Studies on the Antagonism by Chloramphenicol of Carbon Tetrachloride-Induced Damage: Examination of Mitochondrial Protein Synthesis[1]

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Studies on the Antagonism by Chloramphenicol of Carbon Tetra-chloride-Induced Damage: Examination of Mitochondrial Protein Synthesis. BRABEC, M. J., BRADLEY, C., AND BERNSTEIN, I. A. (1976). *Toxicol. Appl. Pharmacol.* **38**, 157–167. The damage induced in the mitochondria of rat liver during intoxication by carbon tetrachloride is prevented by the administration of chloramphenicol early in the intoxication. The possibility that the protective ability of chloramphenicol lies in the compound's ability to inhibit mitochondrial protein synthesis was eliminated by the failure of other inhibitors of mitochondrial protein synthesis, namely Tevenel, a chloramphenicol analog, and oxytetracycline, to prevent mito-chondrial damage. Conversely, the optical isomer of chloramphenicol, which is not an inhibitor of mitochondrial protein synthesis, and does not possess antibiotic activity, does prevent mitochondrial damage during in-toxication by CCl_4. SKF 525A also preserves mitochondrial integrity, albeit not as well as chloramphenicol. Chloramphenicol, early in the course of intoxication, partially prevents the characteristic loss of protein syn-thesis after CCl_4. It was concluded that the protective action of chlor-amphenicol could be due to either an interdiction of a destructive event triggered by CCl_4, or to the preservation of the cellular repair processes being served by protein synthesis in the cytosol.

The hepatotoxic effects of CCl_4 that lead to hepatic necrosis in the rat involve, at least in part, a loss of mitochondrial function (Reynolds *et al.*, 1962). Restoration of mito-chondrial function seems concomitant with animal recovery and involves repair of damaged mitochondrial elements (Brabec *et al.*, 1974). During the study of these repair processes in the CCl_4-damaged rat liver, chloramphenicol was found to be a potent antagonist of the CCl_4-induced mitochondrial lesion, and to ameliorate the hepato-toxicity of CCl_4 in general[2] (Brabec and Bernstein, 1975). Similar observations have since been reported by Huyen and Banaschak (1973).

[2] Brabec, M. J., and Bernstein, I. A., "Recovery of Liver Mitochondria following CCl_4 Exposure," presented to the Biochemistry Section, American Chemical Society, Washington, D.C., September, 1971.

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The etiology of CCl_4-intoxication and the known metabolic effects of chloramphenicol suggest the following possible loci for protection by chloramphenicol. (a) Mitochondrial protein synthesis: Chloramphenicol inhibits mitochondrial protein synthesis, and is reported to bind to the acceptor site of the 50 S ribosomal subunit (Contreras et al., 1974). A novel possibility is that the biosynthetic operations of the mitochondrion in the CCl_4-traumatized hepatocyte are impaired and mitochondrial dysfunction results from the accumulation of protein(s). Chloramphenicol would prevent such an accumulation, and thereby the incipient mitochondrial lesion. Such a mechanism has been offered to explain the protection cycloheximide offers cells of the intestinal crypt after X-irradiation (Lieberman et al., 1970). (b) CCl_4 metabolism: The initial molecular lesion induced by CCl_4 is thought to be the result of the production of an activated metabolite of CCl_4 by microsomal enzymes. The activated metabolite is postulated to bind to an adjacent membrane molecule, disrupting function (Reynolds, 1972), or to initiate a homolytic lipid peroxidation of the unsaturated fatty acids of the phospholipids in the membrane (Recknagel et al., 1974). Chloramphenicol has been reported to be an inhibitor of the N-dealkylation of aminopyrine in vitro (Dixon and Fouts, 1962) and to prolong the sleeping time of hexobarbital-exposed rats (Grogan et al., 1972). Chloramphenicol decreases the formation of $CHCl_3$ from CCl_4 in rats (Huyen and Banaschak, 1973). The theory that toxicity results from the metabolism of CCl_4 would predict a protective action by chloramphenicol due to inhibition of CCl_4 metabolism. The purpose of this study was to elucidate the antagonism of CCl_4-intoxication by chloramphenicol.

