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BIOCHEMICAL CHANGES IN RAT LIVER AFTER ADMINISTRATION OF CARBON DISULPHIDE, WITH PARTICULAR REFERENCE TO MICROSOMAL CHANGES

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Abstract—Rats given an oral dose of carbon disulphide (1 ml/kg) exhibited an increased liver size and protein content 24 hr later, but showed a marked prolongation of the hexobarbitone "sleeping time". After a single oral dose of CS_2, the activity of aniline hydroxylase and nitroanisole demethylase and the level of cytochrome P-450 were found to fall very rapidly in the liver microsomes and remained depressed for at least three days: aniline hydroxylase was more severely affected and recovered at a faster rate than either cytochrome P-450 or nitroanisole demethylase. The level of cytochrome b_5 decreased only slightly. A "P-420" peak was detected in microsomal preparations from poisoned animals in the early stages of the intoxication and the total protohaem content of the microsomes was unchanged at 4 hr but decreased significantly at 24 hr. The most likely interpretation for these findings is that CS_2 causes a rapid alteration in the cytochrome P-450 leading to loss of its spectrum and of its activity in drug oxidation and that the altered cytochrome (or its haem moiety) is lost from the damaged membranes at a slow rate.

The extent of the microsomal changes caused by CS_2 appeared to depend on the activity of the drug-metabolizing enzymes at the time when CS_2 was administered. Pre-treatment with phenobarbitone increased the loss of microsomal enzymes and cytochrome P-450 caused by CS_2, whereas SKF-525A afforded some protection. This may indicate that a metabolite of CS_2, rather than CS_2 itself, is the real toxic agent responsible for the observed picture.

Changes were also noted in the rate of incorporation of labelled leucine into proteins by liver slices from the poisoned rats: an inhibition was observed at 4 hr and this was followed by a stimulation 24 hr after CS_2 administration.

IN THE course of a study of the acute and subacute toxicity of carbon disulphide in the rat a significant and progressive increase in liver size was noted with a rise in the total protein content of the liver. Liver enlargement with increased liver protein content is frequently observed after administration of several lipid soluble compounds and it is usually associated with a stimulation of the activity of microsomal drug-metabolizing enzymes. In contrast, after carbon disulphide administration a marked and prolonged depression of drug metabolism occurred. Experiments were carried out to elucidate the biochemical mechanism underlying these effects of carbon disulphide and are described in the present paper.

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MATERIALS AND METHODS

Treatment of animals. Male albino rats (150–180 g) of the Porton strain, maintained on cube diet MRC 41B[1] were used. They were fasted overnight before carbon disulphide administration and kept with water but no food thereafter, unless otherwise indicated. Carbon disulphide was administered by oral intubation mixed with an equal volume of arachis oil; control animals were given arachis oil alone.

In some experiments the animals were treated with phenobarbitone or fed sucrose *ad lib.* for 2 days before administration of carbon disulphide. Phenobarbitone sodium was given by i.p. injection in two doses (6 hr apart) of 50 mg/kg in the first day and in a single dose of 80 mg/kg in the second, this last dose 24 hr before carbon disulphide. Some phenobarbitone-treated animals were also given a dose of SKF-525A (40 mg/kg) by i.p. injection 45 min before carbon disulphide. The core body temperature was measured with a thermocouple inserted 7–8 cm from the anus. At different times after carbon disulphide administration the animals were killed by decapitation and bled from the severed neck for at least 30 sec. The liver was quickly removed, rinsed with distilled water, blotted and weighed.

Preparation of liver microsomes and estimation of microsomal cytochromes and protohaem. Homogenates of the liver (10% w/v) were prepared in 1·15% (w/v) ice-cold KCl using the homogenizer described by Aldridge *et al.*,[2] as modified by Webster and Smith,[3] with a difference in diameter between pestle and tube of 0·02 in. and revolving at 1100 rev/min. The homogenate was centrifuged in a refrigerated angle centrifuge at 9000 *g* for 20 min and the supernatant centrifuged again in a Spinco ultracentrifuge at 78,000 *g* for 60 min. The 78,000 *g* pellet, henceforth referred to as "the microsomes" was then washed with KCl and suspended in 0·1 M phosphate buffer pH 7·4 so that 1 ml contained the microsomes from 0·12 g of liver. *Cytochrome* b_5 was estimated from the difference spectra between the $Na_2S_2O_4$-reduced and the oxidized microsomal suspension, using an extinction coefficient of 163 mM^{-1} cm^{-1} for the difference in absorbancy at 424 and 409 mμ.[4] *Cytochrome P450* was estimated from the difference spectra between the microsomal suspension reduced with $Na_2S_2O_4$ and subsequently treated with carbon monoxide and the $Na_2S_2O_4$-reduced control: an extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in absorbancy at 450 and 490 mμ[5] was used to calculate the concentration of cytochrome P450. When P420 pigment was present together with cytochrome P450 in the same sample, the amount of P420 was calculated from the difference spectra used for the estimation of P450, as suggested by Omura and Sato.[6] All spectra were recorded with a Unicam SP800 spectrophotometer.

Washed microsomes obtained from livers which had been perfused *in situ* with ice-cold saline were used to determine the *total protohaem* content. The difference spectra between the $Na_2S_2O_4$-reduced and the oxidized haem solutions in 0·15 N-NaOH and 25% pyridine were recorded and an extinction coefficient of 19 mM^{-1} cm^{-1} for the difference in absorbancy at 557 and 541 mμ was used to calculate the concentration of haem (this value was obtained experimentally with crystalline haemin chloride). The microsomes were either directly mixed with pyridine/NaOH or their haem extracted with acetone containing 1% (v/v) conc. HCl, the acetone solution taken to dryness and the haem dissolved in pyridine/NaOH. Essentially similar results were obtained by the two methods, but in most experiments the total haem recovered from both

normal and phenobarbitone-treated rats was less than the amount expected from the combined concentrations of cytochrome P-450 and b_5.

