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PREVENTION OF CARBON TETRACHLORIDE-INDUCED NECROSIS BY INHIBITORS OF DRUG METABOLISM—FURTHER STUDIES ON THEIR MECHANISM OF ACTION*

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Abstract—Liver necrosis caused by CCl_4 is known to be decreased by the administration of SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate), Sch 5705 (ethyl 2-diethylaminoethyl-2-phenyl-2-ethyl malonate), Sch 5706 [ethyl *N*-(2-diethylaminoethyl) 2-phenyl-2-ethyl malonate], Sch 5712 (ethyl 2-diethylaminoethyl 2-ethyl-2-butyl malonate), CFT 1201 [2-diethylaminoethyl 2-phenyl (2-propene) 4-penten-1-oate], Lilly 18947 (2,4-dichloro-6-phenyl phenoxyethyl diethylamine) and DPEA (2,4-dichloro-6-phenyl phenoxyethylamine). Although these substances are known to inhibit cytochrome P-450 dependent drug-metabolizing enzymes in liver microsomes, they apparently do not evoke their protective effects by slowing the elimination of CCl_4. In fact, SKF 525-A, but none of the other inhibitors, partially prevents the impairing effects of CCl_4 on cytochrome P-450 in liver. Although SKF 525-A markedly decreased the liver necrosis and the rise in serum isocitrate dehydrogenase (ICD) caused by CCl_4, it only partially prevented the CCl_4-induced decrease in body temperature.

WE PREVIOUSLY reported that administration of 2-diethylaminoethyl 2,2-diphenylvalerate (SKF 525-A) to rats prevented not only CCl_4-induced necrosis but also the early destruction of cytochrome P-450.[1] At that time we thought that SKF 525-A exerted these effects by inhibiting the conversion of CCl_4 to an active metabolite which is thought to mediate CCl_4-induced hepatotoxicity. Marchand *et al.*[2,3] later found that SKF 525-A decreased the absorption of CCl_4 and other compounds from the gastrointestinal tract, and they postulated that the protective effect of SKF 525-A could be explained by the decrease in CCl_4 concentration in the liver. In addition, Cignoli and Castro[4,5] found that several other inhibitors of drug metabolism partially block CCl_4-induced necrosis, but that their effectiveness in blocking necrosis could not be completely explained by either their potency as inhibitors of drug metabolism or their ability to inhibit CCl_4-induced lipid peroxidation *in vitro*.

In the present study, we have compared the effects of several competitive inhibitors of cytochrome P-450 enzymes on the liver levels of CCl_4 administered orally, as well as their effects on various aspects of CCl_4 toxicity, including the plasma levels of isocitrate dehydrogenase, the decrease in the level of cytochrome P-450 in liver microsomes, liver weight and lipid peroxidation.

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EXPERIMENTAL

Chemicals. Reagent grade carbon tetrachloride was employed in these studies. The 2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride (SKF 525-A) was a gift from Smith Kline & French Laboratories. Ethyl 2-diethylaminoethyl-2-phenyl-2-ethyl malonate hydrobromide (Sch 5705), ethyl *N*-(2-diethylaminoethyl) 2-phenyl-2-ethyl malonate hydrobromide (Sch 5706) and ethyl 2-diethylaminoethyl 2-ethyl-2-butyl malonate hydrobromide (Sch 5712) were kindly supplied by Societa Italiana Prodotti Schering, Milan, Italy. The 2-diethylaminoethyl 2-phenyl 2-(2 propene) 4-penten-1-oate hydrochloride (CFT 1201) was a generous gift from Preuss & Temmler Laboratories, Germany. The 2,4-dichloro-6-phenyl phenoxyethyl diethylamine hydrochloride (Lilly 18947) and 2,4-dichloro-6-phenyl phenoxy-ethylamine (DPEA) were gifts from Eli Lilly & Co. The 10-(3-dimethyl amino isopropyl) phenothiazine hydrochloride (promethazine) was kindly supplied by Wyeth Laboratories.

Treatment of animals. Sprague–Dawley male albino rats (200–300 g) were used in these experiments. The animals were maintained on a Purina rat chow diet and food was withdrawn 12–14 hr before CCl_4 administration. SKF 525-A, Sch 5705, Sch 5706, Sch 5712, Lilly 18947 and promethazine, dissolved in a 0·9% NaCl solution, and DPEA, dissolved in distilled water, were administered i.p., at a dose of 50 mg/kg, except for promethazine whose dose was 25 mg/kg. Control animals received either saline or distilled water i.p. Thirty min later, CCl_4 was given either undiluted *per os* at a dose of 2·5 ml/kg or intraperitoneally as a 20% (v/v) or 30% (v/v) solution in olive oil at a dose of 5 ml solution/kg. Control rats received the equivalent amount of olive oil intraperitoneally, when appropriate. The animals were decapitated in a Harvard guillotine and bled. Their livers were removed rapidly and weighed.

