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MASTER

TOXIC EFFECTS OF LITHOCHOLIC ACID
AND RELATED 5 β -H STEROIDS

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Lithocholic acid, and several related steroid metabolites, have significant toxicity for sub-cellular particles, whole cells, tissues and organs, and entire organisms. These steroids are important, endogenous compounds in man, and knowledge of their pharmacological effects has relevance to the understanding of human health and disease. This review will be concerned primarily with their injurious effects, which can result in cellular damage, inflammation, and fever.

The lytic properties of bile and bile acids were well recognized by the early 19th century.¹ They have been studied using a wide variety of single celled species, but most work has been done with erythrocytes. Knowledge of the structural basis for this effect stems from the classical paper by Berliner and Schoenheimer in 1938,² who examined a number of bile acids and sterols and found that those with a 3 α hydroxyl group and a 5 β -H, or A:B ring cis structure, had the greatest hemolytic activity. Within this group, activity varied inversely with the number of oxygen functions. Several years ago we became interested in the structural similarity between lithocholic acid and neutral steroid pyrogens, such as etiocholanolone. This prompted us to compare the hemolytic effects of certain neutral and acidic steroids.³ Since most of these steroids are relatively insoluble in aqueous solutions, we decided to incorporate sufficient methanol in the system to insure steroid solubility.

Steroids in methanol were added to red cells in buffered saline. After incubation at 37°, the suspensions were centrifuged and the hemoglobin in the supernatant determined. The hemolysis produced by several steroids is shown in Fig. 1. Lithocholate and its conjugates, together with deoxycholate, are considerably more hemo-

lytic than chenodeoxycholate and cholate. The neutral steroids are grouped together between chenodeoxycholate and cholate. Fig. 2 depicts the relative hemolytic activity of several neutral steroids, and also illustrates the old findings of Ponder⁴ and others that serum inhibits steroid hemolysis. The open bars represent hemolysis produced by the steroid in saline, and the hatched bars represent the hemolysis produced in 16% human serum. Cholesterol, not shown, seems to be less effective in protecting against neutral steroid hemolysis than against bile acid hemolysis.

Steroid hemolysis of all kinds is extremely temperature dependent. The increasing hemolytic activity of progesterone with relatively small increments in temperature is shown in Fig. 3. This finding, together with the absence of methemoglobin production during steroid hemolysis, suggests an effect on the cellular membrane, rather than a generalized inhibition of metabolism.

The toxicity of these steroids also extends to sub-cellular particles. Wiseman^{5,6} has shown that their ability to disrupt lysosomes parallels their hemolytic activity. Similarly, their ability to lyse "liposomes", which are artificial membranous structures composed of cholesterol and phospholipid, also correlates well with their hemolytic properties.^{5,7} Finally, Lee and Whitehouse have studied the inhibitory effects of bile acids on mitochondrial oxidative phosphorylation.⁸ Again, the structure-activity relationship mirrors that for hemolytic activity.

The precise way in which these steroids interact with membranes of cells and organelles to cause their disruption remains to be clarified. Certainly solubilization of membrane cholesterol, a theoretical possibility with some bile salts, is not a factor in lithocholate and neutral steroid hemolysis. Pethica and Schulman⁹ have presented

data indicating that a critical amount of bile salt, about 45 million molecules, must be bound to each red cell to produce lysis; however, the significance of this observation is still not clear. Despite the effect of bile salts on oxidative phosphorylation, metabolic processes are probably not involved. In addition to the evidence from red cell studies, lysis of bacteria at 0°, or of heat killed bacteria, is presumably unrelated to enzymatic processes. It is therefore reasonable to suppose that some physical phenomenon, perhaps involving a phase-transition, occurs within the membrane.

These cytotoxic effects of bile acids also render them harmful to tissues and organs, although this direct cellular activity may not be the only way by which they cause injury. There has been much interest in their effects on the epithelium of the gall bladder and gastro-intestinal tract. Dr. Scheiner has reviewed very nicely some of the more recent work concerning their effects on small intestinal mucosa. Bile salts have also been implicated in the etiology of pancreatitis; however, earlier work often failed to take into consideration the wide spectrum of bile acid toxicity, from lithocholate and deoxycholate on the one hand to taurocholate and dehydrocholate on the other. Hansson et al. have emphasized the difference between dihydroxy and trihydroxy bile salts in their ability to produce experimental pancreatitis; cheno-deoxycholate and deoxycholate were much more toxic than cholate. They also observed that 2 out of 5 patients with acute pancreatitis had high levels of unconjugated bile salts in their bile. The importance of conjugation in detoxifying some, though not all, bile acids has been emphasized by Dawson and Isselbacher,¹¹ among others. To my knowledge, adequate investigations on the presence or absence of lithocholate and its conjugates in acute hemorrhagic pancreatitis have not been performed.

