Alterations in biliary lipids of mice during dehydrocholic acid feeding

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Abstract Mice were fed a lithogenic diet consisting of Purina chow and 0.5% dehydrocholic acid (DHA group). Controls received Purina chow. Every 2 wk for 20 wk animals were killed, and biliary phospholipid, cholesterol, and bile salt concentrations were determined, as well as the extent of gallstone formation. With time there was a gradual, significant decline in the concentration and the relative composition of phospholipid in both groups compared with initial values. There was a significant increase in biliary cholesterol concentration and relative amount in the DHA group compared with the control. No significant differences were found in the relative amounts of bile salt or phospholipid between the two groups. Feeding DHA resulted in an increased concentration of bile salts and the sum of measured lipid compared with controls. After 8 wk, gallstones were found in approximately 60% of autopsied animals and correlated with increased cholesterol concentration. Our data support the hypothesis that there is a component of cholesterol secretion that may not be bile salt- or phospholipid-dependent. Our data also suggest that biliary phospholipid secretion decreases with age.

Supplementary key words gallstones · bile · cholesterol · phospholipid · bile salts · aging · micelles

Three methods have been published in recent years on the induction of cholesterol gallstones in mice. The earliest was the model described by Tepperman, Caldwell, and Tepperman (1) in which a normal diet is made lithogenic by the addition of cholesterol and cholic acid. The substantial amount of data published on the model emanates from several independent laboratories and confirms Tepperman's work.

The most recently published model is that of Besancon, Marche, and Parrot (2) in which the addition of cholic acid to a normal diet was, by itself, sufficient to produce gallstones, although the time required for stone formation was greater than for the Tepperman model. This work was recently confirmed in a publication by Gerolami et al. (3).

A third model was described by Besancon et al. (4) as early as 1965 and was confirmed in the laboratories of Duteil, Rambert, and Desgranges (5). This model uses dehydrocholic acid as the lithogenic component of the diet. At the time our study was initiated, the only data published on the alterations in biliary lipid concentrations resulting from dehydrocholic acid feeding was a twofold increase in the cholesterol content of the gallbladder bile (6). Therefore, we decided to quantitate biliary lipids periodically to determine the time sequence of any changes in biliary lipid concentrations and to correlate any changes with the formation of gallstones in this model. After we had submitted our results for publication, Gerolami et al. (7) reported that the concentrations of hepatic biliary cholesterol and phospholipids in mice were increased after 20 days of feeding dehydrocholic acid.

In the present study, we noted age-related changes in phospholipid concentrations which we believe to be of sufficient interest to discuss in this paper.

METHOD

Female Charles River mice, weighing 25–30 g, were randomly divided into control and lithogenic diet groups. Control mice were fed Purina chow ad lib. The lithogenic diet group received, ad lib., Purina chow supplemented with 0.5% dehydrocholic acid (Steraloids, Inc., New York). Beginning at zero time and every 2 wk for 20 wk, 10 or 20 animals in each group were killed after being fasted overnight. Gallbladders were excised and bile was examined for evidence of gallstones or crystals visible to the unaided eye. In the cases where these were present, animals were considered to have developed "stones."

Average body weight of a group of mice was determined at the start of the experiment. Thereafter, on the day of autopsy, the average body weight was determined for each group of animals being killed, and the average body weight gain was calculated.

10 μl of bile was collected from each animal in a group and pooled for lipid determinations. Two to four analyses

Abbreviations: DHA, dehydrocholic acid.

were carried out on each group, with three to five animals usually comprising one analysis. In the dehydrocholic acid-fed group (DHA), bile was pooled from animals with and without stones. An attempt was made to collect bile free from stones, but in some cases crystals were carried over into the bile aliquot.

Biliary lipids were extracted into 95% ethanol, using the method of Nakayama (8), and quantitated in duplicate. Bile salt concentrations were determined enzymatically by the Admirand and Small (9) modification of the procedure of Talalay (10). The hydroxysteroid dehydrogenase (EC 1.1.1.50 and 1.1.1.51) used for this assay was an enzyme preparation from *Pseudomonas testosteroni* and was purchased from Worthington Biochemicals Corp., Freehold, N.J. For the cholesterol assay, sufficient water was added to the 95% ethanol extract to produce a 70% ethanol solution from which cholesterol was partitioned into petroleum ether. Cholesterol was determined using the Rosenthal, Pfluke, and Buscaglia modification (11) of the Zlatkis, Zak, and Boyle procedure (12).