Assay of microsomal drug-metabolizing enzymes. Their activity *in vivo* was assessed by comparing the "sleeping time" of some carbon disulphide-treated animals with that of control animals, after intraperitoneal injection of 150 mg hexobarbitone sodium/kg. The "sleeping time" was taken as the time elapsing between the onset of anaesthesia and the regaining of the righting reflex. Liver aniline hydroxylase and nitroanisole demethylase were assayed *in vitro*[7] using the 9000 *g* supernatant of a 10–20% (w/v) liver homogenate as the source of the enzymes.

Incorporation of [^{14}C]*-leucine into liver and blood serum proteins*

Twenty-four hr after carbon disulphide administration rats were injected i.p. with 2 μmoles (20 μc)/kg [U-^{14}C]-L-leucine and killed 30 min later by decapitation. Blood was collected from the severed vessels of the neck, allowed to clot at room temperature and the serum separated by centrifugation. The incorporation of [^{14}C]-leucine into liver proteins was also measured *in vitro* 4 hr and 24 hr after carbon disulphide. Liver slices approximately 0·4 mm thick were cut by hand from the left lobe of the liver, gently blotted and weighed. The slices (200 $\pm$ 5 mg) were incubated at 38° in 5 ml of Krebs–Ringer phosphate solution, pH 7·4[8] containing $CaCl_2$ (1·35 mM) and glucose (55 mM), with O_2 as the gas phase and with shaking (95–100 double oscillation/min). After exactly 10 min, 5 μmoles (0·2 μc) [U-^{14}C]-L-leucine were added to each flask and the reaction stopped after a further 30 min by addition of 1 ml 50% (w/v) trichloroacetic acid. Under these conditions the incorporation of radioactive leucine into liver proteins proceeded at a linear rate for at least 1 hr and the amount of label recovered in the total proteins at the end of the incubation was proportional to the weight of liver slices taken over the range examined (100–250 mg). In one experiment the incorporation of labelled leucine into protein was studied by using the post-mitochondrial supernatant of liver homogenates.[9] The livers were homogenized in 2·5 volumes of an ice-cold medium containing 0·225 M sucrose, 0·13 M KCl, 0·01 M $MgCl_2$ and 0·05 M Tris buffer pH 7·5 and the large cytoplasmic particles removed by centrifugation at 9000 *g* for 15 min. The incubation mixture contained in a total volume of 2·1 ml, 1·4 ml of 9000 *g* supernatant, 2 μmoles ATP, 20 μmoles Na-phosphoenol pyruvate and 40 μg of pyruvate kinase (Type II, Sigma Chemical (London) Co.). The incubation was carried out in test tubes at 38° with shaking (60 double oscillations/min). The reaction was started by the addition of 200 mμmoles (2 μc) of [U-^{14}C]-L-leucine and stopped 10 min later by the addition of trichloroacetic acid to a final concentration of 5%.

The livers from animals injected with labelled leucine and the liver slices from experiments *in vitro* were homogenized in 7% (w/v) ice-cold trichloroacetic acid and the proteins isolated essentially as described by Villa-Treviño *et al.*[10] A similar procedure was followed for the isolation of the labelled serum protein and of the proteins from the incorporation experiment with the post-mitochondrial supernatant. Weighed duplicate samples of the dry protein powder were taken into scintillation vials and dissolved in hyamine; 10 ml of a scintillator fluid [a 1:1 (by vol.) toluene–ethyl cellosolve mixture containing 0·25% of 2,5-diphenyloxazole and 0·015% of 1, 4-*bis*-(4-methyl-5-phenyloxazol-2-yl) benzene] were added to the vials for counting. The radioactivity of the trichloroacetic acid-soluble fraction of the livers from animals

injected with labelled leucine was also measured: duplicate aliquots (0·5 ml) of the 7% trichloroacetic acid supernatant of the liver homogenates were taken into scintillation vials and 10 ml of a scintillator fluid [a 1:3:3 (by vol) toluene–dioxan–ethyl cellosolve mixture containing 1% of 2, 5-diphenyloxazole, 0·05% of 1, 4-*bis*-(4-methyl-5-phenyloxazol-2-yl) benzene and 8% of naphthalene] added to the vials. This latter procedure was also followed to determine the radioactivity of the free leucine isolated from the trichloroacetic acid-soluble fraction of the liver of animals injected with labelled leucine (see later).

All samples were counted in a Packard Tri-Carb liquid-scintillation spectrometer and corrected for quenching by the use of internal standards.

Analytical methods. Proteins were determined by the method of Lowry *et al.*[11] using bovine serum albumin fraction V (Armour Laboratories, London) as the standard. Nucleic acids were extracted into 5% (w/v) trichloroacetic acid according to Schneider;[12] DNA was estimated by the diphenylamine reaction[13] and RNA by the orcinol reaction.[14] Purified yeast RNA (Type XI, Sigma Chemicals Co., London) and calf thymus DNA (Worthington Biochem. Corp., Freehold, New Jersey, U.S.A.) were employed as standards. Total liver lipids were determined according to Folch *et al.*[15] and liver glycogen using the anthrone reagent.[16] The water content of the liver was determined by drying a small sample overnight at 105°.

The amount of free leucine was determined in the acid-soluble fraction of the liver by ion-exchange chromatography. Trichloroacetic acid was removed by treating the acid soluble extract (10 ml) with five 10 ml lots of ether and an aliquot of the extract equivalent to 330–460 mg wet liver taken to dryness under a stream of air, dissolved in 1 ml of 0·1 N-HCl (containing 0·1 μmole *nor*-leucine as the internal standard) and applied on the ion-exchange column of a Technicon aminoacid analyser. Known portions of the effluent were collected for determination of radioactivity.

RESULTS

Liver enlargement and prolonged hexobarbitone "sleeping time". The effect of a single oral dose of carbon disulphide (1 ml/kg) on body and liver weight is shown in Fig. 1. Some rats were fasted for the whole length of the experiment, while others were returned to food 24 hr after CS_2 administration. Poisoned animals lost weight on fasting at a rate which was comparable to that of their controls, but regained weight on re-feeding at a slightly lower rate. Twenty-four hr after carbon disulphide administration treated animals exhibited a significant increase in liver size which persisted on re-feeding for at least 3 days. In another experiment rats were fasted and given CS_2 (0·5 ml/kg/day) for 3 days: this treatment also resulted in liver enlargement and chemical analysis showed that the increased liver size was accompanied by a significant rise in the total hepatic protein and RNA, with no significant change in the total DNA content of the liver (Table 1).

