Mitochondrial Fatty Acid Oxidation and Susceptibility to Endotoxin in Acute Liver Injury

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ABSTRACT

FARRAR, W. EDMUND, JR. (Walter Reed Army Institute of Research, Washington, D.C.), LAURENCE M. CORWIN, AND THOMAS H. KENT. Mitochondrial fatty acid oxidation and susceptibility to endotoxin in acute liver injury. *J. Bacteriol.* **90**:1365-1372. 1965.—Acute liver injury was produced in guinea pigs with three chemically unrelated hepatotoxins: CCl₄, allyl alcohol, and DL-ethionine. The effects of these agents on liver morphology, susceptibility of animals to *Escherichia coli* endotoxin, endotoxin-inactivating ability of tissue homogenates, and substrate oxidation by liver mitochondria were studied. CCl₄ markedly reduced oxidation of all substrates studied except succinate, impaired the ability of liver homogenates to detoxify endotoxin in vitro, and increased the susceptibility of animals to the lethal effect of endotoxin by 150-fold. Allyl alcohol produced a severe morphological lesion in the liver but did not impair fatty acid oxidation by mitochondria, diminish endotoxin detoxification by liver homogenates, or greatly enhance susceptibility of the animals to endotoxin. Ethionine showed an effect intermediate between the other two agents. These findings are consistent with the hypothesis that the liver performs an important function in the detoxification of endotoxin by the oxidation of fatty acid residues in the endotoxin molecule.

It is widely believed that the endotoxins of gram-negative bacteria are responsible for at least some of the clinical manifestations of diseases caused by these microorganisms in man and animals (Spink, 1954, 1960; McKay, Jewett, and Reid, 1959; Greisman et al., 1961). Despite intensive investigation over the past several decades, the physiological mechanisms by which endotoxin is rendered nontoxic in the mammalian organism have not been clearly defined. The reticuloendothelial system (RES) appears to play a major role in removal of endotoxin from the circulation (Braude, Cary, and Zalesky, 1955; Cremer and Watson, 1957; Carey, Braude, and Zalesky, 1958), and it is this system which has attracted most attention.

Several groups of investigators, using several different animal species, have demonstrated the ability of serum and plasma and of homogenates of various tissues to detoxify bacterial endotoxins in vitro (Landy et al., 1957; Keene, Landy, and Shear, 1961; Trapani et al., 1962; Keene, 1962; Smith et al., 1963; Farrar, 1965). Such activity has been found in organs containing predominantly parenchymal cells, such as kidney, as well

as in those containing a large complement of reticuloendothelial cells, such as liver and spleen. That this activity, at least in the liver, may be of physiological significance in the response of animals to endotoxin is suggested by the finding that production of acute liver injury with carbon tetrachloride (CCl₄) renders guinea pigs abnormally susceptible to the lethal effect of endotoxin (Formal, Noyes, and Schneider, 1960; Farrar and Magnani, 1964). Liver tissue removed from animals poisoned with CCl₄ was only about 0.4% as active as normal liver tissue in its ability to enzymatically detoxify endotoxin in vitro. Healing of the morphological lesion in the liver was associated with an increase in endotoxin-inactivating capacity of liver tissue and a return of normal resistance to endotoxin challenge in the animals.

The process by which liver homogenates inactivate endotoxin in vitro appears to involve activation and subsequent oxidation of the fatty acid portion of the endotoxin molecule (Corwin and Farrar, 1964). This observation provides evidence that the lipid component of the lipopolysaccharide may be responsible, or at least necessary, for its toxicity. If hepatic enzymes which oxidize fatty acid substrates are involved in the detoxification of endotoxin in vivo, it should be

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possible to demonstrate, in various types of experimental liver injury, a correlation between hepatic enzyme activity in vitro and susceptibility of the animals to the lethal effect of endotoxin. To explore this relationship, acute nonfatal liver injury was produced in guinea pigs by three chemically unrelated hepatotoxins: CCl_4 , allyl alcohol, and DL-ethionine. The effect of each of these agents on hepatic oxidation of fatty acids, in vitro inactivation of endotoxin by tissue homogenates, and susceptibility of the animals to endotoxin injection was determined.

