Lithocholate metabolism during chenotherapy for gallstone dissolution

2 Absorption and sulphation

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\textbf{SUMMARY} The amount of lithocholate input and the size of the total lithocholate exchangeable pool were measured using isotope dilution in three patients ingesting chenodeoxycholic acid for gallstone dissolution and two healthy control subjects. Because the specific activity decay curve was biexponential in appearance, input was calculated using a stochastic analysis employing the Stewart-Hamilton equation. By this method, the lithocholate input and the size of the exchangeable pool in gallstone patients during chenic acid ingestion was four to five times that observed in the healthy control subjects. It was calculated that about one-fifth of the newly formed lithocholate was absorbed in both gallstone patients and healthy control subjects. The extent of sulphation of absorbed lithocholate was then measured in seven gallstone patients who had been ingesting chenodeoxycholic acid for gallstone dissolution by defining the chemical form of radioactivity in bile after intravenous administration of labelled lithocholate. Similar studies were carried out in eight healthy control subjects, but, in addition, the extent of sulphation of injected lithocholyglycine and lithocholytaurine was also defined. When lithocholate was injected, the majority of radioactivity in bile (50-60\%) was present as sulphated conjugates, both in gallstone patients and healthy control subjects. The degree of sulphation was greater for glycine than taurine conjugates, and these findings, which suggested preferential sulphation of the glycine conjugates of lithocholate, were confirmed by showing that injected lithocholyglycine was sulphated to a much greater extent than injected lithocholytaurine. These findings indicate that in patients receiving chenic acid there is effective sulphation of lithocholate, especially its glycine conjugates, despite a considerable increase in lithocholate absorption; they may provide an explanation for the lack of hepatotoxicity of chenic acid in man.

In the preceding paper (Allan et al., 1976), we reported that the majority of lithocholate in blood was sulphated in gallstone patients receiving chenotherapy. Although these data suggested that lithocholate absorption was increased in such patients, they did not prove it, as relative concentrations of bile acids cannot be used to infer input rates, and, for secondary bile acids, these values can be measured only by isotope dilution (Hofmann and Hoffman, 1974a). Accordingly, we have extended these studies by measuring, using isotope dilution, the amount of lithocholate entering the bile acid pool as well as the size of the exchangeable lithocholate pool in gallstone patients on chenotherapy. We also carried out experiments aimed at measuring the extent of sulphation of the absorbed lithocholate by defining the metabolism of intravenously administered radio-labelled lithocholate and its glycine or taurine conjugates. Similar studies were carried out in healthy subjects.

\textbf{Methods}

\textit{Experimental design}

\textit{Lithocholate kinetics} Labelled lithocholate was administered intravenously and the specific activity decay curve of all lithocholate species present in bile...
was defined in order to calculate input and the size of the exchangeable pool.

Lithocholate biotransformation  Labelled lithocholate or one of its conjugates was administered intravenously in the evening and a bile sample obtained the following morning.

SUBJECTS
Patients with radiolucent gallstones and radiologically visualising gallbladders who had been taking chenic acid (mean dose 11-0 mg/kg/day) for a minimum of six months were studied. Clinical features of these patients are summarised in Table 1. All had normal serum levels of bilirubin, SGOT, SGPT, and alkaline phosphatase. Healthy subjects in this study were adult men with normal liver function as described. Informed consent was obtained.

PROCEDURE
Lithocholate kinetics  A nasoduodenal tube was inserted under fluoroscopic control. 24-13C-lithocholic acid, 40 μCi, was administered intravenously at 7.00 am on the first day. Bile samples were obtained at 12.00 noon, 5.00 pm, and 10.00 pm that day, as well as in the morning and evening of the second and third days using intravenous cholecystokinin (75 Ivy dog units, Karolinska Institute, Stockholm, Sweden) to induce gallbladder contraction. At least 30 ml bile was aspirated, thoroughly mixed, and a 2 ml aliquot retained and mixed with 18 ml ethanol; the solution was stored at 4°C before analysis, and the remainder was reinserted into the gastrointestinal tract. Three patients with gallstones and two normal subjects were studied. In earlier studies conducted in three gallstone patients and three healthy controls, bile samples were not collected during the first 24 hours. Data from these studies has not been presented since, as discussed below, a valid specific activity decay curve cannot be constructed unless samples are obtained during the initial 24 hours after isotope administration.