In some fasted rats the hexobarbitone "sleeping time" was determined at different intervals after a single dose of carbon disulphide and compared with that of similarly treated controls (Table 2). Carbon disulphide administration was followed by a prolongation of the "sleeping time" for at least 3 days, suggesting that the livers of the poisoned animals, in spite of their increased size and protein content, could oxidize the barbiturate *in vivo* at a slower rate than the normal livers.

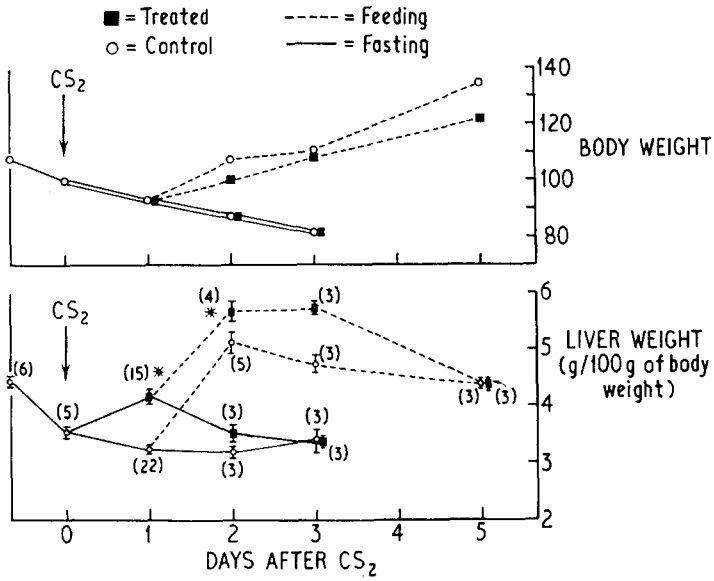

FIG. 1. The effect of a single oral dose (1 ml/kg) of CS_2 or oil (given at the time indicated by an arrow) on the body weight and liver weight of the rat. Some animals were fasted for the whole length of the experiment (continuous line), while others were returned to food 24 hr after CS_2 administration (dotted line): O, control rats; ■, CS_2-treated rats. The body weights are expressed as percentage of the weights observed at the time of dosing and are the means of the observed values. The liver weights are the means ± S.E.M. of the number of observations in parentheses. *These livers contained significantly more protein than their corresponding controls.

TABLE 1. LIVER WEIGHT AND COMPOSITION AFTER TREATMENT WITH CARBON DISULPHIDE

	Liver (g/100 g body wt.)	mg/total liver per 100 g body wt		
		DNA	RNA	PROTEIN
Control	2·75 ± 0·05	6·6 ± 0·5	23·9 ± 1·0	391.7 ± 22·6
Treated	3·57 ± 0·06	7·7 ± 0·5	37·1 ± 1·5	499·3 ± 10·6
P	< 0·01	> 0·2	< 0·001	< 0·001

Rats were given CS_2 or oil (0·5 ml/kg daily) for 3 days and fasted for the whole length of the experiment. Values given are the means ± S.E.M. of six observations.

Activity of microsomal drug-metabolizing enzymes and levels of microsomal cytochromes. Fasted rats were killed at various time intervals after a single oral dose of carbon disulphide and their liver preparations tested *in vitro* for the activity of aniline hydroxylase and nitroanisole demethylase. The liver microsomes were also obtained from both treated and control rats and microsomal cytochromes estimated. In agreement with the results of the *in vivo* "sleeping time" experiment, poisoned animals showed a decreased activity of both enzymes and lower levels of cytochromes P-450 and b_5 at 24 hr, and all values returned to normal only at a slow rate and were still depressed 3 days after carbon disulphide administration (Fig. 2). A difference was

TABLE 2. THE EFFECT OF A SINGLE DOSE OF CARBON DISULPHIDE ON THE HEXOBARBITONE "SLEEPING TIME" OF THE RAT

		Time after CS_2 (days)		
		1	3	6
"Sleeping time" (min)	Control	49·6 ± 3 (5)	33·5 ± 7·2 (5)	33·0 ± 6 (6)
	CS_2	120·6 ± 9 (6)	57·5 ± 6·8 (5)	29·4 ± 2·4 (4)
	P	< 0·001	< 0·05	> 0·2

Rats were fasted overnight before CS_2 administration (1 ml/kg) and returned to food, 24 hr after dosing. The same animals were used for all four "sleeping time" tests. Values given are the means ± S.E.M. of the number of observations in parentheses.

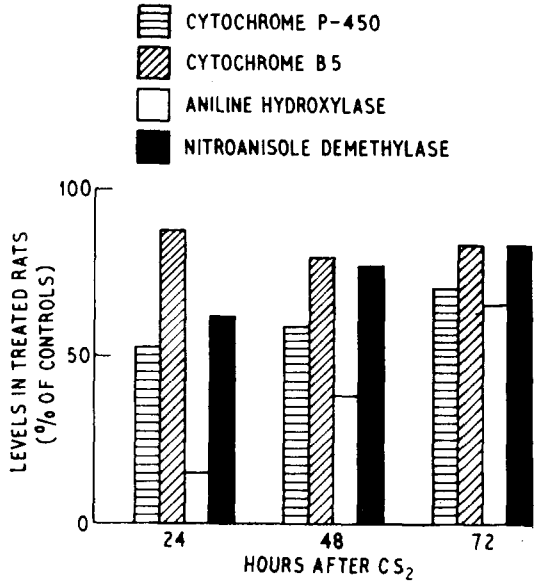

FIG. 2. The effect of a single oral dose (1 ml/kg) of CS_2 on the levels of microsomal cytochromes P-450 and b_5 and on the activity of aniline hydroxylase and nitroanisole demethylase. All values refer to the content of the total liver of 100 g body weight and are the means of at least three observations. The actual values observed at 24 hr are given in Table 4. All animals were fasted overnight before administration of CS_2 and their fasting continued for the whole length of the experiment.

noted in the behaviour of the two enzymes tested: aniline hydroxylase was more markedly affected and recovered at a faster rate than nitroanisole demethylase.