METHODS

White laboratory rats, CFN strain, weighing 180–250 g, were used. CCl_4 was injected ip, without carrier, at 1.2 ml/kg, a dose that permitted 80% of the animals to survive the intoxication. The sodium succinyl salt of chloramphenicol and SKF 525A[3] were dissolved in distilled water, 100 mg/kg, and given ip at the indicated dose regimen. Typically, the animals received CCl_4 followed immediately by 100 mg/kg of chloramphenicol, and were sacrificed 40 hr later. D-CAP, L-CAP (the respective optical isomers of threo-chloramphenicol) and Tevenel (a sulfamoyl analog of chloramphenicol) were dissolved, 50 mg/ml, in propylene glycol and administered ip at selected doses as indicated. Oxytetracycline dissolved in propylene glycol, 50 mg/ml, was also injected ip[4].

Mitochondria, prepared from rat livers, were assayed polarographically in an YSI Model 53 Biological Oxygen Monitor as previously described (Brabec et al., 1974). The respiratory control ratio was calculated from the State 3 (+ADP) and State 4 (−ADP) oxygen uptake rates as described by Estabrook (1967). Mitochondrial protein was determined by the Biuret method (Jacobs et al., 1956).

Incorporation of amino acids was determined in animals injected ip with 50 μCi per 200 g body weight [3,4-^{3}H]leucine (sp act 60 Ci/mmol) either 4.5 or 25.5 hr after CCl_4 administration (30 min prior to sacrifice). The mitochondria were prepared, and acid-insoluble radioactivity was determined by a modification of the method of Mans and Novelli (1961). The mitochondrial suspension was diluted with 0.05 M L-leucine to a concentration of 10 mg/ml and sonicated 1 min at 8 A on a Bronson Sonifier, Model LS-75. Approximately 0.8 mg of mitochondrial protein was streaked on 1- $\times$ 6-cm strips of Whatman $\#$1 filter paper and immediately submerged in ice-cold 10% TCA–0.025 M L-leucine for 30 min. The strips were then successively transfered at 15-min intervals through the following solutions at the indicated temperatures (C°): 10% TCA, 0°; 10% TCA, 90°; 5% TCA, 0°; ethanol–ether, 20°. The strips were then dried in an oven, placed in 8 ml of a scintillation cocktail (Packard Permablend I), and counted in a Packard Tri-Carb liquid scintillation counter. Net counts per minute were recorded. Controls, which had received 100 mg/kg of D-CAPS 4.5 or 26.5 hr prior to [3,4-^{3}H] leucine, were not significantly different from nonintoxicated controls in incorporation rates.

Incorporation of amino acids into acid-insoluble protein by isolated mitochondria was determined by the procedure of Simpson *et al.* (1967), as described previously (Brabec *et al.*, 1974). Mitochondria were prepared aseptically from animals which had been pretreated with D-CAPS or oxytetracycline at selected doses and times. After incubation with [U-^{14}C]leucine (sp act 326Ci/mol), the mitochondrial suspension was washed free of soluble [^{14}C]leucine and the acid-insoluble radioactivity was determined as described above. Bacterial contamination, monitored by streaking an aliquot of the incubation mixture on nutrient agar plates and incubating for 24 hr at 37°C was always less than 500 colonies/ml of incubation mixture.

Statistical analysis. The ability of a selected drug to prevent mitochondrial damage suffered during exposure to CCl_4 was evaluated by use of the Student's *t* test (Schefler, 1969).

RESULTS

The respiratory control ratio (RCR) was monitored as an indicator of mitochondrial integrity. The appearance of mitochondrial damage was marked by a decline of the RCR, due to an increase in the State 4 (−ADP) rate, and a decrease in the State 3 (+ADP) rate, as is shown in Fig. 1. Since maximum damage was noted at 40 hr after administration of CCl_4, and recovery in the RCR values began at 40–50 hr, mitochondria for these studies were prepared from rats after 36–40 hr of intoxication. The RCR values for animals similarly treated with CCl_4, but having received 100 mg/kg of D-CAPS at 0 time, are also displayed in Fig. 1. Only a slight decline in the RCR was noted. Mortality of the experimental animals provides another estimation of protection. During these experiments, 72% of the intoxicated animals (53 of 73 experimental animals) and 98% of the protected animals (59 of 60) survived at least 40 hr.

Protection by D-CAPS as a function of dosage is shown in Fig. 2. Below 50 mg/kg protection declined rapidly, with slight protection offered by 25 mg/kg D-CAPS. Since mitochondrial dysfunction is a late development during CCl_4 intoxication (RCR values are normal for the first 10 hr after CCl_4 exposure), the ability of D-CAPS to

protect as a function of time after the administration of CCl$_4$ is critical to the consideration of possible protective mechanisms. As seen in Fig. 3, for any protection, D-CAPS had to be delivered to the intoxicated rat within 7 hr after the animal had received the CCl$_4$.