MATERIALS AND METHODS

Female Hartley strain guinea pigs weighing 300 to 400 g were used. Endotoxins of *Serratia marcescens* and *Escherichia coli* O26:B6 were obtained from Difco.

The hepatotoxins CCl_4 , allyl alcohol, and DL-ethionine were administered in the highest dosage producing less than 5% mortality. For CCl_4 , this dose was 0.15 ml subcutaneously; for allyl alcohol, 0.042 ml/kg intragastrically; and for DL-ethionine, 100 mg intraperitoneally. Animals used in experiments with ethionine were fasted for 12 hr before administration of the drug and for the next 72 hr thereafter.

Morphological effects of the hepatotoxic agents were assessed by killing animals at various times from 6 hr to 7 days after their administration. Tissues from all organs except brain were fixed in 10% neutral formalin and stained with hematoxylin and eosin. Determination of substrate oxidation by liver mitochondria was carried out in a Warburg respirometer. Liver was homogenized in 0.25 M deionized sucrose to make a 10% suspension. This was centrifuged at $600 \times g$ for 10 min to remove nuclei and cell debris. The supernatant was then centrifuged at $10,000 \times g$ for 10 min. The mitochondrial precipitate was washed twice with sucrose solution and taken up in sucrose solution to make a suspension equivalent to 20 g of liver per 100 ml.

Endotoxin-detoxifying activity of tissues was determined as described previously (Farrar, 1965), by use of the chick embryo assay (Farrar and Magnani, 1964).

Susceptibility to endotoxin was determined from the proportion of animals dead 24 hr after endotoxin challenge. The method of Reed and

Muench (1938) was used to determine LD_{50} values for intravenously administered endotoxin.

RESULTS

Morphological effects of CCl_4 , allyl alcohol, and DL-ethionine. The histological changes seen after CCl_4 administration did not differ significantly from those described in a previous report (Farrar and Magnani, 1964). There was progressive central lobular necrosis and fatty metamorphosis which reached a peak at 48 hr (Fig. 2). The necrotic areas rapidly resolved and were largely replaced by regenerated liver by 96 hr. Slight fatty metamorphosis was the only residuum at 7 days. Variable necrosis of the zona reticularis of the adrenal gland was also seen and was maximal at 48 hr.

In allyl alcohol-treated animals, the liver exhibited necrosis and hemorrhage of the peripheral part of the lobule as early as 6 hr, and this increased progressively through 48 hr (Fig. 3). There was little fatty metamorphosis and the central portion of the lobule appeared well preserved. Increased mitoses were evident by 24 hr, but regenerative activity reached a peak at 72 hr. At this time, there was extensive bile duct proliferation, and many mitoses were present in bile duct epithelium and parenchyma. Healing was progressive, and by 7 days only small areas of residual necrosis and mild prominence of bile ducts were apparent.

The changes produced in the liver by DL-ethionine were similar to those described by Koch-Weser, Farber, and Popper (1951) in the rat. These changes were less consistent than those seen after administration of CCl_4 or allyl alcohol. There was variable fatty metamorphosis which generally did not have a zonal distribution. This reaction was maximal at 48 hr (Fig. 4). Focal areas of necrosis were also present in some animals. Some fatty metamorphosis persisted to 7 days. Focal degeneration and necrosis were seen in the pancreas.

Susceptibility of animals with liver damage to lethal effect of endotoxin. Previous studies with this *E. coli* endotoxin revealed that guinea pigs

FIG. 1. Normal guinea pig liver. Hematoxylin and eosin stain. $\times 180$.

