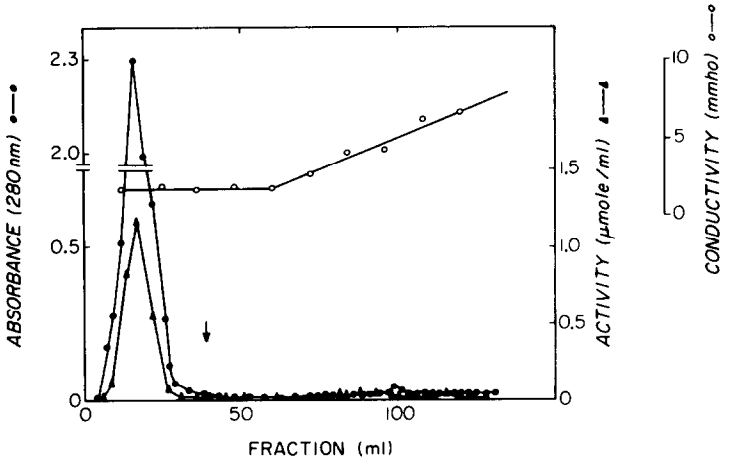

Fig. 3. CM52 column chromatography of serum glutathione *S*-transferases after chloroform treatment. The Sephadex G-100 fraction (18 mg protein) with glutathione *S*-transferase activity obtained from pooled serum (as described in Materials and Methods) was applied to the CM52 column (0.9 × 22 cm) and eluted with the same conditions as shown in Fig. 1.

Table 2. Effects of cysteine and SKF 525-A treatments on chloroform-induced alterations in serum bilirubin concentration and in serum and hepatic glutathione S-transferase activities*

Treatment	Serum bilirubin (mg/100 ml)	Serum transferase (μmoles/ml)	Liver transferase (μmoles/mg)
Control (N = 5)	0.10 ± 0.05	0.09 ± 0.02	2.04 ± 0.30
$CHCl_3$ (N = 5)	0.21 ± 0.08†	2.73 ± 1.31†	0.55 ± 0.15†
$CHCl_3$ + SKF 525-A (N = 4)	0.14 ± 0.02	0.11 ± 0.01‡	1.06 ± 0.09†‡
$CHCl_3$ + cysteine (N = 5)	0.17 ± 0.04	0.14 ± 0.03‡	0.85 ± 0.10†

* Phenobarbital (75 mg/kg) was injected i.p. once daily for 3 days. Chloroform (0.2 ml/kg) was given 24 hr after the last injection of phenobarbital, and the rats were killed 5 hr later. SKF 525-A (75 mg/kg) was injected i.p. 1 hr before giving chloroform. Cysteine (150 mg/kg) was given i.p. 15 min before and 60 min after chloroform treatment. Serum transferase activity is expressed as μmoles product formed/ml of serum with CDNB as the substrate; liver transferase activity is expressed as μmoles product/mg cytosolic protein with CDNB as the substrate. Values are shown as mean ± S.D.

†‡ Analysis of variance: (†) control vs treated, $P < 0.05$; and (‡) chloroform vs chloroform + SKF 525-A or chloroform + cysteine, $P < 0.05$.

S-transferase D + E. The recovery of glutathione S-transferase activity and protein in the *in vitro* experiments are summarized in Table 3.