Whenever blood samples were taken, the animals were kept under light ether anesthesia and blood was collected from the inferior vena cava with a syringe containing heparin.

Enzymatic and chemical determinations. The microsomal fraction of liver was isolated as described by Castro and Gillette.[6] Ethylmorphine *N*-demethylase activity was measured according to the procedure described by Castro and Gillette,[6] except that the ethylmorphine concentration in the incubation mixture was 10 mM, which is about 20–40 times the K_m of ethylmorphine *N*-demethylase in liver microsomes of male Sprague–Dawley rats. Thus any competitive inhibition caused by the residue inhibitors present in the liver microsomes would have been negligible in these assays. Activity is expressed in nmoles formaldehyde formed/mg of protein in 15 min at 37°.

The amount of cytochrome P-450 in liver microsomes was determined by the method described by Schenkman *et al.*[7] Peroxidation of liver microsomal lipids *in vivo* was determined by estimating diene conjugation in lipid extracts of the microsomal fractions, as described by Klaassen and Plaa.[8] The results are expressed as the change in absorbance at 243 nm $\times$ 1000 for a solution having 1 mg microsomal lipid/ml.

Carbon tetrachloride concentrations in liver were estimated according to the procedure described by Recknagel and Litteria.[9] Results were corrected for loss of CCl_4 during the analysis which, under our experimental conditions, was about 50 per cent.

Protein concentrations were estimated either by the procedure described by Lowry *et al.*[10] for diluted microsomal suspensions or by the biuret method[11] for concentrated microsomal suspensions.

NADP-linked isocitric dehydrogenase activity in plasma (plasma ICD) was

measured according to Sterkel *et al.*;[12] one unit of enzyme is the amount of enzyme required to form 1 nmole NADPH/ml of plasma/hr at 25°.

Histological techniques. Small portions of liver from the left and central lobes were immediately fixed in Bouin's solution, embedded in paraffin and stained with hematoxylin–eosin and Mallory's trichrome stain.

Statistics. The significance of the difference between two mean values was assessed either by Student's *t*-test[13] or by the Mann–Whitney U test.[14] The significance of differences involving more than two mean values was assessed by two-way analysis of variance, according to the method of Brownlee.[15]

Temperature. The rectal temperature of the rats was recorded with a small animal thermistor probe (tele-thermometer, Nihon-Kohden).

RESULTS

CCl_4 concentrations in liver at different times after either oral or intraperitoneal administration to rats pretreated with various inhibitors. Prior treatment of the rats with Sch 5705, Sch 5706, Sch 5712, Lilly 18947 or promethazine did not significantly alter the liver levels of CCl_4 at any time after its oral administration (Fig. 1). In agree-