FIG. 2. Guinea pig liver 48 hr after CCl_4 , 0.15 ml subcutaneously. There is a zone of necrosis connecting two central areas at the bottom. Fatty metamorphosis is present adjacent to the necrotic areas. A portal area is at the top. Hematoxylin and eosin stain. $\times 180$.

FIG. 3. Guinea pig liver 48 hr after allyl alcohol, 0.042 ml/kg intragastrically. Large areas of necrosis and hemorrhage surround portal areas. The parenchyma about the central vein (lower left) appears unaltered. Hematoxylin and eosin stain. $\times 75$.

FIG. 4. Guinea pig liver 48 hr after ethionine, 100 mg intraperitoneally. Diffuse fatty metamorphosis is present throughout the lobule. A central vein is at the bottom and portal area at the top. Hematoxylin and eosin stain. $\times 180$.

Figs. 1-4

challenged when the necrotic lesion produced in the liver by CCl_4 was at its maximum were approximately 150 times more susceptible to the lethal effect of endotoxin than were normal controls (Farrar, 1965). Similar results were obtained with the endotoxins of *Shigella flexneri* and *Serratia marcescens* (Farrar and Magnani, 1964).

The effect of allyl alcohol on susceptibility to endotoxin is shown in Table 1. The LD_{50} for endotoxin in animals showing a maximal degree of liver necrosis (at 48 hr) was decreased only threefold in comparison with normal controls. This is in marked contrast to the effect of CCl_4 on response to endotoxin, even though the areas of necrosis produced in the liver by allyl alcohol appeared to be as extensive as those which followed administration of CCl_4 .

Susceptibility of animals given ethionine

TABLE 1. Susceptibility of guinea pigs to lethal effect of *Escherichia coli* endotoxin after administration of allyl alcohol

Endotoxin dosage mg/kg	Normal animals	Interval between allyl alcohol administration* and intravenous endotoxin challenge				
		6 hr	12 hr	24 hr	48 hr	144 hr
15	12/13†	6/6	6/6	6/6	5/6	4/4
5	3/13	2/6	3/6	7/10	5/10	4/4
1.67	2/14	0/6	2/6	3/10	4/10	0/4
0.56			0/6	2/10	2/10	0/4
0.19				1/10	2/12	
0.063					1/12	
LD_{50} (mg/kg)	6.9	6.7	3.6	2.3	2.2	2.9

* Dosage of 0.042 ml/kg by gastric tube.

† Deaths/total.

appeared to be correlated with the extent of the hepatic lesion (Table 2). This agent was intermediate between CCl_4 and allyl alcohol in its effect on susceptibility to endotoxin, lowering the LD_{50} of endotoxin approximately 12-fold (in comparison with fasted control animals) at the time when hepatic fatty metamorphosis was maximal. It is evident from the data given in Table 2 that the degree of starvation imposed in these experiments did not greatly affect response to endotoxin. The nearly twofold difference in LD_{50} for normal control animals in Table 1 as compared with Table 2 is within the range of variation observed in these experiments.

Effects of hepatotoxic agents on endotoxin-detoxifying activity of guinea pig tissues. Guinea pig liver, spleen, and kidney contain enzymes capable of detoxifying endotoxin in vitro (Farrar, 1965). Previous studies have shown that administration of CCl_4 results in a profound reduction in this activity by liver tissue (Farrar and Magnani, 1964), but not by spleen (Farrar, 1965).

Table 3 compares the endotoxin-detoxifying activity of these three tissues in normal animals and in animals given allyl alcohol. It is evident that allyl alcohol, although producing severe morphological damage to the liver, did not depress the endotoxin-detoxifying ability of liver tissue in vitro. Kidney tissue from allyl alcohol-treated animals showed diminished activity when 1% homogenate was used but not when the concentration was 10%.

A similar comparison between the tissues of normal animals and animals given ethionine is shown in Table 4. Administration of ethionine did not significantly reduce the endotoxin-detoxifying activity of any of the three tissues studied.

