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Hepatic and Serum Lipid Patterns During Development of Phenobarbital Induced Fatty Livers in Rats[1] (38226)

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(Introduced by H. Baker)

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Aside from ethanol and choline deficiency, several drugs and hepatotoxic agents are known to produce fatty infiltration in the liver. Recently, it has been demonstrated by Sorrell *et al.* (1) that phenobarbital administration to rats over a 5-day period produced a fatty liver. It is accepted that most agents which produce fatty livers do so by affecting one of the mechanisms that facilitate secretion of plasma lipoproteins (lipid transport) from the liver (2–5). In this study, serial determinations of serum and liver lipids were made both during and following phenobarbital injections in an attempt to determine the role of lipid accumulation and removal in the pathogenesis of the phenobarbital induced fatty liver.

Materials and Methods. Male, Sprague–Dawley rats weighing approximately 250 g were maintained on the modified diet of French (6) described in our initial study (1) which contained 35% fat, 24% protein and 0.7% choline dihydrogen citrate. All animals were pair-fed with an animal of equal age and size in each time period of the experiment. Those animals receiving phenobarbital were injected intraperitoneally with 50 mg/kg sodium phenobarbital every 12 hr. At 3 days and 5 days, groups of control and phenobarbital injected animals were killed and the livers removed and frozen in air-tight plastic containers at −50° until analyzed. Blood samples were also taken from these animals and serum was prepared for analysis. The chylomicrons were removed from the serum samples by centrifuging them at 9500*g* for 30 min in a 40.3 rotor using a Model L Beckman-Spinco Ultracentrifuge (7). The infranatant solution was then frozen until analyzed. Preparative to the analyses for free cholesterol esters, phospholipids and triglycerides in liver and serum, lipids were extracted by homogenization with chloroform–methanol 2:1 (v/v) according to Folch *et al.* (8). Free cholesterol and cholesterol esters were determined by the method of Searcy and Berquist (9); triglycerides were measured by the method of Soloni (10) and phospholipids levels were estimated by the method of Bartlett (11). The levels of free fatty acids in the infranatant solution were determined by the procedure of Soloni and Sardina (12).

Following the fifth day of phenobarbital injection, the remaining animals in the control and phenobarbital injected groups were killed 3 days following withdrawal (8 day rats) and 5 days following withdrawal (10 day rats) of phenobarbital. Sera and livers from these groups were analyzed for lipid components as described above.

To avoid reference unit artifacts arising from the dilution of the liver tissue by fatty infiltration, all comparisons in this study were based on the expression of data in

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μmoles/total liver (13). The student *t* test was used throughout to determine the significance of differences between the means of groups being compared.

Results. During the course of the 10 day experiment, the animals in the control and experimental groups gained weight but no significant changes were observed between these groups at any of the time periods at which rats were killed. Shortly after each injection of phenobarbital the rats slept for varying periods of time. These sleeping periods, however, did not effect the eating habits of the animals as was indicated by their normal food intake and steady growth rate.

The effects of phenobarbital administration on the liver weights in this study are shown in Table I. Phenobarbital produced significant increases in liver weight in 3 and 5 days. Following withdrawal, however, a trend towards normal liver wieght is apparent within 3 days. The data in Table II confirm that obtained earlier (1) showing a doubling of the phospholipid in liver and a 6–7 fold increase in hepatic triglyceride due to the drug. Cessation of phenobarbital administration brings a return toward normal levels in both families of these glycerolipids in 5 days. Both free cholesterol and cholesterol esters appear to reach increased levels (Table II) in the liver in 3 days on phenobarbital with no further increase in 5 days. Withdrawal again allows both members of this lipid family to revert to normal levels.

Analysis of the serum demonstrates that the blood lipid pattern does not change significantly either during the development of the fatty liver or following withdrawal when liver lipids approach normal levels. From Table III, it is evident that serum phospholipids, triglycerides, cholesterol esters and free fatty acids remain unchanged throughout the phenobarbital injection as well as after withdrawal of the drug. Only serum free cholesterol appears to increase slightly on the fifth day of phenobarbital administration (Table III) but returns to normal upon removal of the drug.

