Biliary Lithocholate and Cholestasis during and after Total Parenteral Nutrition: An Experimental Study (43946)

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Abstract. **To evaluate the role of lithocholic acid (LCA) in the etiology of parenteral nutrition-associated cholestasis (PN-AC), we studied (i) the changes in the percentage of biliary LCA and (ii) the emergence and resolution of cholestatic changes in the liver after total parenteral nutrition (TPN) and after 6 weeks of oral feeding following the TPN. We compared these changes in rabbits on TPN support (via a central vein) for 14 days (TPN, $n = 8$) with those after 6 weeks of refeeding (Post-TPN, $n = 8$). Age-matched rabbits on lab chow served as controls (CHOW, $n = 8$). At the end of the diet regimens, the common bile duct was cannulated under anesthesia, and hepatic bile collected for measurements of bile flow and bile acid (BA) secretion rates, and BA profiles. The 60-min biliary excretion of sulfobromophthalein (BSP) after an intravenous bolus (5 mg/kg) was determined. A liver biopsy was taken for light microscopy. After 14 days of TPN, bile flow was reduced by 60%, bile acid secretion by 52%, and BSP excretion by 38%. On refeeding, only the BSP excretory rate recovered fully. In the TPN group, histology of the liver showed hepatocellular degeneration and portal tract inflammation; these resolved after refeeding leaving a mild portal fibrosis in 4/8 rabbits. Total colonic stasis occurred during TPN. With TPN, a decrease in the percentage of biliary glycochenodeoxycholate and an increase in LCA% were seen, whereas after refeeding the increase was in the percentage of glycoursodeoxycholate. An LCA% ≥ 6 was associated with liver cell damage. After 6 weeks of refeeding, the structural cholestasis disappeared, but the decreases in basal bile flow and bile acid secretion (functional cholestasis) persisted. These data associate an increase in biliary LCA with the emergence of cholestasis during TPN in rabbits.** [P.S.E.B.M. 1995, Vol 210]

Since the introduction of total parenteral nutrition (TPN), an iatrogenic syndrome, parenteral nutrition-associated cholestasis (PN-AC) has been seen with disturbing frequency in infants and children maintained on long-term intravenous feeding (1). Rager and Feingold (2) suggested that the enteral fast enforced during TPN rather than the parenteral feeding contributed significantly to the hepatobiliary dysfunction.

During TPN in the human adult with inflammatory bowel disease, Fouin-Fortunet *et al.* (3, 4) have reported an increase in biliary lithocholic acid (LCA) levels associated with the appearance of cholestatic changes in the liver. But it is not clear whether the changes in biliary LCA and liver histology were due to the enteral fast enforced during TPN, the factors responsible for the bowel disease, or the TPN infusate. To evaluate the role of LCA in the etiology of PN-AC, we studied (i) the changes in the percentage of biliary LCA and (ii) the emergence and resolution of cholestatic changes in the liver during and after TPN in rabbits. We compared the changes after 14 days of TPN with those after 6 weeks of oral refeeding following a similar regimen of total intravenous feeding.

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Materials and Methods

Coccidia-free female NZW rabbits (body weight 1.8–2.4 kg; age 9–11 weeks; Hazleton Inc., Denver, PA) were housed individually in cages with large mesh floors, under controlled conditions of an alternate 12:12-h light:dark cycle, at a room temperature of 21°–23°C. The animals were allotted to control and experimental groups. The control group of animals (CHOW, $n = 8$) ate standard laboratory rabbit chow *ad libitum* for 14 days. Of the two subsets of rabbits in the experimental group, one sub-set (TPN, $n = 8$) was nourished by TPN for 14 days, and then sacrificed. Rabbits in the second subset (Post-TPN, $n = 8$) were also maintained on TPN for 14 days, after which they had a liver biopsy and were returned to a diet of lab chow for 6 weeks.