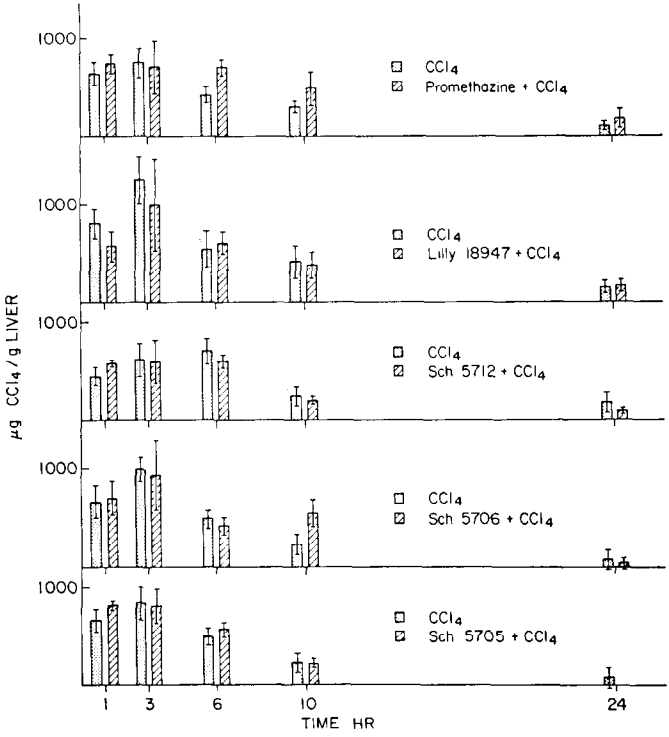

FIG. 1. CCl_4 concentrations in liver at different times after administration. Sch 5705, Sch 5706, Sch 5712, Lilly 18947 and promethazine dissolved in a 0·9% NaCl solution, and DPEA dissolved in distilled water, were administered i.p. at a dose of 50 mg/kg, except for promethazine whose dose was 25 mg/kg. Control animals received either saline or distilled water i.p. Thirty min later CCl_4 was given *per os* at a dose of 2·5 ml/kg. Results in both groups were significantly different at 1 h for Lilly 18947 ($P < 0·05$) and at 6 hr for promethazine ($P < 0·01$). Five animals per group were used. Vertical bars represent S.D.

ment with previous results from other laboratories,[2,3] however, SKF 525-A decreased the CCl_4 levels in liver at early times after its oral administration (Fig. 2). On the other hand, pretreatment of rats with SKF 525-A 30 min before the intraperitoneal administration of CCl_4, either 20 or 30% in olive oil, led to significantly decreased levels of CCl_4 in liver. The effect was significant at 1 and 3 hr after the injection of the 20% solution of CCl_4 in oil, but only at 1 hr after the 30% solution was administered (Fig. 2).

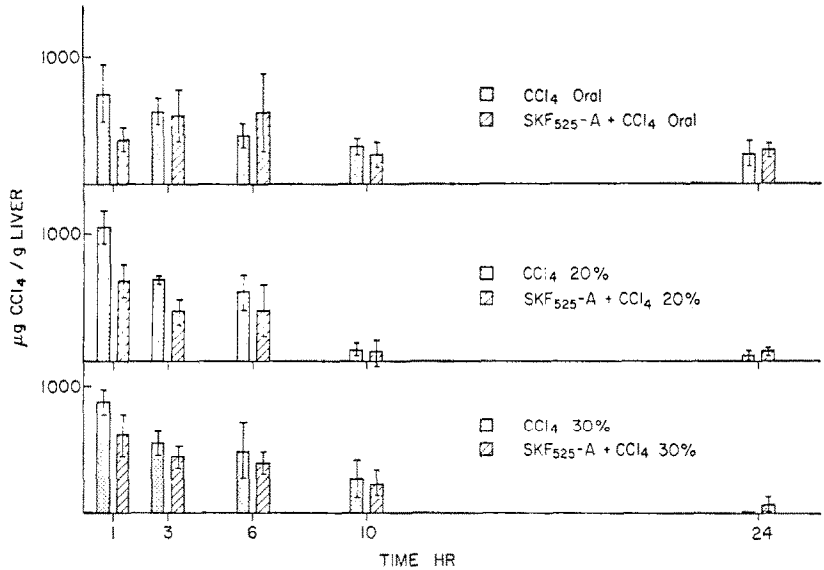

FIG. 2. CCl_4 concentrations in liver at different times after administration in SKF 525-A-pretreated rats. SKF 525-A was given i.p. as a saline solution at a dose of 50 mg/kg 30 min before CCl_4. Controls received saline. CCl_4 was given either orally as in Fig. 1 or i.p. at a dose of 5 ml solution in olive oil/kg at two different concentrations, either 20 or 30%. Results in both groups were significantly different at 1 hr for the oral and both i.p. doses of CCl_4 ($P < 0{\cdot}01$), and at 3 hr for the i.p. administration of 20% CCl_4 ($P < 0{\cdot}01$). Five animals per group were used. Vertical bars represent S.D.

Destruction of microsomal cytochrome P-450 by CCl_4 in rats pretreated with various inhibitors. Neither the level of cytochrome P-450 in liver microsomes nor the *N*-demethylation of ethylmorphine as measured at high substrate concentrations (10 mM) was altered at 3 hr after the administration of Lilly 18947, Sch 5705, CFT 1201, promethazine or DPEA. Moreover, pretreatment of rats with these inhibitors did not prevent the decreases caused by CCl_4 in either cytochrome P-450 or ethylmorphine demethylation. By contrast, the administration of SKF 525-A alone caused an apparent decrease in both the level of cytochrome P-450 in liver microsomes and ethylmorphine demethylation. But pretreatment with SKF 525-A decreased the impairing effects of CCl_4 on both cytochrome P-450 and ethylmorphine metabolism (Table 1).