Discussion. The most common response of the liver to injury is the accumulation of fat especially triglycerides (2, 3). Hepatotoxins, in general, seem to induce fatty livers by a common mechanism which involves impairment of triglycerides release from the liver (4, 5). Preceding and accompanying the increase in liver triglycerides by most hepatotoxins, there is a fall in the concentration of plasma triglycerides (2, 14). Since triglycerides are secreted from the liver primarily in the form of very low density lipoproteins, an impairment in the synthesis of release of these lipoproteins is responsible for the accumulation of lipid in the liver parenchymal cell. However, even though the general mechanism may be the same, the specific process by which these various toxic agents induce fatty livers are probably quite different because the synthesis and release of the very low density lipoproteins are complex processes. Inhibitors of protein synthesis such as puromycin (15) and aflatoxin B (16) inhibit lipoprotein formation by impairing the synthesis of the apoprotein moiety. It has been suggested that ethionine

TABLE I. Liver Weights During Phenobarbital (Pb) Regimen in Rats.

Time Period	No. of animals per group	Mean liver weights ± S.D. (Grams)		
		Control	Pb	*P*
3 Days	5	12.5 ± 1.7	16.5 ± 0.9	<0.002
5 Days	5	11.7 ± 0.5	17.7 ± 1.1	<0.001
Withdrawal				
8 Days	5	11.5 ± 0.9	14.0 ± 0.8	<0.01
10 Days	5	12.0 ± 1.1	14.2 ± 1.0	<0.02

TABLE II. Hepatic Phospholipid, Triglyceride Free Cholesterol and Cholesterol Esters During Phenobarbital (PB) Regimen in Rats.

Time period	No. of animals	Phospholipid[a]	Triglyceride[a]	Free cholesterol[a]	Cholesterol esters[a]
3 Days					
Controls	5	425.1 ± 21.5	206.8 ± 91.8	74.1 ± 9.6	84.3 ± 23.1
PB	5	727.4 ± 37.6[b]	923.6 ± 101.3[b]	118.0 ± 8.7[b]	128.7 ± 12.1[c]
5 Days					
Controls	5	423.2 ± 29.9	169.5 ± 70.8	72.5 ± 8.2	70.4 ± 16.7
PB	5	816.1 ± 90.6[b]	950.1 ± 242.4[b]	112.8 ± 8.2[b]	125.8 ± 16.3[e]
Withdrawal 8 Days					
Controls	5	445.7 ± 12.8	210.1 ± 64.4	89.2 ± 4.6	80.2 ± 20.9
PB	5	592.4 ± 35.8[b]	480.0 ± 213.8[d]	109.1 ± 5.2[b]	91.7 ± 22.5[f]
10 Days					
Controls	5	451.8 ± 38.8	206.7 ± 62.9	91.5 ± 8.3	82.1 ± 5.8
PB	5	560.1 ± 62.7[d]	402.2 ± 57.6[b]	103.2 ± 11.1[f]	84.6 ± 15.1[f]

[a] Values expressed as mean μmoles/total liver ± S.D.
[b] $P < 0.001$ with respect to control values.
[c] $P < 0.01$ with respect to control values.
[d] $P < 0.02$ with respect to control values.
[e] $P < 0.002$ with respect to control values.
[f] Not significant with respect to control values.

TABLE III. Serum Phospholipid, Triglyceride Free Cholesterol, Cholesterol Esters and Free Fatty Acids During Phenobarbital (PB) Regimen in Rats.

Time period	No. of animals	Phospholipid[a]	Triglyceride[a]	Free cholesterol[a]	Cholesterol esters[a]	Free fatty acids[a]
3 Days						
Control	5	2.28 ± 0.42	0.77 ± 0.27	1.08 ± 0.21	3.27 ± 0.27	0.59 ± 0.30
PB	5	2.39 ± 0.25[b]	0.80 ± 0.21[b]	1.15 ± 0.09[b]	3.09 ± 0.18[b]	0.55 ± 0.15[b]
5 Days						
Control	5	1.90 ± 0.40	0.82 ± 0.34	0.81 ± 0.03	2.62 ± 0.37	0.46 ± 0.04
PB	5	2.37 ± 0.46[b]	0.64 ± 0.24[b]	1.04 ± 0.18[c]	2.91 ± 0.77[b]	0.44 ± 0.15[b]
Withdrawal 8 Days						
Control	5	1.38 ± 0.25	0.50 ± 0.17	0.68 ± 0.11	1.83 ± 0.33	0.37 ± 0.05
PB	5	1.30 ± 0.13[b]	0.49 ± 0.17[b]	0.73 ± 0.12[b]	1.95 ± 0.19[b]	0.28 ± 0.07[b]
10 Days						
Control	5	1.48 ± 0.18	0.56 ± 0.17	0.79 ± 0.10	2.12 ± 0.21	0.53 ± 0.13
PB	5	1.57 ± 0.23[b]	0.49 ± 0.13[b]	0.84 ± 0.19[b]	2.14 ± 0.33[b]	0.58 ± 0.19[b]

[a] Values expressed as mean μmoles/total liver ± S.D.
[b] Not significant with respect to control values.
[c] $P < 0.05$ with respect to control values.

