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Changes Associated with the Production of Fatty Livers by White Phosphorus and by Ethanol in the Rat

BY ANNE SEAKINS* AND D. S. ROBINSON*
External Staff of the Medical Research Council, Sir William Dunn School of Pathology, University of Oxford

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Evidence has been obtained in previous studies that the development of a fatty liver, in association with a lowered concentration of plasma lipids, after the administration of ethionine, carbon tetrachloride or puromycin, may be the result of inhibition of the formation of the protein moiety of the plasma lipoproteins in the liver (Harris & Robinson, 1961; Robinson & Harris, 1961; Robinson & Seakins, 1962; Seakins & Robinson, 1963). In the present work the possibility that inhibition of plasma lipoprotein formation could also account for the experimental fatty livers produced in the rat by white phosphorus and by ethanol has been investigated.

METHODS

Female albino rats (Wistar strain) weighing 180–200 g. were used. In the experiments with white phosphorus they were starved for 16 hr. before being given, by stomach tube under light ether anaesthesia, either 1·5 mg. of white phosphorus dissolved in 0·3 ml. of olive oil (test groups) or 0·3 ml. of olive oil alone (control groups). In the experiments with ethanol the rats were fed on their normal diet until either 3 ml. of ethanol–water (1:1, v/v) (test groups) or an isocaloric amount (4 ml.) of a 50% (w/v) solution of glucose in water (control groups) were given, also by stomach tube. All the rats were starved thereafter until they were killed.

Incorporation of DL-[1-^{14}C]*leucine into the proteins of rat liver and plasma* in vivo. At either 2 hr. (white phosphorus groups) or 2·5 or 14 hr. (ethanol groups) after feeding, rats were injected by the tail vein with 20 μc of DL-[1-^{14}C]-leucine (8·3 mc/m-mole) and killed 1·5 hr. (white phosphorus groups) or 2 hr. (ethanol groups) later by exsanguination from the abdominal aorta. The low-density lipoproteins (d < 1·063), high-density lipoproteins (d 1·063–1·21) and the residue proteins (d > 1·21) of the plasma were separated by ultracentrifugal techniques and their specific activities, as well as those of the liver proteins, were determined by methods described by Seakins & Robinson (1963).

Incorporation of [^{32}P]*orthophosphate into the phospholipids of rat liver and plasma.* At either 2 hr. (white phosphorus groups) or 16 hr. (ethanol groups) after feeding, rats were injected by the tail vein with 6 μc of [^{32}P]orthophosphate and killed 3 hr. later by exsanguination. The specific activities of the liver and plasma phospholipids were determined as described previously (Seakins & Robinson, 1963) except that no separation of plasma lipoprotein fractions was carried out.

Incorporation of sodium [1-^{14}C]*acetate into the free cholesterol of rat liver and plasma.* Rats were injected by the tail vein with 20 μc of sodium [1-^{14}C]acetate (13 mc/m-mole) at 2 hr. after feeding with white phosphorus in olive oil or olive oil alone and were killed at various intervals thereafter by exsanguination. Samples of plasma and the livers from individual animals were combined into appropriate test and control groups (three animals/group) and the specific activities of the plasma and liver free cholesterol were determined (Seakins & Robinson, 1963).

Concentration of lipids and free amino acids in plasma and liver. The concentrations of esterified fatty acid, cholesterol and phospholipid in liver and plasma were determined on duplicate samples of lipid extracts prepared as described by Harris & Robinson (1961) with the following modifications. In the preparation of the lipid extracts of liver the whole organ was first homogenized in 240 ml. of ethanol and then 80 ml. of ether was added. The resulting mixture was boiled and filtered while hot and the precipitate was washed twice with 50 ml. portions of ethanol–ether (3:1, v/v). Cholesterol determinations in both liver and plasma were carried out by the method of Pearson, Stern & McGavack (1953) on the

* Present address: Cell Metabolism Research Unit, Medical Research Council, Department of Biochemistry, University of Oxford.

residue obtained after drying suitable samples of the lipid extracts *in vacuo*.