Effect of pretreatment with SKF 525-A on CCl_4-induced necrosis and fatty liver. As shown in Table 2, SKF 525-A administration almost completely prevented the increase in plasma ICD found 24 hr after the intraperitoneal administration at either the 20 or 30% solution of CCl_4, but did not significantly affect the CCl_4-induced increase in liver weight.

TABLE 1. ETHYLMORPHINE *N*-DEMETHYLATION *in vitro* AND LEVELS OF MICROSOMAL CYTOCHROME P-450 AT 3 hr AFTER ADMINISTRATION OF CCl_4 TO RATS PRETREATED WITH VARIOUS COMPOUNDS*

Treatment	EM-ase (Mean ± S.D.)	Decrease (%)	Cytochrome P-450 (Mean ± S.D.)	Decrease (%)
Control	82 ± 15	36·6	0·70 ± 0·08	50
CCl_4 (oral)	52 ± 6		0·35 ± 0·06	
Lilly 18947	92 ± 12	50	0·72 ± 0·13	54·2
Lilly 18947 + CCl_4 (oral)	46 ± 12		0·33 ± 0·06	
Control	87 ± 20	43·7	0·60 ± 0·06	46·7
CCl_4 (oral)	49 ± 13		0·32 ± 0·07	
Sch 5712	92 ± 8	52·5	0·53 ± 0·05	49·1
Sch 5712 + CCl_4 (oral)	44 ± 16		0·27 ± 0·07	
Control	68 ± 9	25	0·54 ± 0·03	35·2
CCl_4 (oral)	51 ± 15		0·35 ± 0·05	
Sch 5705	76 ± 12	36·8	0·51 ± 0·10	39·2
Sch 5705 + CCl_4 (oral)	48 ± 6		0·31 ± 0·03	
Control	51 ± 3	35·3	0·81 ± 0·02	37
CCl_4 (oral)	33 ± 1·4		0·51 ± 0·01	
CFT 1201	50 ± 1·8	42	0·64 ± 0·02	39
CFT 1201 + CCl_4 (oral)	29 ± 1·5		0·39 ± 0·01	
Control	86 ± 7	36	1·00 ± 0·02	38
CCl_4 (oral)	55 ± 2		0·62 ± 0·02	
Promethazine	92 ± 2	39	0·01 ± 0·01	44
Promethazine + CCl_4 (oral)	56 ± 7		0·56 ± 0·04	
Control	108 ± 3	39·8	0·58 ± 0·03	46·6
CCl_4 (oral)	65 ± 5		0·31 ± 0·01	
DPEA	114 ± 8	53·5	0·41 ± 0·02	48·8
DPEA + CCl_4 (oral)	53 ± 4		0·41 ± 0·02	
Control	76 ± 8·5	51·5	0·55 ± 0·07	49·1
CCl_4 (30%)	37 ± 7·3		0·28 ± 0·04	
SKF 525-A	41 ± 9	31·7	0·43 ± 0·09	34·9
SKF 525-A + CCl_4 (30%)	28 ± 5		0·28 ± 0·04	

* The inhibitors and CCl_4 were given as indicated in Figs. 1 and 2. EM-ase activity is given in nmoles formaldehyde formed in 15 min at 37° per mg of microsomal protein; the P-450 content is given in nmoles/mg of microsomal protein. Significance for the overall effect of the inhibitors was established by two-way analysis of variance. The effect of the pretreatment with inhibitors in decreasing the impairing effects of CCl_4 was significant only in the case of SKF 525-A ($P < 0·001$ for the effect on EM-ase and $P = 0·01$ for P-450). Five animals per group were used, except in the experiment on SKF 525-A in which ten animals per group were employed. A similar small but significant preventive effect of SKF 525-A on the destruction of EM-ase and P-450 caused by CCl_4 was also observed in two other experiments using five animals per group.