Effect of treatment with phenobarbitone and of feeding sucrose on the toxicity of carbon disulphide. The liver toxicity of carbon disulphide was found to be influenced by treatments which are known to alter the activity of liver drug-metabolizing enzymes. The microsomal enzymes were stimulated by giving phenobarbitone or depressed by feeding sucrose[17] or by injecting SKF-525A before administration of carbon disulphide.

TABLE 3. EFFECT OF FEEDING SUCROSE AND OF TREATMENT WITH PHENOBARBITONE OR SKF-525A ON THE LEVELS OF MICROSOMAL CYTOCHROMES AND PROTEIN 4 hr AFTER CS_2 ADMINISTRATION

Treatment before CS_2	cyt. P-450 (mμmoles/total liver per 100 g)			cyt. b_5 (mμmoles/total liver)		microsomal protein (mg/total liver)	
	Control	CS_2-treated	Loss of P-450 due to CS_2 (%)	Control	CS_2-treated	Control	CS_2-treated
None	72·4 ± 5·6 (4)	37·1 ± 4* (4)	49	42·7 ± 3·6 (4)	42·7 ± 3·4 (4)	51·8 ± 2 (4)	42·9 ± 4 (4)
Sucrose	53·7 ± 4·3 (4)	34·2 ± 1·9* (4)	36	43·0 ± 4·6 (4)	38·8 ± 2·8 (4)	44·2 ± 3 (4)	41·5 ± 1 (4)
Phenobarbitone	211·0 ± 22 (8)	71·8 ± 8* (7)	64	71·8 ± 7·7 (8)	69·6 ± 8·9 (7)	103 ± 10 (7)	91·0 ± 5 (8)
Phenobarbitone† + SKF-525A	154·0 ± 8·2 (6)	97·0 ± 10·7* (6)	37	45·1 ± 3·2 (6)	51·6 ± 3·4 (6)	62·2 ± 3 (6)	56·2 ± 3 (6)

All the rats, with exception of the ones fed sucrose, were fasted for 16–24 hr before being given CS_2 or oil (1 ml/kg). Values given are means ± S.E.M.' with number of observations in parentheses.

* $P < 0.001$, when compared with corresponding control values.

† The animals of this group had been recently acquired from an outside colony.

After feeding sucrose for 2 days the loss of cytochrome P-450 caused by carbon disulphide was less marked (Table 3).

Pre-treatment with phenobarbitone, on the contrary, greatly enhanced the loss in microsomal enzymes and cytochromes caused by carbon disulphide (Tables 3 and 4). In addition, 24 hr after carbon disulphide, the liver appeared very enlarged and pale, had a greatly increased water content and exhibited centrolobular zone necrosis on histological examination. [18] These livers contained significantly more lipids and less glycogen than the livers from control animals; their total protein content was not significantly different from the control values, but the amount of protein recovered in the microsomal pellet was significantly decreased (Tables 4 and 5).

Some phenobarbitone-treated rats were given SKF-525A 45 min before CS_2, in a dose which is known to inhibit drug metabolism in the intact animal.[19] SKF-525A afforded some protection against the loss of P-450 caused by carbon disulphide (Table 3). It also protected against the late appearance of liver damage (Table 5): both liver size and water content were less markedly affected and the histological changes, though qualitatively similar, were much less pronounced.[18]

Further studies on the changes produced by carbon disulphide in microsomes

Further experiments were carried out in rats pre-treated with phenobarbitone in order to gain more information on the mechanism by which carbon disulphide decreases the levels of P-450 and the activity of drug-metabolizing enzymes of the liver microsomes. The fall in the concentration of P-450 after a single dose of carbon disulphide was found to be very rapid: a very marked decline was already apparent after 30 min and most of the loss had taken place by 4 hr (Fig. 3). A similar time-course was observed for the fall in activity of aniline hydroxylase and nitroanisole demethylase (Fig. 4), whereas the decreases in microsomal protein content and in cytochrome b_5 levels, which were less pronounced, took place at a later stage (between 4 and 24 hr) (Table 3 and 4). Similarly, in uninduced animals the fall in P-450 levels was an early event, whereas the drop in b_5 which was only slight, took place later (Tables 3 and 4); no decrease in microsomal protein was observed in these animals.

Cycloheximide was injected into phenobarbitone-treated rats in a dose which inhibits the incorporation of labelled amino acids into proteins *in vivo* by 90 per cent.[20] This treatment only caused a small and not significant drop in the concentration of P-450 over a period of 6 hr (Fig 3). In agreement with previously reported findings[21] a depression of microsomal drug-metabolizing enzymes was also observed after cycloheximide, but the extent of this depression was less marked than observed after CS_2 (Fig. 4). As noted in both uninduced and phenobarbitone-treated rats given CS_2, the loss of enzyme activity caused by cycloheximide was more pronounced for aniline hydroxylase than for nitroanisole demethylase.

The difference spectra obtained after gassing the microsomal suspension with carbon monoxide showed a significant increase in the 420 mμ absorption in the animals killed during the first hours after CS_2 administration. This was most often in the form of a distinct peak with maximum at 420 mμ, the height of which varied appreciably from animal to animal up to about half the height of the 450 mμ peak; in no instance was the 420 mμ peak high enough to account for the loss in P-450 cytochrome. This 420 mμ peak was usually not observed at 24 hr after CS_2 nor was it seen in any of the

TABLE 4. EFFECT OF PRE-TREATMENT WITH PHENOBARBITONE ON THE LEVELS OF MICROSOMAL CYTOCHROMES AND PROTEINS AND ON THE ACTIVITY OF DRUG-METABOLIZING ENZYMES 24 hr AFTER ADMINISTRATION OF CS_2