The concentrations of free amino acids in liver and plasma were determined as described by Robinson & Harris (1961).

Counting procedure. The counting procedure was as described by Harris & Robinson (1961) but a Nuclear–Chicago gas-flow counter was used. Duplicate samples were counted and the counts did not differ by more than 5%. All counts were corrected to zero mass.

Statistical analysis. Where the significance of the differences between means has been calculated, the Behrens test has been used (Fisher & Yates, 1957). A probability value (P) of less than 0·05 has been considered significant.

RESULTS

Effect of white phosphorus on the lipid content of rat plasma and liver. Twenty-four hours after feeding rats with white phosphorus, the total amounts and the concentrations of esterified fatty acids in the livers were increased markedly above those in control rats (Table 1) and the livers were fatty to the naked eye. The total amounts and the concentrations of cholesterol in the livers were also significantly higher ($P < 0·01$). Similar increases in the liver cholesterol concentration have been found after the administration of carbon tetrachloride and puromycin to rats (Seakins & Robinson, 1963, and unpublished work).

The total amount of phospholipid in the liver was increased significantly ($P < 0·01$) but, as a result of the accompanying rise in the liver wet weight, the concentration fell.

The effect of white phosphorus on the plasma lipid concentration is also shown in Table 1. Twenty-four hours after feeding, the concentrations of all the lipids studied were markedly reduced, the mean concentrations of esterified fatty acid, phospholipid and cholesterol being respectively 35·5, 33 and 12·5% of those in the control animals. A fall in the triglyceride concentration in the plasma after white phosphorus administration has been reported (Lombardi & Recknagel, 1962) and this presumably accounts, in part, for the reduction in esterified fatty acid concentration found here.

Effect of white phosphorus on the incorporation of DL-[1-^{14}C]*leucine and* [^{32}P]*orthophosphate into the proteins and phospholipids of rat liver and plasma.* When DL-[1-^{14}C]leucine was injected into rats 2 hr. after they had been given white phosphorus and olive oil, the incorporation of ^{14}C into the proteins of the liver and into the low- and high-density lipoproteins and the residue proteins of the plasma was significantly less than in corresponding animals given olive oil alone (Table 2).

No effect of white phosphorus on the plasma free amino acid concentration was observed. Thus 3·5 hr. after feeding either white phosphorus in olive oil, or olive oil alone, to groups of five rats the mean plasma amino acid value was, in both groups, 3·1 ± 0·2 μmoles/ml. In the liver, the mean concentrations of free amino acids were 23·5 ± 2·7 μmoles/g. of liver wet weight (white phosphorus group) and 19·3 ± 0·9 μmoles/g. of liver wet weight (control group).

The mean relative specific activities of the liver and plasma phospholipids in a group of rats injected with [^{32}P]orthophosphate 2 hr. after they had been fed with white phosphorus and olive oil and killed 3 hr. after the injection were not significantly different from those in rats given olive oil alone (Table 3).

Effect of white phosphorus on the incorporation of sodium [1-^{14}C]*acetate into the cholesterol of rat liver*

Table 1. *Effect of the administration of white phosphorus on the total esterified fatty acids, phospholipids and cholesterol of rat plasma and liver*

Rats were starved for 16 hr., fed with 1·5 mg. of white phosphorus in 0·3 ml. of olive oil or 0·3 ml. of olive oil alone and killed at 24 hr. after feeding. Each group consisted of five rats; values are means ± S.D.