Histological examination of the livers also indicated that the prior treatment with SKF 525-A decreased the liver necrosis caused by intraperitoneally administered CCl_4. At 24 hr after treatment with SKF 525-A alone, the architectural pattern of the liver was normal (Fig. 3). On the other hand, 24 hr after the administration of 30% CCl_4 in corn oil to animals, there was an intense centrolobular necrosis associated with hemorrhage and edema. In the periportal zones, the liver cells were enlarged and the normal architectural pattern was lost (Fig. 4). By contrast, pretreatment with SKF 525-A markedly decreased the extent of the necrosis produced by CCl_4 24 hr after its administration, but did not prevent the fatty infiltration (Fig. 5). Although congestion,

TABLE 2. EFFECT OF INTRAPERITONEAL CCl_4 24 hr AFTER ADMINISTRATION ON PLASMA ISOCITRIC DEHYDROGENASE (ICD) AND LIVER WEIGHT IN RATS PREVIOUSLY TREATED WITH SKF 525-A*

Treatment	ICD (Mean ± S.D.)	Liver wt/100 g body wt (Mean ± S.D.)
Control	150 ± 92	2·99 ± 0·15
CCl_4 (20%)	93,000 ± 56,900	3·86 ± 0·43
SKF 525-A	225 ± 53	3·11 ± 0·08
SKF 525-A + CCl_4 (20%)	9,915 ± 16,525	3·74 ± 0·29
Control	180 ± 30	2·83 ± 0·27
CCl_4 (30%)	99,000 ± 34,900	3·75 ± 0·26
SKF 525-A	174 ± 45	3·02 ± 0·22
SKF 525-A + CCl_4 (30%)	18,000 ± 10,500	3·53 ± 0·09

* CCl_4 was given i.p. as either a 20 or 30% solution in olive oil at a dose of 5 ml/kg to rats fasted 12–14 hr. SKF 525-A was given as indicated in Fig. 2. Isocitric dehydrogenase activity is given in units (see Methods for details) and liver weight is given in relation to 100 g of body weight. Five animals per group were used. The significance for the overall effect of SKF 525-A on the CCl_4 action on both parameters was obtained by two-way analysis of variance and it was significant only for the effect on ICD, when CCl_4 was either 20% ($P < 0·005$) or 30% ($P < 0·001$).

edema and focal areas of centrolobular necrosis were observed, areas with a normal architectural pattern were more extensive than those of the unprotected animals.

Pretreatment with SKF 525-A also markedly decreased the necrosis found 72 hr after administration of the 30% solution of CCl_4 (Figs. 6 and 7).

TABLE 3. CCl_4-INDUCED LIPID PEROXIDATION *in vivo* IN ANIMALS PRETREATED WITH VARIOUS INHIBITORS OF DRUG METABOLISM*

Treatment	3 hr	6 hr	10 hr	24 hr
Control	185 ± 9	275 ± 22	185 ± 25	183 ± 18
CCl_4 (30%)	292 ± 13	515 ± 59	297 ± 56	398 ± 54
SKF 525-A	173 ± 19	287 ± 28	208 ± 22	205 ± 24
SKF 525-A + CCl_4 (30%)	249 ± 16†	440 ± 43†	269 ± 30	266 ± 30†
Control	172 ± 33			
CCl_4 (oral)	250 ± 39			
Sch 5705	183 ± 44			
Sch 5705 + CCl_4 (oral)	263 ± 44			
Control	220 ± 25			
CCl_4 (oral)	265 ± 22			
Sch 5712	171 ± 23			
Sch 5712 + CCl_4 (oral)	293 ± 50			

* Inhibitors and CCl_4 were given as indicated in Figs. 1 and 2. The animals were sacrificed at different times after CCl_4 as indicated. The lipid peroxidation value is expressed as Δ absorbance at 243 nm × 1000 for a solution having 1 mg microsomal lipid/ml. Significance for the overall effect of the inhibitors was established by two-way analysis of variance. A significant effect was obtained only for SKF 525-A at 3, 6 and 24 hr. Five animals per group were used.

† $P < 0·05$.