(5, 17–19) and carbon tetrachloride (20–22) also decrease lipoprotein release by their interference with protein synthesis, in addition, changes in ATP levels and lipid peroxidation have also been shown to occur during administration of these two toxins. Orotic acid administration (23, 24) results in a fatty liver without inhibition of protein synthesis but apparently interferes with the coupling of lipid to the protein in the liver cell and thereby interferes with lipid transport.

This study suggests that the action of phenobarbital in producing a fatty liver is unlike that of other known hepatotoxins. The accumulation of liver lipids due to phenobarbital administration and the fall in these same lipids upon removal of the drug, concomitant with steady serum lipid levels, would indicate that impaired lipid transport is not the major mechanism involved. The fact that serum free fatty acids remained constant throughout the entire experiment suggests that increased lipid mobilization from adipose tissue also is not a factor.

Although more experimental work will have to be conducted for more conclusive evidence, it would appear from these data that impaired lipid transport and increased peripheral mobilization of fat do not play a major role in the pathogenesis of the phenobarbital induced fatty liver. Other causitive mechanisms might include abnormal fatty acid oxidation and lipid synthesis.

Summary. Lipids were measured in the livers and sera of rats that had received phenobarbital injections for 3 days and 5 days. The same lipid patterns including free cholesterol, cholesterol esters, phospholipids, triglyceride and fatty acids were determined in the livers and sera of rats 3 days and 5 days following withdrawal of the drug. Liver lipids increased with time while the rats were injected with phenobarbital and decreased toward normal levels once the drug was withdrawn. During phenobarbital administration as well as during withdrawal the serum lipid levels did not change. These results suggest that decreased lipid transport and enhanced lipid mobilization may not play major roles in the induction of the phenobarbital fatty liver.

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1. Sorrell, M. F., Tuma, D. J., Noffsinger, J. K., and Barak, A. J., Proc. Soc. Exp. Biol. Med. 143, 839 (1973).

2. Lombardi, B., Fed. Proc. 242, 1200 (1965).

3. Judah, J. D., McLean, A. E. M., and McLean, E. K., Amer. J. Med. 49, 609 (1970).

4. Farber, E., Advan. Lipid Res. 5, 119 (1967).

5. Glaser, G., and Mager, J., Biochim. Biophys. Acta 261, 487 (1972).

6. French, S. W., J. Nutr. 88, 292 (1966).

7. de Pury, G. G., and Collins, F. D., Lipids 7, 225 (1972).

8. Folch, J., Lees, M., and Sloan Stanley, G. H., J. Biol. Chem. 226, 497 (1957).

9. Searcy, R. L. and Berquist, L. M., Clin. Chim. Acta 5, 192 (1960).

10. Soloni, F. G., Clin. Chem. 17, 529 (1971).

11. Bartlett, G. R., J. Biol. Chem. 234, 466 (1959).

12. Soloni, F. G., and Sardina, L. C., Clin. Chem. 19, 419 (1973).

13. Barak, A. J., Clin. Chem. 16, 222 (1970).

14. Masoro, E. J., "Physiological Chemistry of Lipids in Mammals," 253 pp. W. B. Saunders Co., Philadelphia (1968).

15. Robinson, D. S. and Seakins, A., Biochim. Biophys. Acta 62, 163 (1962).

16. Suryanarayana Rao, K., Biochem. Pharmacol. 20, 2825 (1971).

17. Robinson, D. S. and Harris, P. M., Biochem. J. 80, 361 (1961).

18. Farber, E., Lombardi, B., and Costillo, A. E., Lab. Invest. 12, 873 (1963).

19. Glaser, G. and Mager, J., Biochim. Biophys. Acta 261, 500 (1972).

20. Seakins, A. and Robinson, D. S., Biochem. J. 86, 401 (1963).

21. Recknagel, R. O. and Goshal, A. K., Exp. Mol. Pathol. 5, 413 (1966).

22. Recknagel, R. O., Pharmacol. Rev. 19, 145 (1967).

23. Roheim, P. S., Switzer, S., Girard, A., and Eder, A., Biochem. Biophys. Res. Comm. 20, 416 (1965).

24. Pottenger, L. A., and Getz, G. S., J. Lipid Res. 12, 450 (1971).

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