The TPN infusates were a solution of dextrose and amino acids and a 10% soybean oil/egg phospholipid emulsion. Together they provided carbohydrates 14.4 g, protein (as amino acids) 3.6 g, and lipid 4 g/kg/day. The energy intake was 110 kcal/kg day. Electrolytes, vitamins and trace elements were added to the amino acid mixture. These rations are considered adequate for the normal growth of young rabbits (5). All animals had unlimited access to water.

Venous Cannulation. Under sterile conditions and general anesthesia with ketamine (40 mg/kg, im) and xylazine (4 mg/kg, im), a silicone infusion cannula was implanted in the jugular vein of the rabbits in the TPN groups (6). A swivel-cable infusion assembly was attached to the cannula. The amino acid-dextrose mixture was delivered at 6 ml/kg/hr and the lipid emulsion at 1.7 ml/kg/hr as continuous infusions by separate metered pumps. Initiation of TPN was delayed for 24 hr to allow for recovery from the cannulation procedure.

Terminal Study. On the 15th morning of the diet regimen, the rabbits in the CHOW and TPN groups underwent a laparotomy under general anesthesia and mechanical ventilation. The TPN infusion was terminated 2 hr before laparotomy. The cystic duct was occluded with a hemoclip. The proximal bile duct was cannulated with polyethylene tubing (70-cm long) for gravity drainage of bile into tared cryo-tubes. Serial 10-min collections of bile were made, and the bile volumes were determined gravimetrically. Fluid balance was maintained with an intravenous infusion of 0.15 *M* NaCl solution at 25 ml/hr. Core temperature was monitored by a rectal probe and maintained between 38° and 39°C with an electric heating pad.

Bile flow stabilized over a 40-min baseline period. Basal bile flow (μl/kg/min) was calculated as the average of the bile collections between the 21st and 40th min. Then, a bolus of sulfobromophthalein (BSP) in 5% dextrose in water (BSP dose: 5 mg/kg) was given intravenously. Bile samples were collected over the following 60 min. Finally, a wedge biopsy was taken from the right anterior lobe of the liver for light microscopy; and the animal was sacrificed with pentobarbital (150 mg/kg, iv). In the Post-TPN group, on the last morning of TPN, only a liver biopsy was done. These animals were returned to lab chow. After 6 weeks of oral feeds, the terminal study including a second liver biopsy was done. A necropsy was done on all animals at the end of the terminal study. The histopathology of the liver samples was evaluated by the pathologist (I. L. U.) in a blinded fashion.

Chemical Analyses. Bile *BSP* concentrations were assayed by colorimetry after alkalinization of the bile samples (7). Total bile acids in hepatic bile were measured enzymatically with 3-α-hydroxysteroid dehydrogenase (kits from Nycomed AS, Oslo, Norway).

High-performance liquid chromatography. The distribution of conjugated bile acids in hepatic bile was determined according to the isocratic high-performance liquid chromatography (HPLC) technique of Rossi *et al.* (8). Conjugated bile acid standards were obtained from Steraloids Inc. (Wilton, NH) and Calbiochem (La Jolla, CA), and the HPLC-grade reagents from Sigma Chemical Co. (St. Louis, MO). The HPLC unit consisted of a Waters M6000-A pump and U6K sample injector coupled with an Ultrasphere C_{18} octadecylsilane (25 cm × 4.6 mm) column (Beckman, Fullerton, CA). A BioRad 1306 UV-monitor and a Hewlett-Packard 3390-A integrator completed the system. The solvent system was methanol-phosphate buffer. The solvent flow rate was 1.1 ml/min under an operating pressure of 2300–2500 psi. Diluted bile samples were processed through C_{18} BondElut (Analyt Internat, Harbor City, CA) cartridges under vacuum; the eluted samples were concentrated to 1 ml under N_2, and passed through a 0.45 μm nylon filter (8). Five to ten microliters of the filtrate were injected into the HPLC unit. The relative concentrations of six conventional bile acids were detected by their absorbances at 200 nm. Testosterone was the internal standard.

Statistical Analysis. The grouped data were expressed as means and standard errors (SEM). The differences between means were tested by one-way analysis of variance (ANOVA) and Dunnett's tests. When variances were nonhomogeneous, nonparametric (Kruskal-Wallis) tests and Dunn's multiple comparison tests were done (InStat program, version 2.0; Graph Pad software, San Diego, CA). Statistical significance was set at $P < 0.05$.