All subjects were given a standardised 30 calorie/kg-day diet containing 20% protein, 40% carbohydrate, and 40% fat; this was given as three meals.

Lithocholate biotransformation  Labelled bile acids were administered by intravenous injection in the late evening, at least four hours after the last meal. The following morning, after an overnight fast, a bile sample was obtained as described above, divided into two aliquots diluted 1:5 in ethanol, and stored at 4°C before analysis. Seven studies with lithocholate were carried out in eight healthy subjects. In other healthy subjects, three studies were carried out with lithocholylglycine and three with lithocholyltaurine; two studies were also carried out with sulpholithocholylglycine.

ANALYSES
Lithocholate kinetics  Five millilitres of the diluted bile sample was decolourised in ultraviolet light, evaporated to dryness, and the bile redissolved in 0.5 ml 0.05 N NaOH. After the addition of 10 ml of a toluene-based detergent scintillant cocktail (Readi-Solv, Beckman, Fullerton, CA), radioactivity was measured in a Beckman LS-250 liquid scintillation counter with external standardisation and automatic quench correction. The lithocholate radioactivity measured by this technique is present in four species: (1) lithocholylglycine, (2) lithocholyltaurine, (3) sulpholithocholylglycine, and (4) sulpholithocholyltaurine (Cowen et al., 1975a).

For mass determination 2 ml diluted bile sample were evaporated to dryness and hydrolysed using 2 N NaOH and 50% methanol for four hours at 115°C; nordeoxycholic acid was used as an internal standard. To correct for losses during hydrolysis, a reference mixture of conjugated bile acids was analysed simultaneously, with loss being assessed by recovery relative to that of nordeoxycholate. After saponification, the samples were acidified with HCl and the bile acids extracted into ether. Complete

<table>
<thead>
<tr>
<th>Study of lithocholate</th>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Chenic acid dose</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetics</td>
<td>Ki</td>
<td>72</td>
<td>F</td>
<td>750</td>
<td>82-4</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>Ca*</td>
<td>58</td>
<td>F</td>
<td>1500</td>
<td>71-0</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>Lo</td>
<td>53</td>
<td>F</td>
<td>500</td>
<td>61-4</td>
<td>139</td>
</tr>
<tr>
<td>Biotransformation</td>
<td>Bi</td>
<td>53</td>
<td>F</td>
<td>500</td>
<td>65-0</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>Lu*</td>
<td>65</td>
<td>M</td>
<td>1000</td>
<td>81-4</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>No*</td>
<td>59</td>
<td>F</td>
<td>500</td>
<td>62-4</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>Qu</td>
<td>52</td>
<td>M</td>
<td>750</td>
<td>81-8</td>
<td>175</td>
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<tr>
<td></td>
<td>Ek</td>
<td>42</td>
<td>F</td>
<td>750</td>
<td>60-0</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td>60</td>
<td>M</td>
<td>1000</td>
<td>94-5</td>
<td>178</td>
</tr>
</tbody>
</table>

*Also used for biotransformation studies.

Table 1  Details of gallstone patients ingesting chenic acid
Solvolysis occurs in the ether phase (Allan, Hofmann, Ng, van Berge Henegouwen, unpublished) so that the mass of lithocholate measured represents the sum of both sulphated and unsulphated bile acids. The bile acids were then methylated with diazomethane. Acetate derivatives were made and gas chromatography was carried out using a 3% cyanosilicone stationary phase (AN 600 Analabs, Hamden, CT) as described (Hofmann and Poley, 1972).