One of the more interesting examples of bile acid toxicity is the "ductular cell reaction". This was first described by Holsti, in Helsinki.¹²⁻¹⁴ In 1954 he reported that rabbits fed dessicated hog bile develop cirrhosis of the liver (Fig. 4), and, after three or four months, exhibit jaundice and ascites. Figures 5 and 6 show the histology of a normal rabbit liver and the liver of a rabbit with cirrhosis. In the cirrhotic liver in Fig. 6, cells at the periphery of the nodule are degenerating, and the portal fields are greatly enlarged, with a copious inflammatory exudate. The proliferated bile ducts have thickened epithelium. Dense connective tissue bands connect adjacent portal fields. Most of the changes are completely reversible, and animals taken off the diet retain only a few delicate fibrous septa connecting the portal tracts.

In an elegant series of experiments, Holsti showed: first, that the active principle resided in the bile acid fraction of bile, and second, that the active bile acid was lithocholate.¹³ He then tested a large series of bile acids, and found that glycolithocholate and chenodeoxycholate also produced cirrhosis.¹⁴ It is still not clear whether chenodeoxycholate produces this effect per se, or whether it is first metabolized to lithocholate and glycolithocholate, but the latter possibility seems more likely. Since these studies, several investigators have fed lithocholate to a large number of species, including reptiles,¹⁵ amphibia,^{16,17} birds,^{18,19} rodents,²⁰⁻²⁴ and primates.²⁵ While the morphological changes vary slightly in detail, bile duct and ductular cell hyperplasia is a consistent finding. It is only reasonable to assume that lithocholate would have similar effects in humans.

Rats have been found to be relatively resistant to the cirrhotic activity of lithocholate. Since polyhydroxylated bile salts are less toxic, in general, it seemed

that the resistance might be related to the well-known ability of rat liver to hydroxylate bile acids. In attempting to overwhelm this hydroxylating capacity with large amounts of lithocholate, we unexpectedly found that the rats developed large common duct gallstones. Similar observations have been reported by Eysen²¹ and Carey.²⁴

Fig. 7 shows the common duct of a rat fed 1% lithocholic acid in a low protein diet. The duct is filled with yellow-green stones, which consist predominantly of the calcium salts of free or glycine conjugated lithocholate and its 6 β -hydroxy metabolite. The color of the stones and the proportion of free and conjugated bile salts may vary, depending on the presence or absence of infection in the obstructed and inflamed common duct.

Figures 8 and 9 show a portal field with impressive bile duct proliferation. Proliferation begins within 12 hours of lithocholate administration, as determined by the incorporation of tritiated thymidine. The intense acute and chronic inflammatory reaction seems to consist predominantly of eosinophiles. The common duct is involved in a similar process, and its ductular glands proliferate extensively.

Numerous ductular cells appear to slough off into the lumen (Figs. 10 and 11), where they degenerate to form eosinophilic debris. (One of the important questions about the liver pathology induced by lithocholate is whether proliferation is simply an attempt to replace these injured cells, or whether some more fundamental effect on cell differentiation and growth is involved.) The eosinophilic material apparently condenses around precipitated bile salt to form microcalculi. Stone formation does not occur if the diet contains sufficient sulfur-containing amino acids to permit taurine conjugation of the bile salts; apparently these conjugates are sufficiently polar to stay

in solution. Stone formation can also be inhibited by adding cholic acid or cholesterol to the diet. These substances effectively enlarge the bile acid pool, and Hofmann and Small²⁶ have shown that 1 part of lithocholate can be solubilized by 9 parts of polyhydroxylated bile salt. The gallstones and bile duct proliferation are independent phenomena, and stone formation is primarily of interest as an experimental model.