A system was devised to determine whether the inhibition of mitochondrial protein synthesis was a possible mechanism for protection. If inhibition of mitochondrial protein synthesis were the mechanism by which D-CAP prevents mitochondrial damage
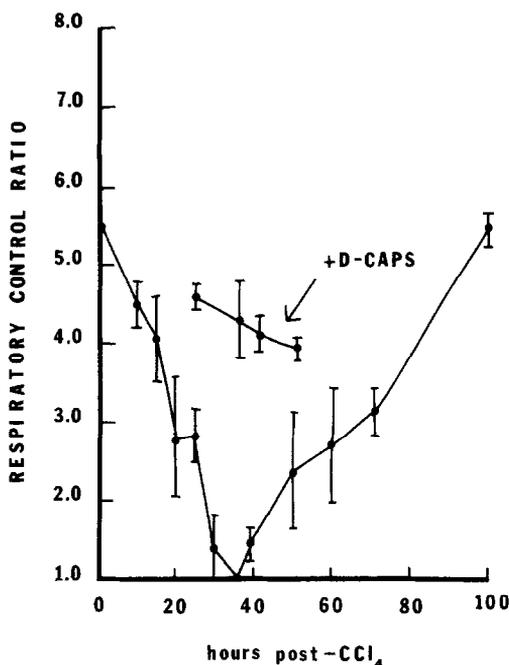

FIG. 1. The course of the hepatic mitochondrial lesion during CCl$_4$ intoxication, in the presence (+D-CAPS) and absence of 100 mg/kg D-CAPS at 0 time. The parameter of mitochondrial integrity displayed is the ratio of the ADP-stimulated to ADP-absent oxidation rate (RCR, respiratory control ratio). Each point represents the mean ± SE of at least seven animals.

during CCl$_4$ intoxication, the model would predict that other inhibitors of mitochondrial protein synthesis would be effective protective agents. The effects of the sulfamoyl derivative of chloramphenicol, Tevenel, which is an antibiotic and an inhibitor of mitochondrial protein synthesis; the optical isomer of chloramphenicol, L-CAP, which is inactive as an antibiotic or as an inhibitor of mitochondrial protein synthesis (Fettes et al., 1972); and oxytetracycline, which is an inhibitor of mitochondrial protein synthesis (DeVries and Kroon, 1970); are compared with the predictions of the model in Table 1. Neither oxytetracycline nor Tevenel would significantly prevent the decline of RCR caused by CCl$_4$. L-CAP, on the other hand, did protect. It is also interesting to note that the effects of L-CAP and D-CAP were not significantly different from each other, but were inferior to the water-soluble monosuccinate derivative of chloramphenicol, D-CAPS.
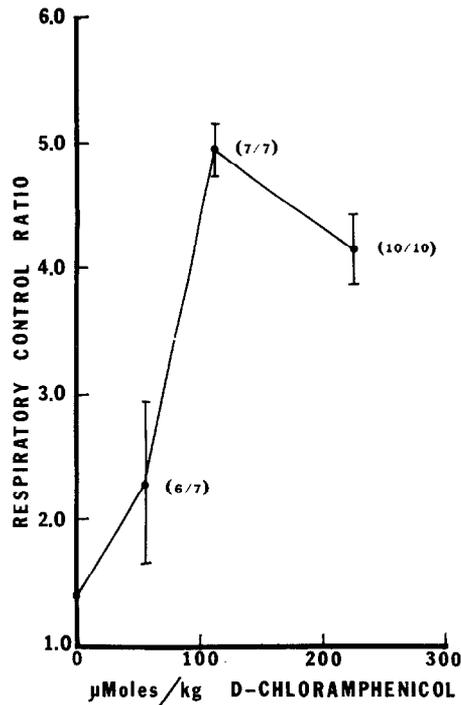
FIG. 2. The effectiveness of D-CAPS as a mitochondrial protective agent as a function of dose. Mitochondria were isolated from animals 40 hr after CCl_4 injection. D-CAPS was injected simultaneously with CCl_4 at 0 time. The numbers in parentheses represent the survival/treated at each time point; each point represents the mean ± SE. Control RCR = 5.1.