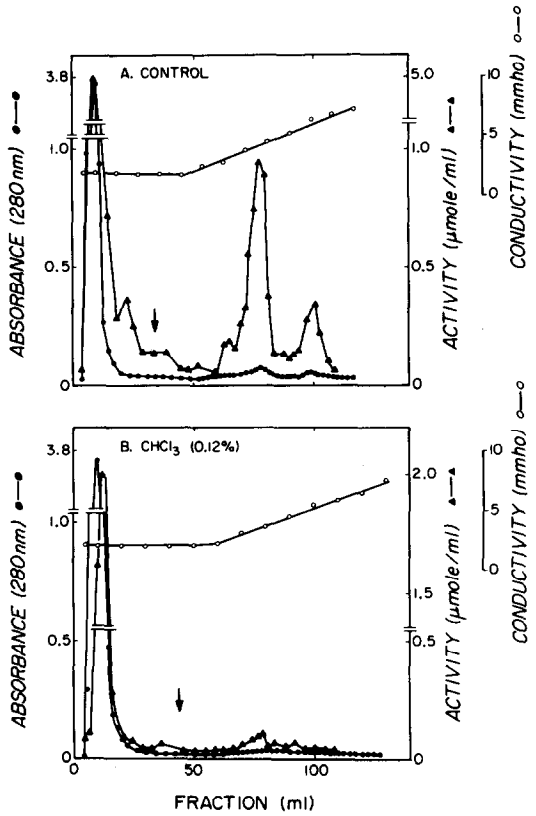

Fig. 4. Chloroform treatment of partially purified hepatic cytosolic glutathione S-transferase *in vitro*. A Sephadex G-100 fraction (5.6 mg) was incubated with chloroform in the presence of microsomes and NADPH at 37° for 10 min and applied to the CM52 column (0.9 × 18 cm) as described in Materials and Methods. The column was eluted with the same conditions as shown in Fig. 1.

DISCUSSION

The available evidence suggests that a reactive metabolite of chloroform is responsible for its hepato- and nephrotoxicity [2]. Recently, phosgene was

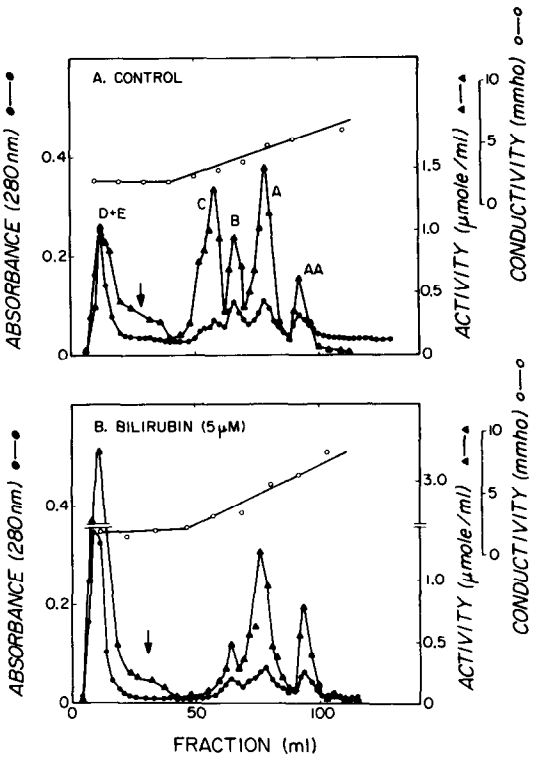

Fig. 5. Bilirubin treatment of partially purified hepatic cytosolic glutathione S-transferase *in vitro*. A Sephadex G-100 fraction (5.6 mg) was incubated with 5 μM bilirubin as described in Materials and Methods and applied to the CM52 column (0.9 × 17 cm). The column was eluted with the same conditions as shown in Fig. 1.

Table 3. Recovery of glutathione *S*-transferase activity and protein from a CM52 column after *in vitro* treatment with chloroform or bilirubin*

Treatment	Glutathione *S*-transferase					
	After incubation	D + E	C	B	A	AA
Control						
Activity	76.1	28.0	1.3	9.1	0.5	2.1
Protein (mg)		4.0	0.1	0.4	0.1	0.1
$CHCl_3$ treatment						
Activity†	16.2	15.2	ND‡	ND	ND	ND
Protein (mg)		4.6	ND	ND	ND	ND
Control						
Activity†	94.8	8.4	8.9	6.0	10.3	3.8
Protein (mg)		1.3	0.4	0.3	0.4	0.2
Bilirubin treatment						
Activity†	63.1	18.4	ND	3.5	15.9	2.4
Protein		2.0	ND	0.2	0.6	0.1

* Partially purified cytosolic glutathione *S*-transferase (Sephadex G-100 fraction, 5.6 mg, total activity 95 μmoles) was incubated with chloroform or bilirubin, as described in Materials and Methods.

† Glutathione *S*-transferase activities are presented as μmoles of product formed/fraction with CDNB as the substrate.

‡ ND = not detectable.

identified as an active metabolite of chloroform, which was produced by microsomal cytochrome P-450-dependent metabolism [2, 11]. Phosgene is highly reactive and binds covalently to cellular macromolecules [12].