Lithocholic acid and related neutral steroids also cause intense inflammation following their intra-muscular injection into man and a number of other species. This property was first described by Kappas in connection with the pyrogenic activity of etiocholanolone.²⁷ The production of fever by etiocholanolone and other pyrogenic steroids is always associated with inflammation, and the relationship between these two activities has been a provocative one.¹ The available evidence now suggests that inflammation is a necessary condition for the febrile response, but that it is not by itself a sufficient condition. Fig. 12 shows the febrile response resulting from the intra-muscular injection of 6 mg of lithocholate.²⁸ Like other steroid pyrogens, it causes a dramatic fever. The fever begins after a prolonged lag period of 4-8 hours, and reaches a peak between 10 and 12 hours. It is preceded by a local inflammatory reaction, characterized by pain at the injection site and a rise in local skin temperature. A systemic leukocytosis develops slightly before the onset of fever, and increases abruptly with the fever. The leukocytosis and the local inflammation both persist long after the fever has subsided.

The chemical structure of a number of 19, 21, and 24 carbon steroids with pyrogenic activity is shown in Fig. 13. The structural basis for pyrogenicity is similar

to that for cytotoxicity, except that it is somewhat more stringent. The 3 α -hydroxy 5 β -H structure is extremely important, and any hydroxyl groups projecting to the rear of the molecule abolish pyrogenicity. The 17 α hydroxyl group of corticoid metabolites and the 7 α and 12 α hydroxyl groups of bile acids are examples. Physiological conjugation of neutral steroid pyrogens also abolishes pyrogenic activity (Fig. 14). Both the sulfate and glucuronide conjugates are non-pyrogenic.

In contrast, as shown in Fig. 15, a number of derivatives of lithocholate retain pyrogenic activity. These include the 3 acetyl derivative, the 24 methyl derivative, and the physiological glycine conjugate. Conjugation with taurine, however, abolishes pyrogenicity, despite the fact that inflammatory activity remains comparable to that of pyrogenic steroids. ^{Figure 16.} This finding emphasizes the point that physiological conjugation does not necessarily detoxify steroids. As a corollary, the type of conjugating substance may determine the physiological and pharmacological properties of the conjugate.

Dillard and Bodel³⁰ have recently presented some most interesting data that make it possible to form a reasonable hypothesis about the mechanism of steroid-induced fever. They found that human leukocytes incubated with pyrogenic steroids release endogenous, or leukocyte, pyrogen. This process is specific for human leukocytes and requires 4 to 8 hours of incubation. These facts probably account for the striking species specificity and prolonged lag period of steroid-induced fever. Local inflammation may be important in delaying steroid absorption and facilitating leukocyte contact with steroid pyrogen. Time does not permit the fuller development of this hypothesis.

Of the several steroids discussed here, lithocholate has been of particular

interest because of its potent and diverse toxic properties, and because it is a major endogenous substance. Six mg are sufficient to give a most uncomfortable fever to a 70 kg man, whereas literally hundreds of mg are produced each day in humans. Furthermore, unlike neutral steroid pyrogens, its physiological taurine and glycine conjugates are also highly toxic.

During investigations on the metabolism of lithocholate in humans, Norman and ¹³¹I found that approximately half of the orally administered C¹⁴-labeled lithocholate recovered from bile was in a form more polar than the taurine and glycine conjugates. These substances have recently been identified as the 3-sulfate esters of glycolithocholate and tauroolithocholate.³² This is, to my knowledge, the first description of an endogenous bile acid sulfate. The origin, fate, and physiological significance of these sulfates is unknown, but they may possibly provide effective detoxification for lithocholate and its conjugates. Preliminary results suggest that endogenously formed lithocholate is not sulfated to a similar extent, but this area will have to be thoroughly investigated under a variety of conditions.

Fig. 17 shows the chromatographic system used to isolate the sulfates by preparative chromatography.³² The taurine and glycine conjugates of bile acids move together, and separation depends primarily on the number of hydroxyl groups on the steroid nucleus. However, tauroolithocholic acid-3-sulfate and glycolithocholic acid-3-sulfate remain near the origin. The behavior of lithocholic acid-3-sulfate in this system is anomalous. In the more commonly used acidic systems, bile acid sulfates move chromatographically in areas associated with other bile acids. Fig. 18 shows the mobility of a number of bile acids in Hofmann's propanol system.³³ It also shows the chromatography

of bile from a patient who had received C^{14} -labeled lithocholate orally. The location of the radioactive metabolites can be compared with the major bile acid constituents in bile. Their detection and identification without the radioactive tracer would be unlikely.