In our initial isotope dilution studies, the first sample of bile was not collected until 24 hours after isotope administration as is customary. Although the specific activity decay curve was monoexponential as we previously reported (Cowen et al., 1975b), the calculated inputs were implausibly large, when calculated using a single pool model (Lindstedt, 1957). In the three gallstone patients ingesting, respectively, 500, 750, and 1000 mg/day of chenic acid, calculated inputs were 629, 374, and 1134 mg/day. The first and last values were clearly implausible, highlighting the necessity of obtaining multiple determinations of specific activity in the immediate 24 hours after isotope administration. Accordingly, in the studies reported here, we obtained three bile samples on the day during which tracer was administered. The specific activity decay curve now obtained was biexponential with a rapid first component. To estimate input and the size of the exchangeable pool, we used a stochastic analysis employing the Stewart-Hamilton equation (Perl and Samuel, 1969; Shipley and Clark, 1972). This 'non-compartmental' analysis gives a figure for the exchangeable pool and input; the method is widely used for estimating cardiac output from indicator dilution curves (Shipley and Clark, 1972). Curves were described by a double exponential as outlined by Samuel and Lieberman (1973).

One assumption of this treatment is that the system is in a steady state, and this would not be true if lithocholate absorption occurred predominantly during the night and the sulphated lithocholate in bile was excreted without appreciable reabsorption. To test whether the proportion of lithocholate in biliary bile acids in fact remained constant throughout the day, we determined the proportion of lithocholate in biliary bile acids using gas/liquid chromatography on samples obtained at 8 am and 5 pm of the same day in six gallstone patients and four healthy controls. There was no significant difference in samples obtained from control or gallstone subjects, when all data were pooled. Nonetheless, in the two gallstone patients with the highest fasting-state lithocholate level, the proportion in the 5 pm sample was about 30% lower. We felt that this difference was not sufficiently great to prohibit the use of the Stewart-Hamilton equation.

**Lithocholate biotransformation** The chemical form of radioactivity in the bile samples was determined by thin layer chromatography on silica gel H using the Butanol 3 system of Palmer and Bolt (1971) followed by zonal scanning of the plates (Snyder and Kimble, 1965). In this system, lithocholyglycine and lithocholyltaurine have similar mobility but are separated from their three sulphate esters, sulpholithocholyglycine, and sulpholithocholyltaurine. Samples were also run in chloroform:methanol:acetic acid:water (65:25:15:9, v/v) on silica gel H containing calcium sulphate (10%, w/w) which resolves unsulphated lithocholyglycine and lithocholyltaurine from each other (Cass et al., 1975). Unconjugated lithocholates are separated from conjugated lithocholates in both systems. All zonal scans were performed in duplicate by analysing both aliquots of the bile sample.

**Tracers** 24-14C-lithocholic acid was purchased (ICN Chemical and Radioisotope Division, Irvine, CA). 24-14C-lithocholyglycine and 24-14C-lithocholyltaurine were prepared by the mixed anhydride method of Norman (1955) and purified by TLC (Cass et al., 1975). 24-14C-sulpholithocholylglycine was prepared as described by Palmer and Bolt (1971). When analysed by thin layer chromatography followed by zonal scanning of the plates, all compounds had a radiopurity greater than 98%.

**Results**

**LITHOCHOLET KINETICS**

The lithocholate specific activity curve did not decline linearly, but biexponentially (Figure). The input and mass of the exchangeable pool were calculated using the Stewart-Hamilton equation for isotope dilution (Table 2). Patients ingesting chenic acid had an exchangeable pool of lithocholates which was four to five times that of the healthy controls; the input of lithocholate in the exchangeable pool was also four to five times larger in the gallstone patients.

We then estimated limits for fractional absorption of newly formed—that is, unlabelled—lithocholate by assuming that all administered chenic acid was converted to lithocholate in the distal intestine and that endogenous synthesis of chenic acid was either unchanged (minimal absorption) or was completely inhibited (maximal absorption). The results (Table 3) indicate that about one-fifth of the lithocholate newly formed in the distal intestine is reabsorbed. A similar fraction of newly formed lithocholate was absorbed in the healthy control subjects. If some chenic acid which is lost from the enterohepatic circulation is not converted to lithocholate, then the fraction will be still greater.
Figure  Biexponential specific activity decay curve of biliary lithocholate after intravenous injection of labelled lithocholate in a gallstone patient receiving chenic acid for gallstone dissolution. The specific activity decay curve for lithocholate in healthy subjects is similar, although the intercept will be higher since the pool size is smaller.