In the present study, it was shown that hepatic cytosolic glutathione *S*-transferase activity was decreased after chloroform treatment and reached a minimum at 5 hr. The decrease of hepatic glutathione *S*-transferase activity was accompanied by an increase in serum glutathione *S*-transferase activity, which reached a peak at 5 hr after treatment and returned to near control values by 24 hr. These results suggest that the decrease in hepatic glutathione *S*-transferase activity is due to a leakage of the hepatic enzymes into the serum. The time course of glutathione *S*-transferase appearance in serum was similar to the time course of covalent binding of chloroform metabolites, as reported by Stevens and Anders [13] who showed that the covalent binding to hepatic microsomal and cytosol proteins is maximal at 6 hr and that hepatoxicity reaches a peak at 18 hr. The appearance of glutathione *S*-transferase activity in serum after chloroform treatment was prevented by treatment with cysteine or SKF 525-A; these agents also inhibit the covalent binding of chloroform metabolites to macromolecules [2, 13]. SKF 525-A inhibits microsomal cytochrome P-450-dependent mixed-function oxidases, and cysteine combines with phosgene to form 2-oxothiazolidine-4-carboxylic acid [14]. Therefore, it is clear that chloroform is metabolized to phosgene, which is associated with the covalent binding of chloroform metabolites and the leakage of glutathione *S*-transferases into serum.

Glutathione *S*-transferases AA, A, B, C, and D + E are normally present in rat liver cytosol. In the time course studies it was observed that, at 2 hr after chloroform treatment, glutathione *S*-transferase B was not detected by CM52 chromatography and by 5 hr the more basic transferases were undetectable, except for glutathione *S*-transferase A. This finding suggests that more basic glutathione *S*-transferases, such as AA, A, B, and C, leaked from the liver into the serum after chloroform treatment. As shown in Fig. 2, the basic glutathione *S*-transferases AA, A, B, and C were not detected in serum; only glutathione *S*-transferase D + E was observed. It was shown that chloroform metabolites were bound to glutathione *S*-transferase D + E in liver cytosol (unpublished data). These data suggested that glutathione *S*-transferases, such as AA, A, B, and C, lost their basic character as a consequence of chloroform treatment and were released into serum.

To clarify the effect of chloroform treatment on transferase activity, experiments were conducted with partially purified cytosolic glutathione *S*-transferases. When cytosolic glutathione *S*-transferases were incubated with chloroform in the presence of hepatic microsomes and NADPH, glutathione *S*-transferase activity was decreased and the more basic glutathione *S*-transferases (AA, A, B and C) were not detected. These results suggest that chloroform is metabolized by the microsomal cytochrome P-450 system, and a metabolite of chloroform causes both a decrease in glutathione *S*-transferase activity and a change in the basic properties of glutathione *S*-transferases. Cresteil *et al.* [15] reported that the active metabolite of chloroform phosgene may be bound to amino groups of macromolecules forming carbamates or substituted ureas. It is, therefore, likely that the amino groups of basic glutathione *S*-transferases in cytosol react with phosgene, resulting in the loss or alteration of their basic properties.

The selective and early alteration of glutathione *S*-transferase B was observed after chloroform treatment *in vivo* (Fig. 1). A similar result was also obtained after bilirubin treatment of glutathione *S*-

transferases *in vitro*. Glutathione *S*-transferase B was scarcely detectable after treatment of Sephadex G-100 fraction with 5 μM bilirubin. When 10 μM bilirubin was used, both glutathione *S*-transferases C and B were undetectable (data not shown). This decrease in the activity and protein of transferases B and C was accompanied by an increase in glutathione *S*-transferase D + E after bilirubin treatment. Thus, it was confirmed that glutathione *S*-transferases B and C are altered by bilirubin and are recovered as glutathione *S*-transferase D + E from CM52 columns. Since chloroform treatment increased serum bilirubin concentrations, it is, therefore, probable that the bilirubin formed *in vivo* after chloroform treatment was responsible for the alteration in glutathione *S*-transferases B and C. The selectivity of this alteration of glutathione *S*-transferases B and C can be explained by the affinity of bilirubin for the transferases; the K_D of binding of bilirubin to glutathione *S*-transferases AA, A, B, and C is 100, 15, 2, and 2 μM [5] respectively. Thus, bilirubin binding causes not only a decrease of glutathione *S*-transferase activity but also an alteration of their chromatographic properties.

In conclusion, chloroform caused a release of hepatic cytosolic glutathione *S*-transferases into serum. Both the active metabolite of chloroform, which was produced by the microsomal cytochrome P-450 system, and bilirubin, which was increased by chloroform treatment, played roles in altering the properties of the glutathione *S*-transferases.

Acknowledgement—This research was supported by National Institutes of Health Grant ES 03126.

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