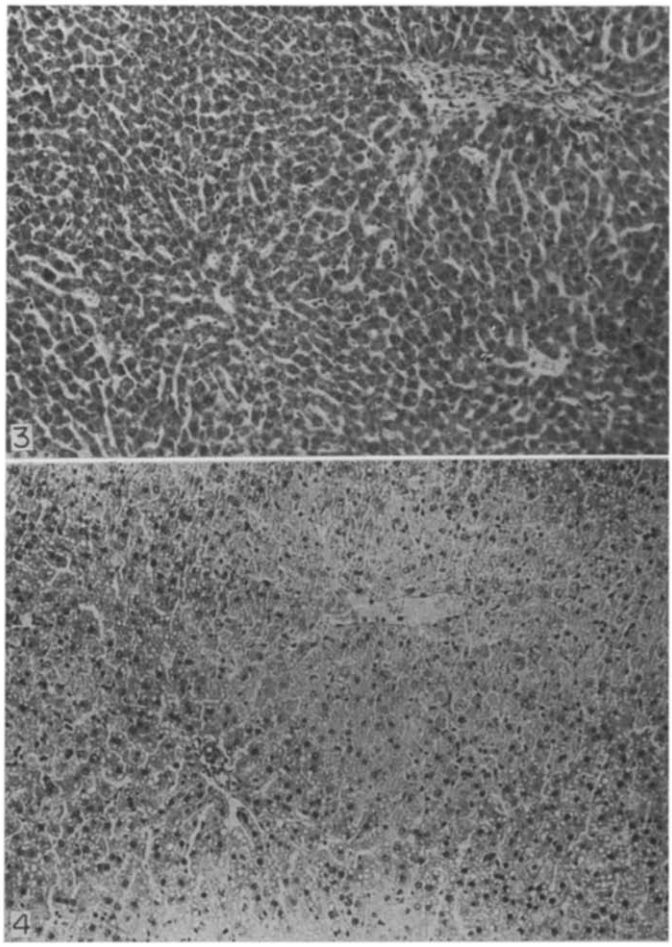

FIG. 3. Liver section taken from a rat which had received SKF 525-A 24 hr before. The liver structure is essentially normal. Hematoxylin and eosin (×158).

FIG. 4. Liver section taken from a rat which had received 30% CCl_4 24 hr before. Note the striking centrolobular necrosis. Hematoxylin and eosin (×158).

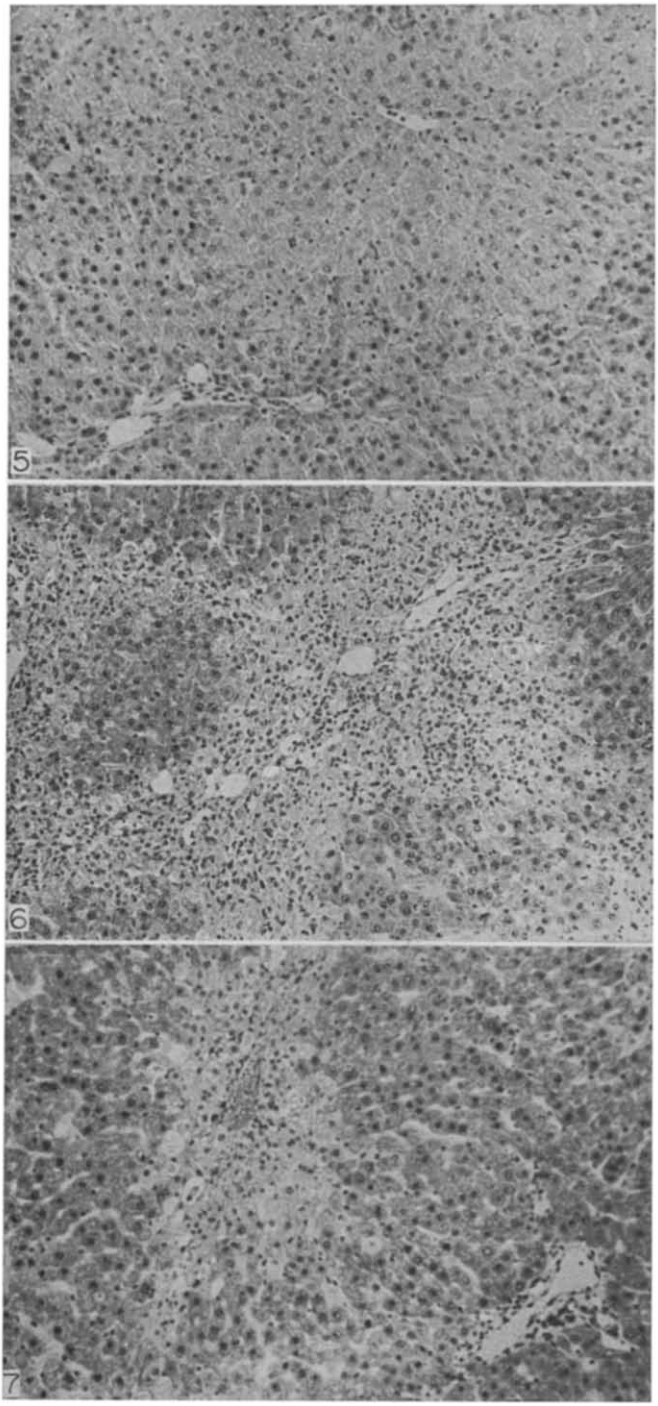

FIG. 5. Liver section taken from a rat pretreated with SKF 525-A and which had received 30% CCl_4 24 hr before. The liver cells of the central areas are swollen and the periportal zones are well preserved. Hematoxylin and eosin (×158).