TABLE 1

THE MITOCHONDRIAL PROTECTIVE CAPABILITIES OF MITOCHONDRIAL AND MICROSOMAL INHIBITORS DURING CCl_4 INTOXICATION[a]

Treatment	RCR ± SD
Control	5.10 ± 0.84 (11)
CCl_4 (1.2 ml/kg)	1.34 ± 0.46 (20)
+D-chloramphenicol, succinyl salt (100 mg/kg)	4.11 ± 0.66 (10)
+D-chloramphenicol (100 mg/kg)	3.27 ± 0.47 (7)[c]
+L-chloramphenicol (100 mg/kg)	2.60 ± 1.15 (7)[c]
+Tevenel (50 mg/kg)	1.50 ± 0.83 (5)[b]
+Tevenel (100 mg/kg)	1.49 ± 0.22 (7)[b]
+Oxytetracycline (50 mg/kg)	2.01 ± 1.14 (5)[b]
+SKF 525A (0 time, 100 mg/kg)	2.58 ± 1.03 (4)
+SKF 525A (30 min prior to CCl_4, 40 mg/kg)	2.91 ± 1.34 (7)

[a] CCl_4-intoxicated rats were injected with the selected drug as described in Methods. Liver mitochondria were prepared after 40 hr of intoxication and the respiratory control ratio (RCR) was determined. Numbers in parentheses indicate numbers of animals used for each determination. D-CAPS, D-CAP, L-CAP, and SKF 525A offered protection, drug-treated vs CCl_4 ($p < 0.001$).

[b] Not significant, drug-treated vs CCl_4 ($p < 0.1$).

[c] Not significant, L-chloramphenicol vs D-chloramphenicol ($p < 0.1$).

To further evaluate the inhibition of mitochondrial protein synthesis as a protective mechanism, the relative effectiveness of D-CAPS and oxytetracycline as inhibitors of mitochondrial protein synthesis *in vitro* was determined. Mitochondria were isolated and assayed for amino acid incorporation rates at selected periods following injection of either D-CAPS or oxytetracycline. As seen in Table 2, oxytetracycline was a more potent and persistent inhibitor of mitochondrial protein synthesis than was D-CAPS
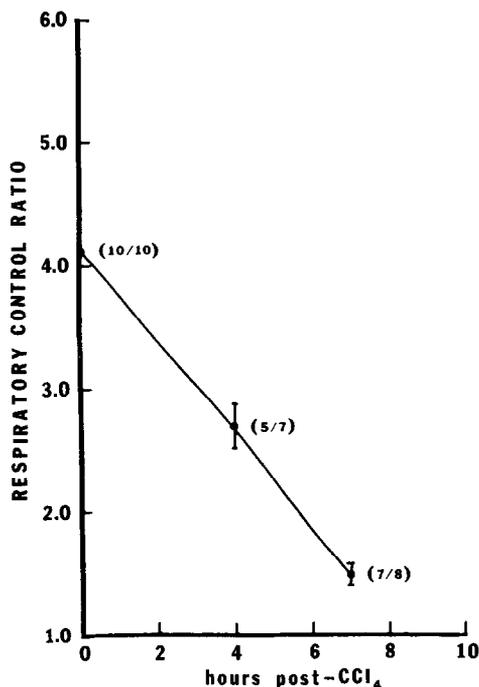

FIG. 3. The effectiveness of D-CAPS as a mitochondrial protective agent as a function of time of delivery after CCl_4. Methods as in Fig. 2. Mitochondria were isolated and assayed 40 hr after CCl_4. D-CAPS (100 mg/kg) was given at the indicated times after CCl_4. Each point represents the mean ± SE.

although neither antibiotic achieved complete inhibition for a prolonged period of time.

The failure of oxytetracycline and Tevenel, and the success of L-CAP in preserving mitochondrial activity during CCl_4 intoxication, suggest that the protective abilities of chloramphenicol are being expressed at some point in the cell other than the mitochondrial ribosome. The reports of the inhibition of the microsomal drug metabolizing system by chloramphenicol (Dixon and Fouts, 1962) and the involvement of the microsomal enzymes in intoxication by CCl_4 (Recknagel et al., 1974) point to the endoplasmic reticulum as a possible site of the protective action of the antibiotic. To examine this possibility, the protective abilities of chloramphenicol were compared with those of SKF 525A, a known antagonist of CCl_4 intoxication (Cignoli and Castro, 1971; Reynolds and Moslen, 1974). SKF 525A is a potent inhibitor of microsomal enzyme activity, a mechanism offered to explain its antagonism to intoxication from CCl_4.