Table 3  Absorption of newly formed lithocholate during chenotherapy*  
*The input is the amount of unlabelled lithocholate entering the exchangeable lithocholate pool and is analogous to a synthesis rate for primary bile acids. Reabsorption—that is, enterohepatic cycling—of the lithocholate moiety of labelled lithocholate conjugates, which have already entered the exchangeable lithocholate pool, is not determined by this method. Therefore, the mass of all lithocholate absorbed daily from the intestine is considerably greater than the input figure given here.  
†The term \( f_{\text{abs,\text{direct}}} \) is equal to the input of unlabelled lithocholate into the exchangeable lithocholate pool divided by the input of labelled lithocholate (equivalent to dose and no endogenous synthesis (for maximal absorption) or dose + endogenous synthesis (for minimal absorption)). Endogenous synthesis was assumed to be 200 mg/day.

BIOTRANSFORMATION OF LITHOCHOLATE (Table 4)  
Gallstone patients  When unconjugated lithocholate was injected, nearly all biliary radioactivity (> 98%) was excreted in products having the chromatographic mobility of the sulphated (52%) or unsulphated (47%) conjugates. Sulphation appeared to occur principally with glycine conjugates (43%) and to a much smaller extent with taurine conjugates (10%). The unsulphated taurine conjugates (24%) and the unsulphated glycine conjugates (23%) were present in similar proportions. In the gallstone

Table 2  Lithocholate kinetics determined using input analysis on samples obtained from 0-72 hours  
*Specific activity decay curve was fitted using the equation \( y = a_1e^{-b_1t} + a_2e^{-b_2t} \).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dose (mg/day)</th>
<th>Input (mmol/day)</th>
<th>( f_{\text{abs,\text{direct}}} )†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki</td>
<td>750</td>
<td>192</td>
<td>21</td>
</tr>
<tr>
<td>Ca</td>
<td>1500</td>
<td>188</td>
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</tr>
<tr>
<td>Lo</td>
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<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>18.3</td>
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</tbody>
</table>

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<table>
<thead>
<tr>
<th>Sulphated</th>
<th>Un sulphated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine conjugates</td>
<td>Glycine conjugates</td>
</tr>
<tr>
<td>Gallstone subjects receiving chenic acid (N = 7)</td>
<td>9.8 ± 1.8</td>
</tr>
<tr>
<td>Healthy subjects (N = 8)</td>
<td>14.6 ± 1.8</td>
</tr>
<tr>
<td>( p )</td>
<td>&gt; 0.05 NS</td>
</tr>
</tbody>
</table>

Table 4  Chemical form of radioactivity (mean % ± SEM) recovered in bile after intravenous injection of unconjugated \( ^{14} \)C-lithocholate
patients the proportion of sulphated and unsulphated
glycine and taurine conjugates was the same in men
and women.

Healthy subjects Unconjugated lithocholate under-
went similar biotransformation in healthy subjects
not ingesting chenic acid. Nearly all biliary radio-
activity ( > 98 %) was excreted in products having
the chromatographic mobility of the sulphated
(58 %) or unsulphated conjugates (42 %). Sulphation
appeared to occur principally with glycine conjugates,
whereas the nonsulphated conjugates were mostly
taurine derivatives.

BIOTRANSFORMATION OF LITHOCHOLYL
CONJUGATES
Healthy subjects (Table 5) The observed difference
in sulphation of lithocholyl conjugates after ad-
ministration of labelled lithocholic acid was con-
firmed in healthy subjects by the separate administra-
tion of labelled taunine and glycine lithocholyl
conjugates.

<table>
<thead>
<tr>
<th></th>
<th><em>H lit-gly</em></th>
<th>*H lit-tau</th>
<th>*H sul-lit-gly</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N = 3)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Sulphated</td>
<td>65.9 ± 4.4</td>
<td>23.4 ± 0.9</td>
<td>93.0 ± 0.8</td>
</tr>
<tr>
<td>Unsulphated</td>
<td>33.4 ± 4.9</td>
<td>74.8 ± 1.2</td>
<td>5.7 ± 1.1</td>
</tr>
</tbody>
</table>

Table 5 Chemical form of radioactivity (mean
% ± SEM) recovered in bile after intravenous injection
of conjugated lithocholate in healthy subjects

*Abbreviations: lit-gly, lithocholyglycine (glycolithocholate); lit-tau,
lithocholytaurine (taurolithocholate); sul-lit-gly, sulfolithocholy-
glycine (glycolithocholate sulphate).