In summary, the extensive biological toxicity of lithocholate and related steroids has been described. Lithocholate and its taurine and glycine conjugates are the most potent steroids known in their ability to disrupt cells and cellular membranes. Lithocholate and glycolithocholate are unique among bile acids in possessing both the structural requirements for pyrogenicity and the capacity to induce bile duct proliferation. Lithocholate and its derivatives are also the only bile acids known to undergo physiological sulfate conjugation, but it is not yet known whether this results in detoxification. Further studies on the metabolism and pharmacological activity of lithocholate and its derivatives may clarify their significance in health and disease.

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FIGURE LEGENDS

Fig. 1. --Relative Hemolytic Activity of Steroids. The system consists of the steroid dissolved in methanol, 0.5 ml; phosphate buffered saline, pH 6.6, 2.0 ml; 50 per cent washed human red cells, 0.05 ml. After centrifugation, the cells were centrifuged, the hemoglobin in the supernatant converted to cyanmethemoglobin with Drabkin's reagent, and the optical density determined.³

Fig. 2. -- Serum Inhibition of Steroid Hemolysis.³

Fig. 3. --Effect of Temperature on Progesterone-induced Hemolysis. The conditions are the same as in Fig. 2.

Fig. 4. --Liver Cirrhosis Induced by Lithocholic Acid Administration. Photograph courtesy of Dr. Paul Holsti, Helsinki.

Fig. 5. --Control Rabbit-Liver Histology. Courtesy Dr. Paul Holsti.

Fig. 6. --Lithocholic Acid Treated Rabbit - Liver Histology. Courtesy Dr. Paul Holsti.

Fig. 7. --Gallstones Induced by Lithocholic Acid. Sprague-Dawley rat fed an 8% protein, 1% lithocholic acid diet for 16 weeks.

Figs. 8 and 9. --Bile Duct Hyperplasia Induced by Lithocholic Acid.²³

Figs. 10 and 11. --Histology of Gallstone Formation.²³

Fig. 12. --Lithocholic Acid Fever.²⁹

Fig. 13. --Pyrogenic Steroids.

Fig. 14. --Effect of Conjugation on Neutral Steroid Pyrogens.²⁹

Fig. 15. --Pyrogenic Activity of Lithocholic Acid Conjugates.

Fig. 16. --Dissociation of Fever and Inflammation.²⁸

Fig. 17. -- Chromatographic System for Isolating Sulfate Conjugates. The system consists of: Butanol 50, 0.01 M Tris Buffer 9.25, propionic acid 0.75. TLS, tauroolithocholic-3-sulfate; GLS, glycolithocholic-3-sulfate; LS, lithocholic-3-sulfate; TC, taurocholate; GC, glycocholate; TD, taurodeoxycholate; GD, glycodeoxycholate; TL, tauroolithocholate; GL, glycolithocholate.

Fig. 18. --Labeled Biliary Metabolites of C¹⁴-Lithocholic Acid. Chromatographic system: n-propanol 10, propionic acid 15, isoamyl acetate 20, water 5. C, cholic acid; D, deoxycholic acid; L, lithocholic acid; TL, tauroolithocholic acid; GL, glycolithocholic acid. Standard spots, detected by spraying with sulfuric acid and heating, are identified with dots. Radioactivity, detected by autoradiography, is identified by solid lines. (The autoradiogram has been shifted slightly to the right.)

O.D.
540m μ

STEROID HEMOLYSIS

(STEROID CONC. 0.04mM)

SERUM INHIBITION OF STEROID HEMOLYSIS

Δ O.D.
540 m μ

(STEROID 0.2 mM; INCUBATION 110 min, 37°C)

TEMPERATURE and STERIOD HEMOLYSIS

BILE ACID FEVER IN MAN

PYROGENIC STEROIDS OF THE 5β (A:B *cis*) SERIES

LITHOCHOLIC ACID CONJUGATES

DISSOCIATION OF FEVER AND INFLAMMATION

TAUROLITHOCHOLIC ACID 25 MG. I.M.

BUTANOL 3

C D L C D L TL GL L
 taurine glycine sulfate BILE C¹⁴

TL S L S GC GD TL GL
 GLS TC TD