TABLE 2

In Vitro Amino Acid Incorporation by Isolated Mitochondria Following In Vivo Exposure to d-CAPS or Oxytetracycline[a]

Treatment	Exposure period	[U-^{14}C]leucine incorporation	
		cpm/mg/hr	% Control
D-chloramphenicol, succinyl salt			
25 mg/kg	30 min	1288	124
—		1040	
100 mg/kg	30 min	978	74
—		1316	
100 mg/kg	60 min	87[b]	51
—		184[b]	
300 mg/kg	60 min	293[b]	61
—		480[b]	
Oxytetracycline			
50 mg/kg	30 min	432	42
Propylene glycol		1040	
100 mg/kg	30 min	419	51
Propylene glycol		820	
100 mg/kg	8 hr	1210	89
Propylene glycol		1360	

[a] Rats were injected with the indicated inhibitor or, in the case of oxytetracycline controls, an equal volume of propylene glycol. Hepatic mitochondria were aseptically prepared at selected times later, and incubated with [U-^{14}C]leucine. The incorporation of radioactivity into acid-insoluble protein was measured.

[b] These experiments contained 0.5 μCi/reaction; all others contained 1 μCi/reaction.

As may be seen in Table 1, SKF 525A provided a low level of protection for mitochondria during CCl$_4$ intoxication. However, if delivery of SKF 525A occurred 30 min prior to the administration of CCl$_4$, protection was much more obvious.

The similarity in the antagonism to CCl$_4$ intoxication provided by SKF-525A and d-CAPS suggests that d-CAPS may be exerting its action on the microsomal enzymes responsible for activation of CCl$_4$. A characteristic membrane lesion of CCl$_4$ intoxication is the degranulation of the rough endoplasmic reticulum, and the concomitant inhibition of protein synthesis (Smuckler et al., 1962; Reynolds and Ree, 1971). The inhibition is transient, however, and normal rates of synthesis are achieved 20 hr after exposure to CCl$_4$. The effect of d-CAPS on this aspect of CCl$_4$ intoxication was examined by monitoring the incorporation of [3,4-^{3}H]leucine into mitochondrial protein in vivo. As seen in Table 3, CCl$_4$ results in 55% inhibition of the incorporation of [3,4-^{3}H]leucine into mitochondrial protein 5 hr after administration of CCl$_4$. d-CAPS ameliorates this inhibition, allowing only a 30% depression. However, when the incorporation of amino acid was examined at a later period (26 hr), animals protected by d-CAPS were incorporating significantly greater amounts of [3,4-^{3}H]leucine than were control or CCl$_4$-intoxicated animals.

TABLE 3

The Effect of D-CAPS on the Inhibition of Protein Synthesis During CCl_4-Intoxication[a]

Animal group	Specific activity of mitochondrial protein			
	Time post-CCl_4			
	5 hr		26 hr	
	cpm/mg ± SE	Control (%)	cpm/mg ± SE	Control (%)
Control (2)	264 ± 11	100	108 ± 4	100
CCl_4 (3)	118 ± 8	45[b]	181 ± 41	167
CCl_4 + D-CAPS (3)	181 ± 17	69[c]	252 ± 17	223[b]

[a] Chloramphenicol was injected, 100 mg/kg, to rat at the same time as CCl_4. Thirty minutes prior to sacrifice, the animals were injected ip with [3,4-^{3}H]leucine. The mitochondria were prepared and the [3,4-^{3}H]leucine incorporation into acid-insoluble protein was determined. The specific activity is expressed as counts per minute (cpm) per mg protein ± SE. The 5-hr experiment used 100 μCi/200 g of [3,4-^{3}H]leucine per animal; the 26-hr used 200 μCi/200 g.
[b] $p < 0.01$, Student's t test, test vs control.
[c] $p < 0.05$, Student's t test, test vs control.