Effects of hepatotoxic agents on substrate oxida-

TABLE 2. Susceptibility of guinea pigs to lethal effect of *Escherichia coli* endotoxin after administration of DL-ethionine

Endotoxin dosage mg/kg	Normal animals	Fasted animals*	Interval between ethionine administration† and intravenous endotoxin challenge				
			6 hr	12 hr	24 hr	48 hr	144 hr
15	6/10	9/10	8/8	6/8	10/10	10/10	6/6
5	2/10	1/10	2/8	4/8	6/10	12/14	4/6
1.67	0/10	0/10	0/8	0/8	1/10	8/14	0/6
0.56					0/10	6/14	
0.19						4/14	
0.063						2/12	
LD_{50} (mg/kg)	12.7	8.7	7.2	6.4	3.8	0.74	3.8

* Animals fasted for 60 hr before endotoxin challenge.

† Dosage of 100 mg intraperitoneally.

TABLE 3. *Inactivation of endotoxin by tissues of normal animals and animals given allyl alcohol**

Tissue	Amt of homogenate	Source of tissue			
		Normal animal		Allyl alcohol-treated animal	
		Mortality	Per cent protection	Mortality	Per cent protection
Liver	mg				
	180	5/20†	72‡	2/20	89
	18	10/20	44	5/20	72
Spleen	180	1/20	95	0/20	100
	18	3/20	83	5/20	72
Kidney	180	3/20	83	6/20	67
	18	4/20	78	13/20	28
Saline control	0	18/20			

* Incubation medium contained 20 µg of *Serratia marcescens* endotoxin and either 180 or 18 mg of tissue homogenate (supernatant fraction after 600 × g centrifugation). Allyl alcohol, 0.042 ml/kg by gastric tube, was given 48 hr before sacrifice.

† Deaths/total eggs.

‡ Per cent protection =

$$\frac{\% \text{ mortality saline} - \% \text{ mortality sample}}{\% \text{ mortality saline}} \times 100.$$

tion by liver mitochondria. In Table 5 are shown the effects of liver damage produced by each of the three hepatotoxic agents on the oxidation of various substrates by liver mitochondria. In each case, the livers were removed at the time of maximal histological change, i.e., 48 hr after administration of the hepatotoxin. Mitochondrial preparations from animals starved for 60 hr were also studied to evaluate the nonspecific effects of starvation, since the animals given ethionine were deliberately starved for 60 hr, and animals given the other agents often showed profound anorexia.

CCl₄ administration reduced the oxidation of octanoate by liver mitochondria by 82% during the first 30-min period, and by 95% during the second 30 min. A highly significant reduction in oxidation of malate and β-hydroxybutyrate, but not of succinate, was also seen. In fact, succinate oxidation was increased in the livers damaged by CCl₄. These findings are in essential agreement with data previously reported by Christie and Judah (1954).

Preparations from animals given allyl alcohol showed no change from untreated controls, except

TABLE 4. *Inactivation of endotoxin by tissues of normal animals and animals given DL-ethionine**

Tissue	Amt of homogenate	Source of tissue			
		Normal animal		Ethionine-treated animal	
		Mortality	Per cent protection	Mortality	Per cent protection
Liver	mg				
	180	3/20	82	4/20	77
	18	8/20	53	7/20	59
Spleen	180	0/20	100	1/20	94
	18	6/20	65	7/20	59
Kidney	180	9/20	47	6/20	65
	18	12/20	29	8/20	53
Saline control	0	17/20			

* Dosage of 100 mg intraperitoneally 48 hr before sacrifice. Both normal and ethionine-treated animals were fasted for 60 hr prior to sacrifice.

for a tendency toward increased oxidation of succinate.