	Liver						
		Esterified fatty acids		Cholesterol		Phospholipid	
	Wet wt. (g.)	mg./whole liver	mg./g. of liver	mg./whole liver	mg./g. of liver	mg./whole liver	mg./g. of liver
White phosphorus-treated group	7·7 ± 0·9	527 ± 113	69·2 ± 18·0	25·3 ± 2·9	3·30 ± 0·33	263 ± 33	33·9 ± 1·1
Control group	5·3 ± 0·1	206 ± 14	39·1 ± 2·6	14·8 ± 0·8	2·78 ± 0·07	206 ± 16	39·0 ± 2·3

	Plasma		
	Esterified fatty acids (mg./100 ml.)	Cholesterol (mg./100 ml.)	Phospholipid (mg./100 ml.)
White phosphorus-treated group	40·0 ± 6·7	4·8 ± 1·3	25·2 ± 5·3
Control group	114·7 ± 9·6	33·5 ± 3·7	75·6 ± 7·7

Table 2. *Effect of the administration of white phosphorus on the incorporation of* DL-[1-^{14}C]*leucine into the proteins of rat liver and plasma* in vivo

Rats, fed as described in Table 1, were injected intravenously with 20 μc of DL-[1-^{14}C]leucine 2 hr. later and killed 1·5 hr. after the injection. Each group consisted of six rats; values are means ± S.D.

	Radioactivity in protein hydrolysates (counts/min./100 μg. of amino N)			
	Plasma low-density lipoproteins (d < 1·063)	Plasma high-density lipoproteins (d 1·063–1·21)	Plasma residue proteins (d > 1·21)	Liver proteins
White phosphorus-treated group	1347 ± 337	950 ± 136	97 ± 15	94 ± 23
Control group	1986 ± 298	1380 ± 396	191 ± 24	179 ± 17
Treated group as percentage of control group	68	69	51	52
P of chance occurrence of the differences	< 0·01	< 0·05	< 0·01	< 0·01

Table 3. *Effect of the administration of white phosphorus on the incorporation of* [^{32}P]*orthophosphate into the phospholipids of rat liver and plasma* in vivo

Rats, fed as described in Table 1, were injected intravenously with 6 μc of [^{32}P]orthophosphate 2 hr. later and killed 3 hr. after the injection. Each group consisted of six rats; values are means ± S.D.

	Ratio: $\frac{\text{sp. activity of phospholipid P} \times 10^2}{\text{sp. activity of liver acid-soluble P}}$	
	Plasma	Liver
White phosphorus-treated group	11·8 ± 3·5	23·0 ± 4·2
Control group	11·3 ± 1·7	21·1 ± 2·3

and plasma. The specific activity of isolated liver and plasma free cholesterol was determined at various times up to 2·5 hr. after the injection of sodium [1-^{14}C]acetate into rats which had been fed with either white phosphorus in olive oil (test groups) or olive oil alone (control groups) 2 hr. previously. Despite the fact that liver and plasma samples from a group of rats were combined (see Methods section), the cholesterol specific activities in different groups of control animals killed at the same time-interval after the injection varied over a wide range; for example, in four groups, the observed values at 0·5 hr. ranged between 200 and 630 counts/min./mg. of cholesterol. Similar large variations have been reported by others (Van Bruggen, Hutchens, Claycomb & West, 1953; Loud & Bucher, 1958).

The specific activities of both the liver and the plasma cholesterol of the test animals were within the range of the corresponding activities in the control animals at all the times studied. However, because of the degree of scatter of the control values, it is not possible from the observations to exclude the possibility of minor changes in the pattern of acetate incorporation after feeding with white phosphorus.

In a previous study (Seakins & Robinson, 1963) the results of experiments similar to those reported here led to the tentative conclusion that carbon tetrachloride treatment caused a change in the time-course of labelling of liver and plasma cholesterol from sodium [1-^{14}C]acetate. The degree of variation in the cholesterol specific activity now found in control animals suggests that this conclusion was unjustified.