DISCUSSION

Chloramphenicol is a potent antagonist of the CCl_4-induced mitochondrial lesion, assuring nearly normal mitochondrial activity during a period of serious disruptive events in the cell. A number of studies relating to antagonism of intoxication by CCl_4 have been published (Cignoli and Castro, 1971; Dianzani and Ugazio, 1973; Reynolds and Moslen, 1974; Slater and Sawyer, 1969), including observations that low doses of CCl_4, prior to a toxic dose, act in a protective manner (Dambrauskas and Cornish, 1970; Glende, 1972). The efficacy of the protective action has been evaluated by morphological, histochemical, and biochemical criteria relating to early events of toxicity, or by increase in the LD50 of CCl_4. Monitoring mitochondrial integrity has not heretofore been a parameter used in evaluating hepatic protective agents, perhaps because the mitochondrial lesion appears late during CCl_4 intoxication and therefore is temporally, and spatially, removed from the initial events of intoxication. However, mitochondrial integrity is a convenient and definitive criterion of protection. Mitochondria begin to show loss of function 12–15 hr after the administration of CCl_4. The dysfunction intensifies, then disappears coincident with recovery of liver function (Reynolds et al., 1962). Therefore, the prevention of mitochondrial damage is relevant to survival of the hepatocyte.

D-CAPS has at least two major sites of interaction in the cell (the mitochondria and the endoplasmic reticulum) and both must be considered in a study of its protective action. The failure of Tevenel and oxytetracycline (inhibitors of mitochondrial protein synthesis), and the ability of L-CAP (inactive as an inhibitor of mitochondrial protein synthesis) to prevent the mitochondrial lesion (Table 1), rule against a mechanism of protection (or damage) involving mitochondrial protein synthesis.

On the other hand, involvement of the endoplasmic reticulum is indicated. Figure 2 shows that the protection declines with time after injection of CCl_4, and is absent by 7 hr, a period that corresponds quite well with the retention of CCl_4 in the tissue (Reynolds and Ree, 1971). D-CAPS is reported to increase the hexobarbital-sleeping time of rats and to inhibit the N-dealkylation of aminopyrine (Dixon and Fouts, 1962). However, comparison of D-CAPS with SKF 525A, a potent and persistent inhibitor of microsomal drug-metabolizing enzymes, indicates that D-CAPS is a superior protective agent. SKF 525A has been reported by several authors to be one of the most effective inhibitors of early microsomal changes during CCl_4 intoxication (Cignoli and Castro, 1971; Reynolds and Moslen, 1974). As shown in Table 1, delivery of CCl_4 must be delayed 30 min past the time when SKF 525A is administered. Since in our studies CCl_4 is delivered ip, the inhibition of the absorption of CCl_4 from the intestine by SKF 525A, as suggested by Marchand et al. (1971), would not be a likely mechanism for protection.

Studies of the antagonism of CCl_4-induced alterations of the endoplasmic reticulum present a complicated picture. The relationship between biochemical and physiological disturbances in these membranes (e.g., the loss of glucose-6-phosphatase and cyto-chrome P-450, conjugated dienes in microsomal lipids) and the subsequent cellular pathologies (lipid infiltration, mitochondrial damage, calcium influx) is not clear. If CCl_4 is metabolized by a cytochrome P-450-requiring enzyme, D-CAPS may inhibit that enzyme system. Cignoli and Castro (1971) could correlate inhibitors of drug meta-bolism with antagonism to CCl_4 toxicity, but noted that exceptions existed. Similarly, destruction of cytochrome P-450 by pretreatment with small doses of CCl_4 markedly increased the LD50 of a subsequent toxic dose. Interestingly, the only microsomal parameter significantly altered in the pretreated animals was a decline in conjugated dienes (Recknagel et al., 1974). Despite the simplicity of the postulate that D-CAPS interrupts CCl_4 metabolism, other explanations can be considered to accommodate existing data. In this regard, the conclusion that D-CAPS prevents mitochondrial damage could be erroneous. Table 3 shows that D-CAPS ameliorates, but does not abol-ish, the CCl_4-induced loss of protein synthesis. Over 90% of mitochondrial proteins arise on cytoplasmic ribosomes (Beattie, 1971) and even a transitory interruption of protein synthesis could jeopardize the mitochondrion. On the other hand, the rates at which amino acids are incorporated in the protected animals exceeded those of controls at 26 hr (Table 3), perhaps reflecting increased repair activity. In this interpretation, the preservation of protein synthesis by D-CAPS does not prevent the mitochondrial lesions, but assures that the cell can repair the incipient damage at a rate equal to its production. This would assume that the processes that lead to mitochondrial damage during CCl_4 intoxication are not the same as those that lead to disruption of protein synthesis, which would be sensitive to interdiction by D-CAPS.

Lastly, observations from longstanding clinical use of D-CAPS predict that few side effects of the drug would be expected at the single, moderate dose (50 mg/kg) that was an effective antagonist to CCl_4 in this study, indicating a real potential for thera-peutic application in cases of acute intoxication from CCl_4.

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