FIG. 6. Liver section taken from a rat which had received 30% CCl_4 72 hr before. A striking centrolobular necrosis is observed. Hematoxylin and eosin (×158).

FIG. 7. Liver section taken from a rat pretreated with SKF 525-A and which had received 30% CCl_4 72 hr before. The necrotic areas are reduced. Hematoxylin and eosin (×158).

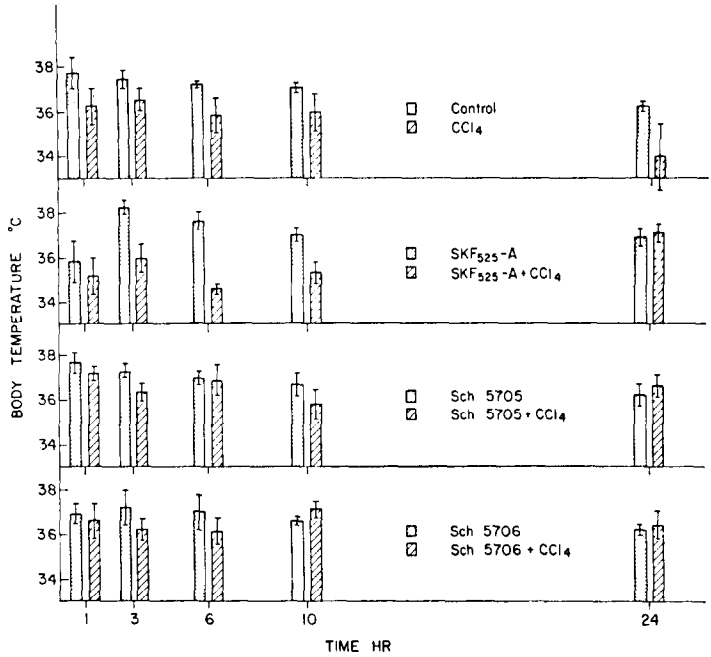

FIG. 8. Effect of inhibitors of drug metabolism on the decrease of body temperature produced by CCl_4. Inhibitors were given as indicated in Figs. 1 and 2; CCl_4 was given orally as described in Fig. 1. Controls received saline. Results were significantly different at 1 hr for SKF 525-A ($P < 0.01$) and at 10 hr for Sch 5706 ($P < 0.05$) with respect to control rats. The P value for the significance of the overall effect of the prior treatment with the inhibitors obtained by two-way analysis of variance was $P < 0.01$ for SKF 525-A and $P < 0.025$ for Sch 5705 at 6 hr; $P < 0.005$ for Sch 5706 at 10 hr and $P < 0.005$ for the three compounds at 24 hr.

CCl_4-induced lipid peroxidation in vivo *in animals pretreated with either SKF 525-A, Sch 5705 or Sch 5712.* As shown in Table 3, SKF 525-A administration to rats produced a small but statistically significant decrease in the extent of the CCl_4-induced lipid peroxidation at 3, 6 and 24 hr after intraperitoneal administration of CCl_4. But no significant effect on the lipid peroxidation caused by CCl_4 was observed when the rats were pretreated with either Sch 5705 or Sch 5712.

Effect of inhibitors of drug metabolism on the decrease of body temperature produced by CCl_4. Since CCl_4 is known to lower body temperature, it was of interest to determine whether the inhibitors of cytochrome P-450 also affected body temperature. When administered alone, SKF 525-A caused a decrease at 1 hr and an increase at 3 hr, and Sch 5705 and Sch 5706 had little or no effect. When administered in combination with CCl_4, however, the inhibitors caused variable effects. Up to 10 hr after CCl_4 administration, the temperature was lower in rats pretreated with SKF 525-A, but tended to be higher in rats pretreated with Sch 5705 or Sch 5706 than it was in rats receiving CCl_4 alone. At 24 hr after CCl_4 administration, however, the temperature in rats receiving any of the inhibitors was normal, whereas the temperature in rats receiving CCl_4 alone was markedly decreased (Fig. 8).

DISCUSSION

Slater *et al.*[16] have reported that pretreatment with SKF 525-A protects against CCl_4-induced necrosis. Studies from our laboratory[1] confirmed these findings and

attributed the protective effect of SKF 525-A to the inhibition of microsomal enzymes that catalyze the conversion of CCl_4 to an active metabolite. However, Marchand *et al.*[2,3] demonstrated that prior administration of SKF 525-A delays the gastrointestinal absorption of CCl_4 and thus suggested that the decrease in CCl_4 concentration in the liver could explain the protective effect of this compound.