	Treatment before CS_2					
	None			Phenobarbitone		
	Control	CS_2-treated	% loss due to CS_2	Control	CS_2-treated	% loss due to CS_2
Cytochrome P-450 (mμmoles)	63·1 ± 5 (6)	33·5 ± 0·7† (6)	46·9	368·7 ± 10 (6)	51·8 ± 4† (4)	85·9
Cytochrome b_5 (mμmoles)	33·1 ± 2·5 (6)	29·1 ± 3·2† (6)	12·1	99·0 ± 3·3 (6)	54·4 ± 2·6† (4)	45·1
Microsomal protein (mg)	32·7 ± 0·8 (9)	38·6 ± 1·4 (9)	—	96·1 ± 6·8 (6)	64·5 ± 9* (4)	32·9
Aniline hydroxylase (mμmoles 4-aminophenol produced/hr)	5·3 ± 0·8 (3)	0·78 ± 0·2* (3)	85·3	15·9 ± 1·9 (6)	0·82 ± 0·1† (4)	94·8
Nitroanisole demethylase (mμmoles *o*-nitropenol produced/hr)	6·73 ± 1·1 (3)	4·2 ± 2·7 (3)	37·6	59·4 ± 3·4 (6)	12·7 ± 1·2† (4)	78·6

Animals were fasted for 16–24 hr before CS_2 (1 mg/kg) and killed after further 24 hr of fasting. Values given refer to levels per total liver per 100 g body wt. and are the means ± S.E.M. of the number of observations in parentheses.

*$P < 0.05$; † $P < 0.001$, when compared with corresponding control values.

TABLE 5. EFFECT OF TREATMENT WITH PHENOBARBITONE OR SKF-525A ON THE HEPATIC SIZE, CHEMICAL COMPOSITION AND HISTOLOGICAL PICTURE SEEN 24 hr AFTER ADMINISTRATION OF CARBON DISULPHIDE

	Treatment before CS_2					
	None		Phenobarbitone		Phenobarbitone + SKF-525A	
	Control	CS_2-treated	Control	CS_2-treated	Control	CS_2-treated
Liver wt. (g/100 g body wt.)	3·25 ± 0·06 (22)	4·17 ± 0·09‡ (15)	4·17 ± 0·09 (17)	6·69 ± 0·18‡ (18)	4·42 ± 0·04 (9)	5·09 ± 0·14‡ (11)
Water content (mg/g wet liver)	694 ± 2·4 (6)	711 ± 0·4‡ (5)	701 ± 3·6 (5)	768 ± 2·5‡ (8)	700 ± 2·9 (5)	722 ± 3·8‡ (9)
Total protein (mg/total liver per 100 g)	580 ± 18 (9)	706 ± 18‡ (8)	716·3 ± 26 (6)	811 ± 50 (4)	—	—
Total lipids (mg/total liver per 100 g)	138·7 ± 7 (4)	147·5 ± 8 (4)	197 ± 23 (4)	357 ± 33† (5)	228·3 ± 21 (3)	238·7 ± 19 (4)
Glycogen (mg/total liver per 100 g)	3·7 ± 1·1 (4)	1·24 ± 0·17 (4)	6·06 ± 1·02 (3)	2·76 ± 0·34* (4)	—	—
Degree of histological changes due to CS_2 (see ref. 18)		− − −		+ + +		+ − −

Rats were fasted overnight before CS_2 (1 ml/kg) and killed after further 24 hr of fasting. Values given are the means ± S.E.M. with number of observations in parentheses.

*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$, when compared with corresponding control values.

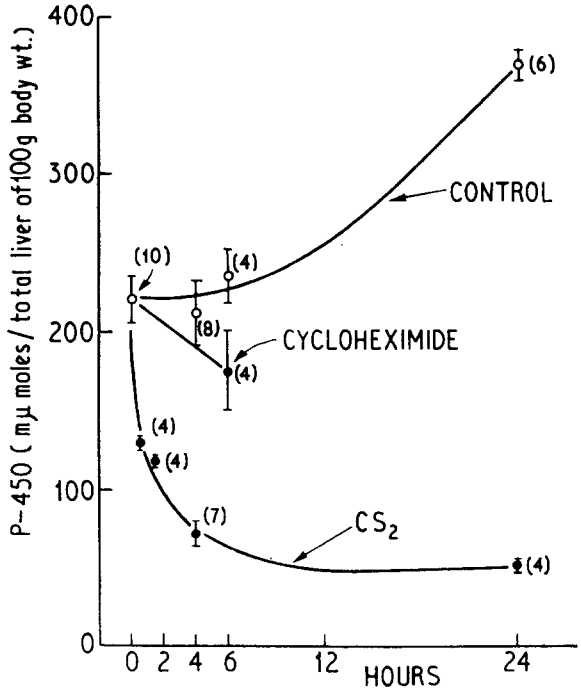

FIG. 3. The effect of a single oral dose of CS_2 (1 ml/kg) or of a single intraperitoneal injection of cycloheximide (40 mg/kg) on the levels of microsomal cytochrome P-450 in phenobarbitone-induced rats. All animals were fasted for 24 hr before CS_2 administration and their fasting continued for the whole length of the experiment. Values given are means ± S.E.M.

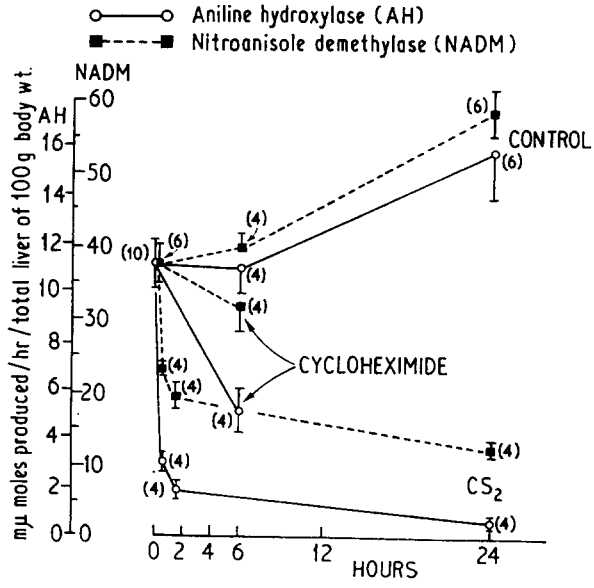

FIG. 4. The effect of a single oral dose of CS_2 or of a single intraperitoneal injection of cycloheximide on the activity of liver aniline hydroxylase (○———○) or nitroanisole demethylase (■– – –■) in phenobarbitone-induced rats. All animals were treated as described in the legend of Fig. 3. Values given are means ± S.E.M.

control animals which did not receive CS_2. Since the spectrum of the carbon monoxide compound of P-420 is almost identical with that of haemoglobin,[6] special care was taken in some experiments to free the microsomal suspensions from blood as much as possible: the livers were perfused *in situ* with ice-cold physiological saline through the portal vein and the microsomes washed twice with isotonic KCl. The height of P-420 peak observed in preparations from perfused livers was not less than observed when microsomes were prepared as usual, without perfusing the liver.