Results

Over the 14 days of TPN, weight gain was minimal (average 6 g/day), with one of the eight rabbits losing weight. In the chow-fed group, the average weight gain was 23 g/day. Over the 6 weeks after TPN, the weight gain was 13 g/day; this was in keeping with the slower

weight gain (15 g/day) in chow-fed rabbits from the 12th to 18th week of life.

At necropsy, the liver was enlarged, pale, and friable in the TPN group. The cecum was distended with both gas and a fetid, dark fluid: fecal pellets were absent from the gut. The liver and cecum appeared normal in the CHOW and Post-TPN groups, and the colon contained numerous fecal pellets.

Hepatobiliary Dysfunction. Hepatobiliary function and serum bile acid concentrations are outlined in Table I. On total intravenous feeding, basal bile flow and bile acid secretion rates and the capacity to excrete the cholephilic dye, BSP, were low after 14 days, compared with chow-fed levels. Serum bile acid concentrations were high. After 6 weeks of oral feeding following the TPN regimen, bile flow and bile acid secretion rates were still low, whereas the capacity to secrete BSP and the concentration of serum bile acids reverted to the normal range.

Conjugated Bile Acids in Hepatic Bile. In our laboratory, the recoveries by HPLC of bile acids added to pooled, normal rabbit bile (over a concentration range of 0.10 to 10.0 m*M*) were 85% (SEM 8%) for glycoursodeoxycholate, 92% (SEM 2%) for glycocholate, and 77% (SEM 5%) for glycodeoxycholate.

The relative percentages of the six conventional bile acids in hepatic bile isolated by HPLC (taurocholic acid [TCA], glycocholic acid [GCA], glycodeoxycholic acid [GDCA], glycochenodeoxycholic acid [GCDCA], glycoursodeoxycholic acid [GUDCA], and glycolithocholic acid [GLCA]) are shown in Table II and in Figure 1. Glycodeoxycholic acid was the major bile acid. The percentages of GDCA and of total (glyco- and tauro-) amidated cholic acid remained essentially unchanged in the three groups. Taurocholic acid was absent in the Post-TPN group of rabbits. This was presumably the result of the switch-over to glycine conjugation which occurs in herbivorous animals as they approach adulthood (9). The percentage of glycochenodeoxycholic acid was low in the TPN group, with a further lowering after oral feeding (TPN vs Post-TPN: $P < 0.05$) (Fig. 1). This stepwise depression was associated with a high glycolithocholic acid fraction in the TPN group (Chow vs TPN: $P < 0.05$), and a sharply elevated glycoursodeoxycholic acid fraction in the Post-TPN group (TPN vs Post-TPN: $P < 0.05$).

Table II. Percentages of TCA, GCA, and GDCA in Hepatic Bile during Chow Feed, TPN, and 6 Weeks after TPN

	%TCA	%GCA	%GDCA
CHOW	2.8 [0.1–6.6]	9.0 [3.3–12.9]	75.1 [70.0–78.7]
TPN	8.6 [5.8–9.8]	3.1 [0.8–7.9]	76.1 [72.8–80.7]
Post-TPN	0.0	13.8 [9.1–17.4]	78.5 [73.2–82.0]
CHOW vs TPN *P*	0.016	NS	NS
CHOW vs Post-TPN *P*	—	NS	NS
TPN vs Post-TPN *P*	—	<0.01	NS

Note. Values are expressed as medians, with 95% confidence intervals in the brackets. TCA, taurocholic acid; GCA, glycocholic acid; GDCA, glycodeoxycholic acid. Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple comparisons test.

Histologic Changes in the Liver. The morphologic changes seen in the liver on light microscopy after 14 days of TPN were dilated central veins, balloon degeneration of hepatocytes, mostly in the centrilobular area and moderate-to-severe in degree, and infiltration of portal triads with mononuclear cells (Table III and Fig. 2 and 3). In two of the eight rabbits in the TPN group (Table IV, PN-123 & 124), sinusoidal congestion was the only abnormality in the liver. Biliary LCAs in these two rabbits did not exceed 3%. However, their bile flow and bile acid secretion rates were low.