When the glycine conjugates were injected, most
of the biliary radioactivity was excreted in products
having the chromatographic mobility of sulphated
glycine conjugates (65 %) and only 33 % as the
unsulphated glycine conjugates.

When the taurine conjugates were injected, only a
minority (23 %) of the biliary radioactivity was excreted
in products having the chromatographic mobility
of sulphated taurine conjugates; the majority (75 %)
chromatographed as nonsulphated conjugates.

Finally, when labelled lithocholylglycine sulphate
was injected, nearly all the biliary radioactivity
(93 %) was excreted in products having the same
chromatographic mobility as the original material.
There was minimal desulphation (6%).

Discussion

LITHOCHOLATE INPUT
These data indicate that there is a marked increase
in absorption of newly formed lithocholate from
the intestine in gallstone patients receiving chenic
acid therapy and suggest that this increase is propor-
tional to dosage. This increase is associated with a
modest increase in the proportion of lithocholate in
biliary bile acids (Allan et al., 1976), but the data
presented here indicate that hepatic sulphation is as
complete in these patients as in healthy control
subjects. Similar conclusions have been reported in

Our data confirm the complexity of lithocholate
kinetics in man. Lithocholate 'specific activity' is
the weighted average mean specific activity of four
species, whereas, for the other major bile acids, it is
the weighted average of only two species—that is,
the glycine and taurine conjugated species (Hoffman
and Hofmann, 1974). For lithocholate the specific
activity decay curve is biexponential, and the single
compartmental model proposed by Lindstedt (1957)
is inadequate; for cholic and chenodeoxycholic, the
specific activity decay curve is monoexponential
(Pomare and Low-Beer, 1974; Duane et al., 1975),
and the single compartmental model is a valid
approximation (Hoffman and Hofmann, 1974). The
specific activity decay curve of lithocholate declines
much more rapidly than that of the other major
primary and secondary bile acids in bile (c.f. Vlahcevic
et al., 1971).

The physiological meaning of the second portion
of the decay curve is uncertain. The flattening of the
specific activity decay curve probably reflects re-
cycling of unconjugated tracer from the distal
intestine (resulting from deconjugation and de-
sulphation of the conjugated lithocholate sulphate
excreted in bile). Because of the uncertainty of the
physiological meaning of the specific activity decay
curve, we have calculated input and total exchange-
able pool using the Stewart-Hamilton equation. The
data could also be interpreted as representing a
rapidly exchangeable and a slowly exchangeable pool.

In our initial studies of lithocholate kinetics in
healthy man (Cowen et al., 1975b), we were unaware
of the biexponential slope of the lithocholate specific
activity decay curve, and it is likely that we over-
estimated the input and pool size of lithocholate. If
it is assumed that daily lithocholate formation is
equal to the daily synthesis rate of chenic acid, and
this is taken as 0.5 mmol/day (Vlahcevic et al., 1971;
Danzinger et al., 1973), then the percentage of newly
formed lithocholate absorbed into the lithocholate
pool in our two healthy subjects was 16 % and 27 %.
These figures are similar to those observed for gall-
stone patients on chenogetherapy. Thus, in five
patients, at least one-fifth of the newly formed litho-
cholate in the distal intestine was absorbed. None-
theless, this fraction is significantly smaller than that
reported for deoxycholate (Hepner et al., 1972;
Quarfordt and Greenfield, 1973). Whether the lithocholate pool remains constant throughout the day is uncertain, but in healthy subjects, we found no significant difference in the proportion of lithocholate in biliary bile acids of fasting-state and late afternoon bile.