The total protohaem content was also determined in microsomes from treated and control animals both at 4 and 24 hr after CS_2. Four hr after treatment the microsomal haem levels in the treated livers were the same as in the control livers or slightly decreased, while at 24 hr microsomes from treated animals consistently contained less haem than those from control animals. Essentially similar results were obtained whether the rats had been treated with phenobarbitone or not before administration of CS_2.

The effect of CS_2 on microsomal cytochrome was also studied *in vitro*: the 9000 *g* supernatant of the liver homogenate from a phenobarbitone-treated animal was incubated at 5° in the presence of large excess CS_2 for either 15 or 60 min. Control incubations were run without CS_2. At the end of these incubation periods the microsomes were isolated and washed, and their cytochrome content determined. CS_2 caused a progressive decrease in P-450, with losses of 30 per cent at 15 min and of 60 per cent at 60 min (as compared with control incubation values). Most of the amount lost at 1 hr could be accounted for as P-420. There was no change in the levels of cytochrome b_5, nor was any P-450 lost in the control incubation.

Incorporation of [^{14}C] *leucine into liver and blood serum proteins* in vivo *and* in vitro. Fasted, uninduced rats were given an i.p. dose of labelled leucine at a time after CS_2 (24 hr) when their body temperature had come back to normal and their liver size started to increase. CS_2 -treated animals incorporated significantly greater amounts of radioactive leucine into their liver proteins than did their controls, but had no significantly different amounts of label in the trichloroacetic acid-soluble fraction of their livers (Table 6). The specific radioactivity of the serum proteins was also increased in the treated animals, though the increase did not prove statistically significant: the observed values were (means $\pm$ S. E. M.) 300 $\pm$ 42 dpm/mg of protein for the controls and 429 $\pm$ 46 for the treated, with six observations in either group. Carbon disulphide is known to react with amino acids *in vivo*[22] and such a reaction with the body leucine might lead to a depletion of the endogenous pool of the free amino acid. If the liver pool of free leucine were significantly depleted 24 hr after CS_2, when the labelled leucine was administered, the radioactive amino acid would be diluted to a lesser extent in the treated animals and this might explain the increased radioactivity of their liver proteins. The free amino acids present in the trichloroacetic acid-soluble fraction of the liver from animals injected with labelled leucine were separated by ion exchange chromatography using a Technicon amino acid analyser and the amount of free leucine and its specific activity determined (Table 6). There was no significant difference between treated and control animals in either the liver content of the free leucine or in its specific radioactivity at 30 min after i.p. injection of labelled leucine. Only 19 to 40 per cent of the total trichloroacetic acid-soluble radioactivity of the liver could be accounted for as free leucine: radioactivity was found in the effluent of the amino acid analyser column in association with peaks tentatively identified as urea and glutamate.

TABLE 6. *IN VIVO* INCORPORATION OF [^{14}C]-LEUCINE INTO LIVER PROTEIN AND LEVELS AND SPECIFIC RADIOACTIVITY OF HEPATIC FREE LEUCINE 24 hr AFTER ADMINISTRATION OF CS_2

	Total radioactivity recovered (dpm per liver per 100 g body wt.) in:		Free leucine	
	trichloroacetic acid soluble fraction	proteins	μmoles/g wet liver	spec. radioactivity dpm/μmole
Control	61,840 ± 6294 (4)	342,000 ± 37,000 (6)	0·29 ± 0·01 (5)	14,760 ± 2015 (4)
CS_2-treated	60,300 ± 2040 (4)	520,000 ± 17,000* (6)	0·26 ± 0·01 (4)	18,673 ± 2758 (4)

Animals were fasted overnight before administration of CS_2 (1 ml/kg). After further 24 hr fasting, they were injected with [^{14}C]-leucine ($4·4 \times 10^6$ dpm/100 g) and killed 30 min later. Values given are the means ± S.E.M. of the number of observations in parentheses.
*$P < 0·01$, when compared with corresponding control value.

This indicated that under the conditions of these experiments appreciable conversion of leucine had taken place and that after injection of [U-^{14}C]-L-leucine, the total trichloroacetic acid-soluble radioactivity of the liver cannot be taken as a measure of the amount of label present as unchanged leucine.

Liver slices were obtained from control and treated animals 4 and 24 hr after CS_2 and examined for their ability to incorporate [U-^{14}C]-L-leucine into proteins. The effect of administering phenobarbitone and SKF-525A, prior to treatment with CS_2, on the *in vitro* incorporation of radioactive leucine into liver proteins was also studied (Table 7). In uninduced rats the incorporation of the label was depressed at 4 hr and

TABLE 7. THE EFFECT OF PRE-TREATMENT WITH PHENOBARBITONE OR WITH PHENOBARBITONE AND dKF-525A ON THE *IN VITRO* INCORPORATION OF [^{14}C] LEUCINE INTO PROTEIN BY LIVER SLICES FROM CS_2-TREATED ANIMALS AND THEIR CONTROLS