Effect of ethanol on the lipid content of rat plasma and liver. The concentrations of lipids in rat liver and plasma 8 hr. and 14 hr. after feeding with ethanol are compared in Table 4 with the corresponding concentrations in the liver and plasma of control animals fed with an isocaloric quantity of glucose. In the liver, the concentration of phospholipid and of cholesterol did not change after ethanol administration but there was a progressive rise in the concentration of total esterified fatty acids. In view of the constancy of the cholesterol and phospholipid concentrations this must represent an accumulation of triglyceride in the liver. The concentrations of the plasma lipids after feeding with ethanol were not significantly different from those in control animals fed with glucose.

Effect of ethanol on the incorporation of DL-[1-^{14}C]*leucine and* [^{32}P]*orthophosphate into the proteins and phospholipids of rat liver and plasma*. The mean specific activities of the proteins of the liver and plasma in groups of rats given ethanol 2·5 or 14 hr. before being injected with DL-[1-^{14}C]-leucine were not significantly different from those in control animals given glucose (Table 5). The incorporation of [^{32}P]orthophosphate into the phospholipids of the liver and plasma, on the other hand, was significantly increased 16 hr. after feeding with ethanol (Table 6).

Table 4. *Effect of the administration of ethanol on the total esterified fatty acids, phospholipids and cholesterol of rat plasma and liver*

Rats were fed with either 3 ml. of ethanol–water (1:1, v/v) or 4 ml. of 50% (w/v) glucose solution in water and were killed 8 or 14 hr. later. Each group consisted of five rats; values are means±S.D.

	Time after feeding (hr.)	Wet wt. of liver (g.)	Total esterified fatty acids: Plasma (mg./100 ml.)	Total esterified fatty acids: Liver (mg./whole liver)	Total cholesterol: Plasma (mg./100 ml.)	Total cholesterol: Liver (mg./whole liver)	Total phospholipids: Plasma (mg./100 ml.)	Total phospholipids: Liver (mg./whole liver)
Ethanol-treated group	8	5·2±0·4	125±26	338±45	48±14	16±1·9	111±26	201±18
	14	5·4±0·4	112±19	436±98	35±6	17±1·5	92±11	201±13
Control group	8	5·5±0·3	116±9	216±13	39±5	15±1·8	92±8	185±8
	14	5·2±0·6	117±24	225±23	38±2	16±2·8	87±11	188±16

Table 5. *Effect of the administration of ethanol on the incorporation of* DL-[1-^{14}C]*leucine into the proteins of rat liver and plasma* in vivo

Rats, fed as described in Table 4, were injected intravenously with 20 μc of DL-[1-^{14}C]leucine 2·5 or 14 hr. later and killed 2 hr. after the injection. Each group consisted of six rats; values are means±S.D.

	Time after feeding (hr.)	Radioactivity in protein hydrolysates (counts/min./100 μg. of amino N): Plasma lipoproteins (d < 1·063)	Plasma lipoproteins (d 1·063–1·21)	Plasma proteins (d > 1·21)	Liver proteins
Ethanol-treated group	2·5	2750±870	1120±239	327±53	273±50
	14	2156±410	1165±153	244±72	218±12
Control group	2·5	2275±380	996±204	272±44	223±18
	14	1876±192	1120±78	282±33	218±16

Table 6. *Effect of the administration of ethanol on the incorporation of* [^{32}P]*orthophosphate into the phospholipids of rat liver and plasma* in vivo

Rats, fed as described in Table 4, were injected intravenously with 6 μc of [^{32}P]orthophosphate 16 hr. later and killed 3 hr. after the injection. Each group consisted of six rats; values are means±S.D.