LITHOCHOLATE BIOTRANSFORMATION

Methodology The technique of administering labelled isotope at night and its recovery from gallbladder bile after giving CCK the following morning was originally conceived as a simple method of determining the extent of sulphation after one hepatic pass. However, this assumes that all nocturnal biliary secretion is retained in the gallbladder. If a fraction of the secreted bile bypassed the gallbladder and entered the duodenum directly, then the unsulphated fraction might be reabsorbed, and if so, undergo further enterohepatic circulation. If such occurred in our study, the chemical changes observed may have occurred during more than one hepatic pass. A recent study in the baboon suggests that less than half of the hepatic bile secreted in the fasting state is retained in the gallbladder, the remainder passing directly into the duodenum (O’Brien et al., 1974), and some studies in intubated man have shown continuous nocturnal secretion of bile (Brunner et al., 1974; Northfield and Hofmann, 1975). Further studies are necessary to define the fate of nocturnal biliary secretion in man.

SULPHATION

Gallstone patients vs healthy subjects In the healthy subjects our technique estimated the proportion of sulphated lithocholate conjugates as 58% and unsulphated conjugates as 42%; these values are similar to those reported by Cowen et al. (1975a) of 60% and 40%, respectively, in healthy subjects from whom complete bile collections were made during four hours after intravenous administration of labelled isotope.

In gallstone patients during chenotherapy, despite the increased lithocholate absorption, a degree of sulphation was observed similar to that seen in healthy subjects. This finding is in agreement with the data in the accompanying paper showing that serum lithocholate is largely sulphated in gallstone patients ingesting chenic acid (Allan et al., 1976). It seems likely that this effective sulphation prevents the hepatotoxic effects which have been observed in a wide variety of experimental animals when lithocholate is fed (Palmer, 1972). This explanation for the protective effect of sulphation is supported by a recent study where we showed that, in the rhesus monkey, which develops hepatic damage during chenotherapy, there is marked impairment of lithocholate sulphation (Gadacz et al., 1976); and, with the isolated perfused liver, sulphated lithocholate is much less hepatotoxic than unsulphated lithocholate, in acute experiments (Javitt, 1973; Liersch et al., 1975).

Sex differences Hepatic sulphation of deoxycorticosterone is five times more efficient in the adult female than the adult male rat and the difference is abolished after administration of testosterone (Carlstedt-Duke and Gustafsson, 1973). However, no sex differences were detected in our gallstone patients, as both the extent of lithocholate sulphation and the proportion of glycine and taurine conjugates sulphated were similar in men and women.

Site of biotransformation The site of lithocholate sulphation has not been clearly established but does occur in the isolated perfused rat liver (Liersch and Stiehl, 1974). In man, after intravenous administration of labelled lithocholylglycine, plasma radioactivity slowly becomes predominantly sulpholithocholylglycine suggesting reflux of the conjugated sulphate from the liver or the biliary tract (Cowen et al., 1975c).

Influence of conjugation on sulphation The greater sulphation of lithocholylglycine than lithocholyltaurine has not been reported previously and is the first reported instance in which a glycine conjugated bile acid is metabolised differently from a taurine conjugated bile acid. The explanation for this difference is not known, but we suggest that lithocholylglycine, being more hydrophobic than lithocholyltaurine, exhibits greater microsomal binding. As a result, more lithocholylglycine than lithocholyltaurine molecules are exposed to the sulphating enzymes.

SAFETY OF CHENOTHERAPY

We think that continuing effective sulphation of lithocholate despite a considerable increase in its absorption provides a simple explanation for the absence of significant hepatotoxic effects observed thus far with clinical experience in more than 1600 patients ingesting chenic acid (Hofmann and Paumgartner, 1975). The strikingly different toxicity of chenic acid in man and the non-human primate (Webster et al., 1975; Goldstein, 1976; Morrissey et al., 1975; Dyrszka et al., 1976), which can now be explained by species differences in the detoxification of lithocholate, suggests that the safety of cheno-thrapy for gallstone dissolution must be judged on the results obtained in man rather than those observed in non-human primates.
We acknowledge the skilful technical assistance of Annie Haddad and aid in data analysis from Dr. Paul Thomas and Dr. Neville E. Hoffman.

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