Treatment before CS_2	Time of killing (hr after CS_2)		Radioactivity of liver protein (dpm/mg)	CS_2-treated / Control
None	4 hr	Control	87·2 ± 4·5 (5)	0·48
		CS_2-treated	41·7 ± 5·5 (5)	
	24 hr	Control	86·8 ± 7·1 (6)	1·47
		CS_2-treated	127·5 ± 15·8 (6)	
Phenobarbitone	4 hr	Control	75·0 ± 7·0 (4)	0·62
		CS_2-treated	46·5 ± 6·6 (4)	
	24 hr	Control	45·2 ± 2·3 (5)	4·36
		CS_2-treated	197·2 ± 3·5 (5)	
Phenobarbitone + SKF-525-A	24 hr	Control	55·8 ± 6·0 (5)	1·90
		CS_2-treated	105·8 ± 13·3 (5)	

The animals were fasted 16–24 hr before administration of CS_2 (1 ml/kg) and their fasting continued until they were killed. Values given are the means ± S.E.M. with number of observations in parentheses.

stimulated at 24 hr, the degree of this stimulation being very similar to that observed in the whole animal. When animals were treated with phenobarbitone before being given CS_2, the degree of inhibition observed at 4 hr, was not greater than that observed in the uninduced animals; the stimulation of the incorporation seen 24 hr after CS_2 (when liver necrosis had appeared) was, however, much more pronounced. This further increase in [^{14}C]-leucine incorporation (attributable to pre-treatment with phenobarbitone) could be largely prevented by administering SKF-525A to phenobarbitone-induced animals 45 min before CS_2.

The slices from necrotic livers might be more permeable than normal slices to either the labelled leucine or to oxygen: a greater uptake of the isotope or a better state of oxygenation of the necrotic slices might explain the increased radioactivity of their proteins after incubation with [^{14}C]-leucine. To exclude both these possibilities, the incorporation of [^{14}C]-leucine into proteins was measured in a cell-free system, using post-mitochondrial supernatant from phenobarbitone-induced animals killed 24 hr after administration of CS_2. Preparations from treated animals incorporated leucine into proteins at a greater rate than those from control rats and the degree of the increase was similar to that observed with liver slices (Table 8).

TABLE 8. THE INCORPORATION OF [^{14}C] LEUCINE INTO PROTEIN BY 9000 *g* SUPERNATANTS OF LIVER HOMOGENATES FROM PHENOBARBITONE-INDUCED ANIMALS TREATED WITH CS_2

		Radioactivity of protein (dpm/mg)		CS_2-treated / control
Control	Rat 1	36·6		
	Rat 2	76·6		
	Average		56·6	
CS_2-treated	Rat 1	200·0		3·8
	Rat 2	230·0		
	Average		215·0	

The animals were fasted for 24 hr before administration of CS_2 (1 ml/kg) and their fasting continued for further 24 hr, when they were killed. The composition of the incubation system is given in the Materials and Methods section.

DISCUSSION

Depression of cytochrome P-450 and of drug-metabolizing enzymes. These were the earliest changes to be detected after administration of CS_2 and in certain respects the most prominent in the whole picture: a very marked depression of P-450 and of microsomal enzymes was observed even when there was no histological evidence of liver damage and persisted long after the general conditions of the animals had come back to normal[18] and the early depression of protein synthesis disappeared.

The assay of cytochrome P-450 depends on the appearance of a strong absorption band with a maximum at 450 mμ in presence of carbon monoxide. The reduced absorption observed after treatment with CS_2 could be due to one or more of the following mechanisms. (1) CS_2 might cause a decrease in the actual amount of cytochrome, either by inhibiting its synthesis or through loss of it from the microsomes. (2) CS_2 might prevent carbon monoxide from binding the cytochrome. (3) It might be responsible for an alteration in the cytochrome molecule (either in the haem or in its protein moiety) making it unable to develop the characteristic spectrum, even though it can still react with carbon monoxide.

CS_2 inhibits the incorporation of labelled leucine into liver proteins within a few hours of administration. The turnover rate of P-450 is not yet known, but if it were very fast, a rapid drop in the concentration of the cytochrome could follow an inhibition of its synthesis. The results of the cycloheximide experiment however suggest that inhibition of synthesis alone cannot be responsible for the very fast decline in P-450 levels nor for the rapid decrease in drug-metabolizing enzymes. The decline of P-450 observed under conditions in which protein synthesis has been reported to be inhibited by 90 per cent[20] would be compatible with a half-life of more than 20 hr (assuming that cycloheximide treatment does not interfere with the degradation of the cytochrome), whereas after CS_2 the levels of P-450 were almost halved in only 30 min.

A direct interference by CS_2 with the binding of carbon monoxide to the cytochrome is also unlikely. The *in vitro* experiment in which the microsomes were incubated with a large excess of CS_2 showed only a relatively slow loss of P-450 and most of the cytochrome which had been lost after 1 hr incubation could be accounted for as P-420, another pigment which requires carbon monoxide for the development of its characteristic spectrum. This latter pigment (P-420) is normally not detectable, but it has been shown to appear when microsomes are subjected *in vitro* to certain treatments, such

as incubation with detergents, degradative enzymes[23] and sulphydryl reagents.[24] The increase in absorption at 420 mμ is then accompanied by a proportional loss in the absorption at 450 mμ, suggesting that P-420 pigment arises from the cytochrome P-450, possibly because of a change in the organization of the microsomal membranes or of the cytochrome molecule.

In the present work P-420 was also seen in microsomal preparations obtained from animals treated with CS_2 and killed during the first hours after treatment. In contrast with the result of the *in vitro* experiment, however, *in vivo* P-420 could only account for a very small fraction of the cytochrome P-450 which had been lost. This suggests that if conversion of P-450 to P-420 is the first stage in the alteration of the cytochrome caused by CS_2, something else follows *in vivo* which leads to the disappearance of a characteristic spectrum: this could either be a loss of the cytochrome from the microsomes or a further alteration in its structure. A significant loss in microsomal haem was only seen 24 hr after CS_2 administration, but not at 4 hr, when most of the P-450 loss had already occurred. This makes it unlikely that the early rapid drop in P-450 concentration caused by CS_2 results from a mere leakage of the pigment out of damaged membranes: it appears more likely that CS_2 causes a rapid alteration in the molecular structure of the cytochrome, making it unable to develop its characteristic spectrum with carbon monoxide and that this altered cytochrome (or its haem moiety) finds its way out of the membranes only at a slower rate. The observation on the haem content of the microsomes also suggests that the main action of the poison is not on the haem portion of the cytochrome but on its protein moiety. It may be relevant to note in this connection that CS_2 has been shown to react with the amino group of amino acids (both of free amino acids and of amino acids present in proteins) to form dithiocarbamate.[22, 25] It should also be noted that a depression in the levels of cytochrome P-450 has been described by Smuckler *et al.*[26] 2 hr after a necrogenic dose of CCl_4 or dimethylnitrosamine, two poisons that cause early electron-microscopic alterations in the endoplasmic reticulum: Smuckler's results and some more recent work[27] which confirmed and extended his observations are also compatible with an alteration in the structure of the P-450 molecule after CCl_4.