Ethionine treatment did not impair oxidation significantly except that octanoate oxidation was reduced by 44% during the first 30 min. An even more impressive reduction was seen during the second 30 min, but this effect was also observed in the starved controls. Succinate oxidation by liver damaged by ethionine was significantly greater than normal.

DISCUSSION

If the liver detoxifies endotoxin by a process of activation and oxidation of fatty acid residues (Corwin and Farrar, 1964), and if this detoxification represents a physiologically significant mechanism in the response of animals to endotoxin, one would expect to find a correlation between the biochemical lesions produced by hepatotoxins and their effect on the susceptibility of animals to endotoxin. Agents which interfere with hepatic oxidation of fatty acid substrates should cause a significant increase in the susceptibility of the animals, whereas those which do not have this action should not.

Such a correlation was found in this study. Administration of CCl₄ caused a pronounced fall in hepatic mitochondrial oxidation of octanoate, malate, and β-hydroxybutyrate, and was associated with a 150-fold increase in the susceptibility of animals to endotoxin. Earlier studies had

TABLE 5. *Effects of hepatotoxic agents on substrate oxidation by liver mitochondria**

Animal	Octanoate		Succinate		Malate		β -Hydroxybutyrate	
	0-30 min	30-60 min	0-30 min	30-60 min	0-30 min	30-60 min	0-30 min	30-60 min
Normal (8)†	9.3 $\pm$ 0.7‡	7.8 $\pm$ 1.0	6.2 $\pm$ 0.5	5.1 $\pm$ 0.5	2.0 $\pm$ 0.1	0.8 $\pm$ 0.1	1.7 $\pm$ 0.2	1.4 $\pm$ 0.2
CCl ₄ (4).....	1.6 $\pm$ 0.2	0.4 $\pm$ 0.1	8.5 $\pm$ 0.8	8.0 $\pm$ 1.2	0.2 $\pm$ 0.2	0.2 $\pm$ 0.1	0.6 $\pm$ 0.2	0.4 $\pm$ 0.1
Allyl alcohol (8).....	7.0 $\pm$ 1.1	5.2 $\pm$ 1.0	7.8 $\pm$ 1.0	6.7 $\pm$ 0.8	2.1 $\pm$ 0.3	0.7 $\pm$ 0.1	1.5 $\pm$ 0.2	1.2 $\pm$ 0.1
Ethionine (8).....	4.9 $\pm$ 0.4	1.7 $\pm$ 0.2	10.4 $\pm$ 1.8	7.6 $\pm$ 1.3	1.4 $\pm$ 0.2	1.0 $\pm$ 0.1	1.4 $\pm$ 0.2	0.9 $\pm$ 0.1
Starved (8)...	8.7 $\pm$ 1.3	3.0 $\pm$ 0.6	8.1 $\pm$ 0.7	5.7 $\pm$ 0.6	2.2 $\pm$ 0.3	0.6 $\pm$ 0.1	2.1 $\pm$ 0.3	0.9 $\pm$ 0.2

* Medium consisted of 40 μ moles of sodium phosphate buffer (pH 7.4), 300 μ moles of NaCl, 12 μ moles of KCl, and 4 μ moles of MgSO₄·7H₂O. The individual substrate concentrations were 60 μ moles for succinate, 30 μ moles for β -hydroxybutyrate or malate, and 5 μ moles for octanoate. NAD was added at 3 μ moles except when succinate was substrate. When octanoate was substrate, 3 μ moles of ATP, 3 μ moles of malate, and 0.1 ml of 1% cytochrome were added. The value for octanoate oxidation was calculated by subtracting the oxidation when octanoate was absent. The final volume was 3 ml. The oxidation was followed for 1 hr at 37 C.

† Number of animals studied.