	Ratio: sp. activity of phospholipid P × 10^2 / sp. activity of liver acid-soluble P: Plasma	Liver
Ethanol-treated group	17·4±5·8	29·0±6·6
Control group	9·6±6·0	18·0±2·4
P of chance occurrence of the differences	<0·05	<0·01

DISCUSSION

In previous studies it has been shown that the fatty livers produced by the administration of ethionine, puromycin and carbon tetrachloride in the rat are accompanied by pronounced falls in the concentrations of all the plasma lipids. It has been suggested that these changes in liver and plasma lipid concentrations may result from the reduction in the rate of formation in the liver of the protein moiety of the low-density lipoproteins of the plasma which appears to precede development of the fatty liver. Thus the low-density lipoproteins normally carry triglyceride from the liver to the extrahepatic tissues and a reduction in their ability to do so, arising from a block in their formation, would presumably lead not only to the accumulation of triglyceride in the liver but also to a fall in the concentration in the plasma of the lipid components of the lipoproteins.

The present results suggest that the action of white phosphorus in producing a fatty liver accompanied by a reduction in lipid concentration in the plasma may be similar to that of the above substances. Its administration is shown to result within 2 hr. in a reduction in the incorporation of DL-[1-^{14}C]leucine into the protein moiety of the plasma low-density lipoproteins, as well as into the remainder of the plasma proteins and the liver proteins, whereas at these times no significant change in the rate of incorporation of [^{32}P]orthophosphate and of sodium [1-^{14}C]acetate into the liver and plasma phospholipids and free cholesterol respectively was observed. Such an explanation would be consistent with the report by Lombardi & Recknagel (1962) that, 24 hr. after feeding white phosphorus to rats, the release of triglyceride from

the livers in response to an injection of Triton WR 1339 was blocked.

No evidence for blockage of triglyceride release was obtained by Lombardi & Recknagel at an earlier time after feeding with white phosphorus, whereas within 2 to 4 hr. of ethionine and carbon tetrachloride administration reduced release of triglyceride after Triton had been observed (Recknagel, Lombardi & Schotz, 1960; Lombardi & Recknagel, 1962). Possibly these differences between the responses to white phosphorus and to carbon tetrachloride or ethionine may be explained by the more drastic reduction in the incorporation of amino acids into the plasma proteins produced by ethionine and carbon tetrachloride than by white phosphorus (Robinson & Harris, 1961; Seakins & Robinson, 1963).

The fatty liver produced by a single dose of ethanol in the rat is not accompanied by a fall in the plasma lipid, nor do the present studies suggest that there is any interference with the formation of either the protein or phospholipid components of the plasma lipoproteins in the liver. On the contrary, the incorporation of [^{32}P]orthophosphate into the liver and plasma phospholipids is significantly increased after 16 hr. Since the effect of ethanol on the liver triglyceride concentration is transitory—after a single dose the concentration returns to normal after 48 hr. (unpublished work)—the effects observed 16 hr. after feeding may be related to the recovery phase concerned with the removal of the excess of triglyceride in combination with the low-density lipoproteins of the plasma.

It should be emphasized that the finding that the fatty liver produced by a single dose of ethanol in the rat does not appear to result from impaired formation of plasma lipoproteins may have little relevance to the development of a fatty liver in chronic alcoholism in man.

SUMMARY

1. The administration of white phosphorus to female rats produced, at 24 hr., a rise in the amount of esterified fatty acid in the liver and large falls in the mean concentrations of cholesterol, phospholipid and esterified fatty acid in the plasma.

2. Two hours after the administration of white phosphorus the incorporation of injected DL-[1-^{14}C]leucine into the liver and plasma proteins was markedly reduced, whereas the incorporations of [^{32}P]orthophosphate and of sodium [1-^{14}C]-acetate into the liver and plasma phospholipid and free cholesterol respectively were not significantly changed.

3. Ethanol increased the liver triglyceride concentration at 14 hr. to almost twice that of control rats. Plasma cholesterol, phospholipid and esterified fatty acid concentrations were not significantly changed.

4. Ethanol did not affect the incorporation of DL-[1-^{14}C]leucine into the liver and plasma proteins at either 2·5 or 14 hr. after its administration. The incorporation of [^{32}P]orthophosphate into the plasma and liver phospholipids was increased at 16 hr.

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