The decreased activity of nitroanisole demethylase and aniline hydroxylase observed in the present study after CS_2-treatment may reflect—at least in part—the lower levels of P-450, since this cytochrome is thought to be involved in the activity of several oxidative drug-metabolizing systems.[28] The observation, however, that aniline hydroxylase and nitroanisole demethylase were affected to a different degree by CS_2-treatment and that their rates of recovery also differed suggests that some other factor concerning each enzyme individually may also be involved in the inhibition of their activity and that the microsomal lesion caused by CS_2 is probably not confined to the cytochrome P-450. This is also indicated by the depression of the incorporation of radioactive leucine into protein seen 4 hr after CS_2 and by the later fall in the concentration of cytochrome b_5, and—in the case of phenobarbitone-induced animals—of microsomal proteins.

A depression of the *in vivo* metabolism of phenytoin[29] and trichloroethylene[30] has been described in man after administration of tetraethylthiuram disulphide (disulfiram) and a marked prolongation of the hexobarbitone "sleeping time" has been observed in mice after treatment with diethyldithiocarbamate.[31] Both disulfiram and diethyldithiocarbamate give rise to CS_2 within the body[32] and it is possible that the

CS_2 originated in the liver from the metabolism of these compounds is responsible for the depression of drug-metabolism observed after their administration.

Effect of treatment with phenobarbitone and SKF-525A and of feeding sucrose. The liver changes caused by CS_2 were found to be influenced by several treatments which alter the activity of the microsomal drug-metabolizing enzymes. Feeding sucrose, which is known to depress the activity of these enzymes,[17] reduced the loss of P-450 caused by CS_2, while treatment with phenobarbitone which stimulates the drug-metabolizing system, enhanced this loss and led to the appearance of liver necrosis.[18] In addition SKF-525A, a powerful inhibitor of the microsomal drug-metabolizing activity reversed the enhancement of liver toxicity caused by phenobarbitone treatment. All these findings are most easily explained by assuming that CS_2 is not the direct toxic agent responsible for the microsomal changes and for the appearance of necrosis, but needs to be converted by the drug-metabolizing system to a derivative which is the real active agent. Similar results have been obtained for the sensitivity of rats to carbon tetrachloride[27, 33] and a similar interpretation put forward to explain them.

Other interpretations, though much less likely cannot be excluded. For example, it is possible that the induction of microsomal enzymes may be associated with the preferential production of a population of microsomal tubules which is particularly susceptible to CS_2 or that it may be accompanied by some other liver change conferring unusual sensitivity to the microsomes and to the cell. In either case the protective effect of SKF-525A might be explained by a competition of this compound with CS_2 for the microsomal binding sites.

A positive correlation was noted in this work between severity of microsomal changes and degree of liver cell necrosis, but it is not possible to conclude from this that the two phenomena are directly related. A loss of P-450 and of drug-metabolizing activity has also been reported after administration of the necrogenic toxins, carbon tetrachloride and dimethylnitrosamine but more work is necessary to establish whether microsomal damage is of regular occurrence in the early stages of all toxic liver cell necrosis and whether it is an important step in the chain of events leading to the death of the cell.

The effect of carbon disulphide treatment on the incorporation of labelled leucine into liver protein and on liver size. A significant depression of the incorporation of labelled leucine into protein was observed 4 hr after administration of carbon disulphide. In the phenobarbitone induced animals the incorporation of leucine into protein was also depressed at 4 hr, but the extent of this depression was not greater than in the uninduced animals, in spite of a considerably greater drop in P-450 and microsomal enzymes and in spite of the appearance of liver necrosis at a later stage.

Twenty-four hr after administration of CS_2 the incorporation of [U-^{14}C]-L-leucine into protein was greater in the CS_2-treated animals than in their controls. The degree of this later increase in leucine incorporation was related to the severity of liver damage. It was greatest in the livers from phenobarbitone-induced rats which had developed necrosis, less marked in the animals also given SKF-525A, where liver damage was present, but to a much smaller extent, and least in the uninduced amimals which did not present any histological evidence of liver damage. A similar behaviour of the incorporation of labelled amino acids into proteins was observed by Arrhenius and Hultin[9] in liver preparations obtained from animals treated with aminofluorene: an early depression of amino acid incorporation was followed by a stimulation 8–48 hr

after the administration of the carcinogen. Since the stimulation did not appear in adrenalectomized animals, Arrhenius and Hultin concluded that adrenal hormones were involved in its production. The finding in the present work that the stimulation of leucine incorporation seen at 24 hr was markedly affected by the degree of liver damage may indicate, on the other hand, that some local factor is involved, connected in some way with the cellular damage.

Liver enlargement was a regular finding in rats given CS_2. Increased liver size is frequently observed after administration of many drugs and lipid-soluble compounds and its toxicological significance has been subjected to discussion.[34-36] When the increased liver size is accompanied by increased protein content and by stimulation of drug-metabolizing enzymes, it has been suggested[34] that it should be regarded as a physiological response to the presence of foreign compounds, an adaptation of the liver to increased production of microsomal enzymes. This was not the case for the liver enlargement which followed CS_2, when the total liver protein increased, but the microsomal protein did not, and the activity of the drug-metabolizing enzymes decreased even in absence of histological evidence of liver damage. These findings indicate that increased liver size and protein content can be dissociated from stimulation of drug-metabolizing enzymes.

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