In the eight animals in the Post-TPN group, the

Table I. Hepatobiliary Function and Serum Bile Acids during Chow Feed, TPN, and after TPN

	Bile flow (μl/kg/min)	BA secretion (μmole/kg/min)	Bile BSP (% dose/60 min)	Serum BA (μ*M*)
CHOW	81.4 (6.2)	0.725 (0.047)	82.5 (1.9)	2.9 (0.5)
TPN	32.0 (3.1)	0.348 (0.040)	50.8 (4.2)	13.2 (2.3)
Post-TPN	52.7 (1.8)	0.314 (0.032)	78.6 (1.9)	4.1 (1.1)
CHOW vs TPN *P*	<0.001	<0.01	<0.001	<0.01
CHOW vs Post-TPN *P*	NS	<0.01	NS	NS
TPN vs Post-TPN *P*	NS	NS	<0.01	<0.1

Note. Values are expressed as means, with SEM in the parentheses. BA, bile acid; BSP, sulfobromophthalein. Because of nonhomogeneity of variances, nonparametric tests were done for bile flow, BSP%, and serum bile acids.

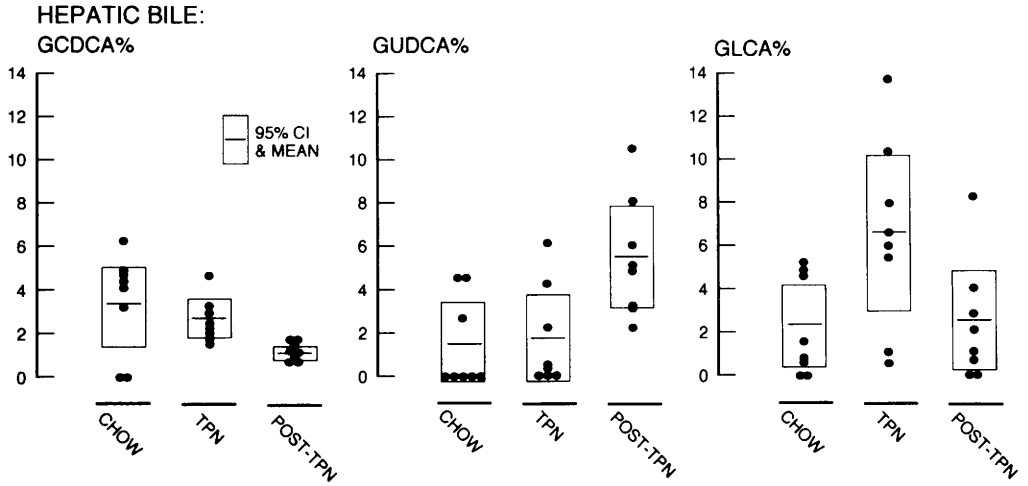

Figure 1. The relative proportions of biliary glycochenodeoxycholic acid (GCDCA), glycoursodeoxycholic acid (GUDCA), and glycolithocholic acid (GLCA) after chow feed, after TPN, and after 6 weeks of oral refeeding. The boxes show the 95% confidence intervals, and the horizontal bars the means.

Table III. Histological Changes in Liver after 14 Days of TPN (A) and after 6 Weeks of Refeeding (B)

Rabbit		Balloon degeneration	Periportal inflammation	Portal fibrosis
PN-539	A:	1	1	0
	B:	0	0	1
PN-541	A:	3	2	2
	B:	0	0	1
PN-542	A:	3	1	2
	B:	0	0	1
PN-543	A:	3	1	2
	B:	0	0	1
PN-544	A:	3	0	1
	B:	0	0	0
PN-545	A:	2	0	1
	B:	0	0	0
PN-546	A:	2	0	2
	B:	0	0	0
PN-547	A:	2	0	1
	B:	0	0	0