The present studies show that even when administered intraperitoneally, SKF 525-A decreases the level of CCl_4 in liver and partially blocks some of the earlier signs of CCl_4 toxicity. They also show that several other compounds previously found to be inhibitors *in vivo* of drug metabolism by liver microsomal cytochrome P-450 enzymes[4] do not significantly alter the CCl_4 levels in liver after oral administration of the toxicant, nor do they affect the impairing effects of CCl_4 as measured by the destruction of cytochrome P-450 or in the case of Sch 5705 and Sch 5712 by lipid peroxidation.

Although the protective effects of SKF 525-A against various aspects of CCl_4 toxicity are apparently related to a decrease in the liver levels of CCl_4, a part of the protective effect of SKF 525-A on liver necrosis could be mediated by inhibition of lipid peroxidation (Table 3). However, the inhibition seems small compared with the marked effect of SKF 525-A in preventing the rise of plasma ICD caused by CCl_4 (Table 2). In contrast to these findings, Rao *et al.*[17] reported that SKF 525-A markedly decreased CCl_4-induced lipid peroxidation, but these workers administered twice as much SKF 525-A as we used in our studies and measured lipid peroxidation 30 min after oral administration of CCl_4, when the effect of SKF 525-A on oral absorption would probably be greater. Moreover, the fact that the administration of Sch 5705, Sch 5706, Sch 5712, CFT 1201 or Lilly 18947 followed by oral administration of CCl_4 can partially prevent necrosis[4,5] without changing the levels of liver CCl_4 (Fig. 1) or lowering body temperature (Fig. 8) or inhibiting lipid peroxidation either *in vitro*[5] or *in vivo* (Table 3) may tend to support our previous view that protection by these compounds was in some way related to their ability to inhibit a minor pathway of CCl_4 metabolism. So many other factors are known to influence CCl_4-induced necrosis, however, that this interpretation of the results should be accepted only tentatively.

REFERENCES

1. J. A. CASTRO, H. SASAME, H. SUSSMAN and J. R. GILLETTE, *Life Sci.* **7**, 129 (1968).
2. C. MARCHAND, S. MCLEAN and G. L. PLAA, *J. Pharmac. exp. Ther.* **174**, 232 (1970).
3. C. MARCHAND, S. MCLEAN, G. L. PLAA and G. TRAIJER, *Biochem. Pharmac.* **20**, 869 (1971).
4. E. V. CIGNOLI and J. A. CASTRO, *Toxic, appl. Pharmac.* **18**, 625 (1971).
5. E. V. CIGNOLI and J. A. CASTRO, *Expl molec. Path.* **14**, 43 (1971).
6. J. A. CASTRO and J. R. GILLETTE, *Biochem. biophys. Res. Commun.* **28**, 426 (1967).
7. J. B. SCHENKMAN, H. REMMER and R. ESTABROOK, *Molec. Pharmac.* **3**, 113 (1967).
8. C. KLAASSEN and G. L. PLAA, *Biochem. Pharmac.* **18**, 2019 (1969).
9. R. RECKNAGEL and M. LITTERIA, *Am. J. Path.* **36**, 521 (1960).
10. O. LOWRY, N. ROSEBROUGH, A. FARR and R. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
11. E. LAYNE, in *Methods in Enzymology* (Eds. S. COLOWICK and N. KAPLAN), Vol. 3, p. 450. Academic Press, New York (1957).
12. R. STERKEL, J. SPENCER, S. WOLFSON and H. WILLIAMS-ASHMAN, *J. Lab. Clin. Med.* **52**, 176 (1958).
13. H. BANCROFT, in *Introduction to Biostatistics*, p. 205. Eudeba, Buenos Aires (1960).
14. C. L. RUMKE and H. DE JONGE, in *Evaluation of Drug Activities: Pharmacometrics*, Vol. 1, p. 80. Academic Press, New York (1964).
15. K. BROWNLEE, in *Statistical Theory and Methodology in Science and Engineering*, p. 379. Wiley, New York (1960).
16. T. F. SLATER, B. SAWYER and U. STRAULI, *Biochem. Pharmac.* **15**, 1273 (1966).
17. K. S. RAO, E. GLENDE and R. O. RECKNAGEL, *Expl molec. Path.* **12**, 324 (1970).