‡ Microatoms O/10 mg of protein $\pm$ standard error of mean.

shown that CCl₄-damaged liver has little ability to detoxify endotoxin in vitro. Allyl alcohol, although producing a severe morphological lesion in the liver, did not significantly depress substrate oxidation by mitochondria and lowered the LD₅₀ for endotoxin only threefold. Allyl alcohol-damaged liver tissue was unimpaired in its ability to detoxify endotoxin in vitro. Ethionine exhibited an effect intermediate between that of CCl₄ and allyl alcohol. Octanoate oxidation was significantly reduced, and the LD₅₀ for endotoxin was reduced 12-fold. Ethionine treatment did not appear to alter the ability of liver tissue to detoxify endotoxin in vitro, but the biological assay method by which this detoxification was measured is relatively insensitive in comparison with the oxidation measurements.

The three hepatotoxic agents used in this study were selected because they are chemically unrelated and presumably produce liver damage by different mechanisms.

CCl₄ is a fat solvent which damages intracellular membranes. Recent work has shown that changes in the ultrastructure of the mitochondrial membrane may be demonstrated as early as 20 sec after its intrasplenic administration (Hübner, 1964). Hepatic ribosomes are also damaged and protein synthesis is impaired (Smuckler, Iseri, and Benditt, 1962). Effects on substrate oxidation by liver mitochondria, similar to the ones described herein, were demonstrated by Christie and Judah (1954). CCl₄ treatment had a pronounced inhibitory effect on all the nicotinamide adenine dinucleotide (NAD)-linked substrates studied. Inasmuch as succinate oxidation was not impaired, this may indicate a specific effect

of the compound on that portion of the electron-transport chain between NAD and the cytochromes.

Allyl alcohol is a highly reactive compound of well-recognized hepatic toxicity. Although its biochemical mode of action has apparently not yet been elucidated, it has been shown to damage vessels in the portal area and to produce periportal necrosis and hemorrhage (Eger, 1954, 1955). Ultrastructural studies indicate that it probably does not act directly on the mitochondrial membrane, as does CCl₄ (Hübner, 1964).

The metabolic effects of ethionine, the ethyl analogue of methionine, have been intensively investigated during the past 15 years, and were reviewed recently by Stekol (1963). Administration of ethionine results in trapping of adenosine triphosphate (ATP) as *S*-adenosyl-L-ethionine, with a consequent rapid fall in hepatic ATP concentration. This deficiency of ATP leads to impaired synthesis of protein in the liver. The fatty liver produced by ethionine appears to be due to deficient synthesis of lipoproteins, which interferes with the transfer of triglycerides to plasma and leads to their accumulation in the liver. These effects are prevented by administration of ATP or adenine (Farber, Lombardi, and Castillo, 1963). Inhibition of transmethylation reactions by ethionine is not prevented by ATP or adenine, but can be prevented by administration of methionine (Gordon, Villa-Trevino, and Farber, 1964). The decreased oxidation of certain substrates by mitochondria from ethionine-treated animals observed in the present study may be related to the finding by Stekol, Mody, and Bedrak (1962) that addition of *S*-adenosyl-

L-ethionine to normal rat liver mitochondria in vitro inhibited the oxidation of pyruvate and α -ketoglutarate, but not of succinate.

Succinate oxidation was increased by each of the three hepatotoxins. This may have been due to increased permeability of the mitochondria to succinate in vitro, indicating some damage to the mitochondrial membrane. The fact that succinate oxidation was not impaired by any of the hepatotoxic agents used indicates that the effects observed on oxidation of other substrates were not due to a simple reduction in the number of functional mitochondria in the preparations, as would be found in extensive liver cell necrosis.

Berry and Smythe (1963) showed that endotoxin itself may interfere with the formation of pyridine nucleotides in the liver through an effect on tryptophan pyrrolase. Since endotoxin and CCl_4 both affect NAD-dependent metabolism, the possibility of a synergistic action must be considered. Our data do not provide evidence for such synergism, however, since the effect of CCl_4 on NAD-dependent systems in vitro occurred despite the addition of NAD to the reaction mixture.

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