Grading: 0 = no morphological changes. For balloon degeneration: 1 = ballooned cells extend <25% of distance from terminal hepatic vein to protal triad; 2 = 25%–50%, 3 = >50% of the distance. For periportal inflammation: 1 = inflammatory cells infiltrate <25% of portal triad area, 2 = they fill 25%–50%; 3 = fill 50%–100% of the area. For portal fibrosis: 1 = fibrosis limited to triad area; 2 = fibrosis extending outside the triad; 3 = bridging fibrosis.

periportal inflammation and balloon degeneration of the hepatocytes seen at the end of the TPN regimen (Fig. 3), had disappeared after 6 weeks of oral feeding (Fig 4). But, fibrosis limited to the portal triads was seen in four of the eight rabbits in the Post-TPN group (Table III).

Discussion

Fouin-Fortunet and coworkers (3, 4) reported similarities between the liver lesions produced experimentally in rabbits fed lithocholate and those during TPN in humans with inflammatory bowel disease, viz., round cell infiltration of portal tracts and periportal fibrosis. These lesions were presumed to be due to accumulation of lithocholic acid in bile.

In this study, we show an association between an increase in biliary lithocholic acid and the emergence of cholestasis during the enteral fast of TPN in normal rabbits. We also show resolution of the histopathologic changes *after* resumption of oral feeds. Our data indicate that when the biliary LCA content is $\geq 6\%$, liver damage occurs in the rabbit (Table III).

The significant aspect of our study is the appearance of morphologic changes in the liver during TPN in rabbits, changes similar to those seen in human infants after long-term TPN. To date, the cholestatic changes seen after TPN in other animal models have been mostly functional in nature: decreases in bile flow and bile acid secretion rates, increased lithogenicity of bile in rats (10), and gallbladder stasis in the prairie dog (11) and piglets (12). The structural changes in the hepatocyte have been steatosis confined to the centrilobular region in guinea pigs (13), and "loss of cytoplasmic basophilia of the centrolobular hepatocytes" in rats (14). Inflammation of the portal triad (triaditis), which is considered highly characteristic of TPN-associated cholestasis in the human infant, was not seen in these other species.

In this study, triaditis and centrilobular degeneration of hepatocytes were the histologic lesions found in 12 of the 14 rabbit livers examined immediately after 14 days of TPN. The intense round cell infiltration of the portal triad we had previously described in rabbits after 10 days of TPN (15) had been replaced by portal fibrosis after 14 days of TPN in the current study. A similar progression of the round cell infiltration to fibrosis of the portal tract with time is often seen in

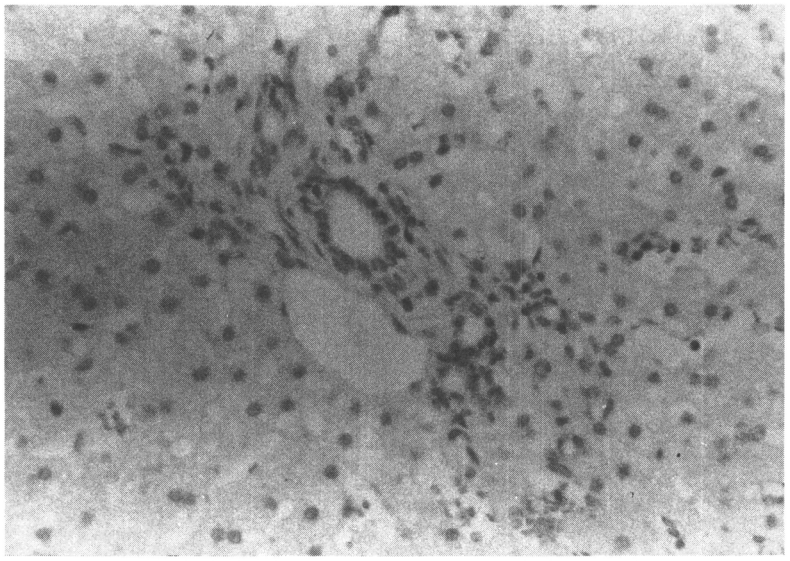

Figure 2. Rabbit PN-541: histopathology of liver after 14 days of TPN shows extensive balloon degeneration (grade 3) with round cell infiltration of the portal tract (grade 1) (×400).

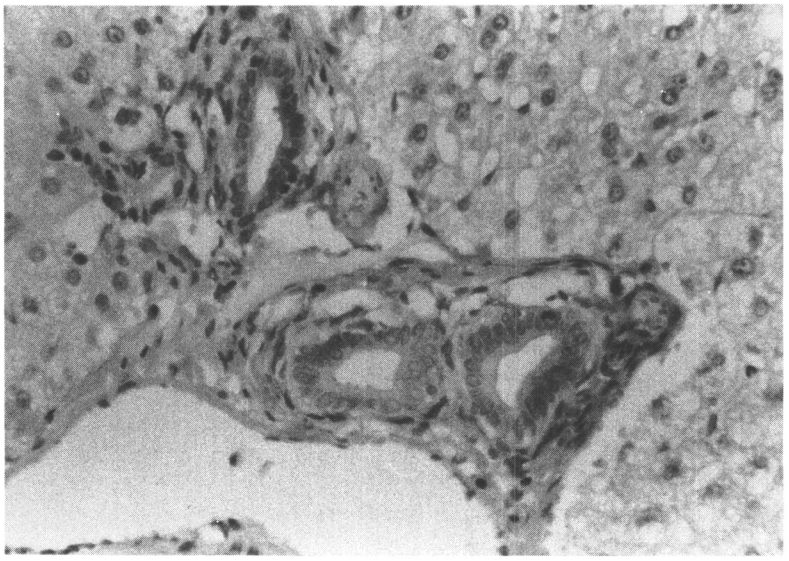

Figure 3. Rabbit PN-542: After 14 days of TPN, the liver shows balloon degeneration (grade 3) of the hepatocytes adjacent to the portal tract. Mononuclear cell infiltration (grade 1 triaditis) and fibrosis (grade 2) are present in the area of the portal tract; but the infiltrate does not extend beyond the limiting plate (×400).

infants with PN-AC. Unlike in the human infant with PN-AC, ductular proliferation was not seen in the rabbits on TPN. The hepatocellular degeneration in the centrilobular area and portal fibrosis during TPN in these rabbits were similar to the structural changes in the liver seen after prolonged oral feeding of rabbits with lithocholate (16); however, foci of hepatocellular necrosis were not seen.

The factors central to the susceptibility of the rabbit liver to damage during TPN are (i) the rabbit's inability to detoxify LCA and (ii) the presence of a large cecum. Detoxification of LCA is effected either through rehydroxylation of the monohydroxy bile acid as in the rat (17), or by its sulfation (and amidation) as in humans and their kindred primate, the chimpanzee (18, 19). Rabbits lack the ability to detoxify LCA by rehydroxylation or sulfation (20). Sulfotransferase, the enzyme which sulfates LCA at the 3 position, is present in the livers of the guinea pig, chimpanzee, and humans. The activity of this enzyme is low in the human fetus (at about 14% of normal adult levels), in the fetal guinea pig, and also in adult man with hepatic disease (21). Therefore, immature newborns and older humans with liver disease are at risk to LCA toxicity during TPN.

In the rabbit, the cecum acts as a hindgut fermenter where water and microorganisms are selectively retained for digestion of cellulose and the syn-

Table IV. Liver Function, Biliary Lithocholic Acid Percentages and Liver Histology during TPN

	Bile Flow (μl/kg/min)	Bile acid secretion (μmole/kg/min)	%GLCA	Liver histology
TPN				
PN-104	50.4	0.311	6.4	BD/PIn
PN-105	21.5	0.262	13.7	HD/PIn
PN-106	34.5	0.271	10.5	No Hist
PN-107	34.9	0.596	5.6	No Hist
PN-118	22.5	0.468	6.6	HD/PIn/PF
PN-119	27.4	0.290	8.0	HD/PIn
PN-123	19.4	0.336	2.9	SinuCong
PN-124	25.1	0.354	1.3	SinuCong
Chow-fed				
Mean	79.9	0.742	2.1	Normal
95% Conf. interval	66.8–92.6	0.622–0.861	0.1–4.1	

Note. GLCA, glycolithocholic acid; BD, balloon degeneration; HD, hydropic degeneration; PIn, portal inflammation; PF, portal fibrosis. SinuCong, sinus congestion; No Hist, no histology available.

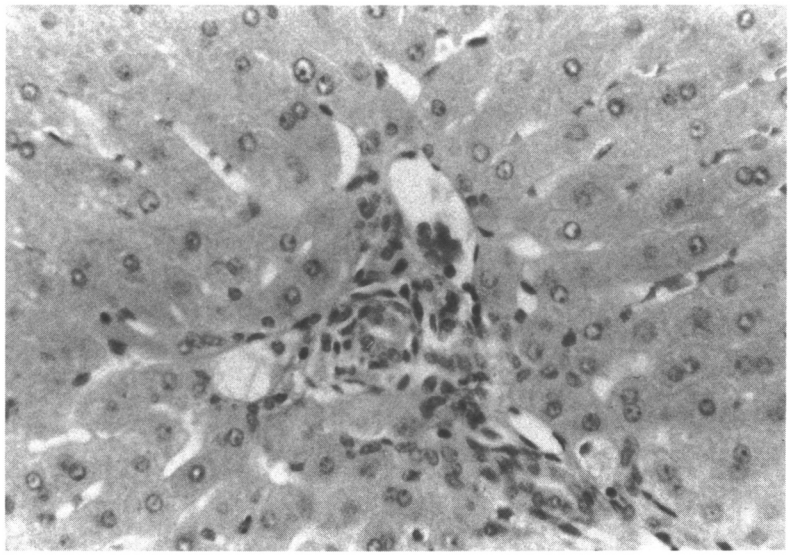

Figure 4. Liver of Rabbit PN-542, 6 weeks after TPN. The hepatocytes are normal in appearance. The triaditis has resolved, but there is some evidence of fibrosis (grade 1) in the portal tract (×400).

thesis of proteins (22). As cecal contents are refluxed continually between cecal base and apex, microbial 7-α-dehydroxylation of cholic acid to deoxycholic acid and of chenodeoxycholic acid to lithocholic acid occurs (23). In the orally fed rabbit, the rapid transit of intestinal contents and their fecal excretion minimize bacterial colonization of the gut (24, 25). Conversely, an enteral fast results in total intestinal stasis in the rabbit, and the proliferation of microflora. Stooling was absent in all the rabbits during the enteral fast of TPN. As fluid and micro-organisms stagnate in the capacious cecum, biotransformation of the bile acids in intestinal chyme by the cecal microflora can proceed unabated. Hepatic injury results with the intestinal absorption and accumulation of unsulfated LCA in bile.

Another interesting finding was the appearance of biliary glycoursodeoxycholate as the major conversion product of glycochenodeoxycholate in the Post-TPN group. 7-α-Dehydroxylation of chenodeoxycholic acid to LCA by fecal bacteroides is the favored process *in vivo* (22, 26). When Clostridia dominate the microbial environment in the cecum, epimerization of chenodeoxycholic acid to ursodeoxycholic acid occurs (27). Major shifts in the dominance of one or other of these two microbial species in the cecum can account for the biotransformation of chenodeoxycholic acid to lithocholic acid during TPN, and of chenodeoxycholic acid to ursodeoxycholic acid in the Post-TPN period (Fig. 1).

We conclude that the enteral fast and its sequela (biliary accumulation of LCA) contributed to the cholestasis associated with TPN in the rabbits. On refeeding, there was resolution of the liver lesions, but bile flow and bile acid secretion were still depressed.

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This project was approved by the Institutional Animal Care and Use Committee at Children's Memorial Hospital, Chicago IL. The animal experiments were conducted in accordance with the NIH/USPHS guidelines for the humane care and use of laboratory animals.

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