For the phospholipid determination, 2 vol of chloroform was added to the 95% ethanol extract. An aliquot of water equal to the volume of ethanol was then added. The samples were centrifuged and the ethanol–water layer was aspirated. This procedure separates inorganic phosphate from phospholipid. An aliquot of the chloroform layer was evaporated to dryness and assayed for phospholipids by the method of Bartlett (13).

Statistical analysis

After determining the pooled variance for each lipid class, comparisons were made at each autopsy period between animals fed a control diet and those fed a lithogenic diet. The results of these analyses are indicated in Figs. 1 and 2 by asterisks.

Examination of all the data suggested that there were time-related alterations in the concentrations and/or relative amounts of the various lipid classes. To evaluate the statistical significance of these changes, the method of least squares was used to find the line of best fit for each lipid as a function of time. Theoretically, if there were no consistent change with time, the slopes of these lines would be close to zero. However, if the slopes differ from zero significantly, then there is a significant correlation between age (time) and the change in lipid. The results of these analyses are expressed as slopes and levels of significance in the text under Results.

RESULTS

Effects of dehydrocholic acid feeding

Feeding DHA to mice resulted in a prompt (2 wk) increase in cholesterol concentration. This increase was significantly different from the controls at every time period

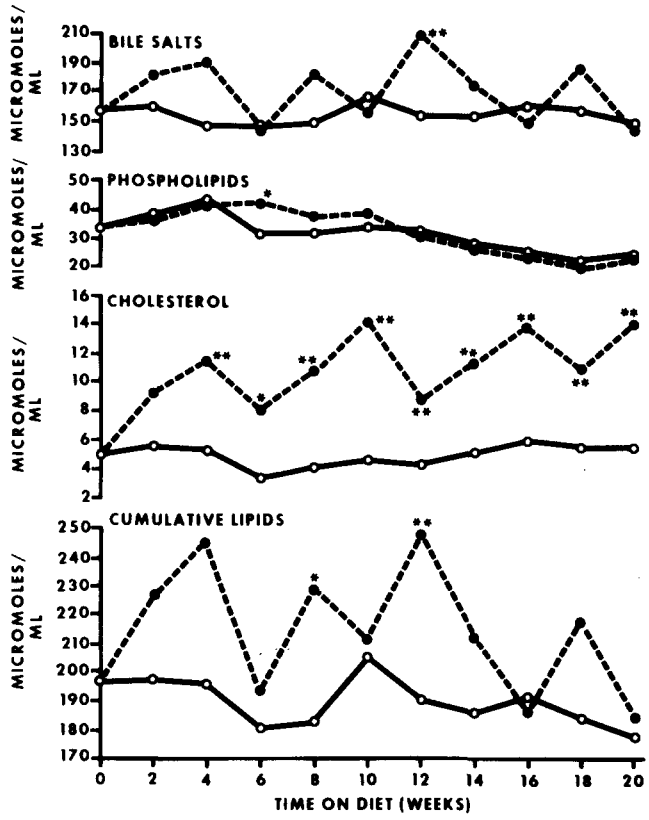

Fig. 1. Concentrations of gallbladder biliary lipids in control and dehydrocholic acid-fed mice. Animals were put on a Purina chow diet (o——o) or Purina chow + 0.5% dehydrocholic acid (●- - -●) at 0 wk. Animals were killed after an overnight fast every 2 wk for 20 wk. Bile was examined for gallstones and assayed for phospholipid, cholesterol, and bile salt concentrations. Cumulative lipids represent the sums of the concentrations of the three lipid classes. Asterisks indicate statistical significance compared with the control for that week: * = $P < 0.05$ and ** = $P < 0.01$. See Results for slopes of lines of best fit.

starting with the 4th wk (Fig. 1). From the 2nd to 20th wk, the average difference in the cholesterol concentration between the two groups was 6.5 μmoles/ml ($P < 0.01$), a greater than twofold increase.

As early as 4 wk after the start of DHA feeding, the relative amount of cholesterol was significantly elevated (Fig. 2). It continued to increase gradually throughout the course of the study. Beginning with the 4th wk, all cholesterol values were significantly greater than the controls at each period except for the 12th wk value.

In mice fed DHA, the concentration of bile salts was greater than in the control group. Although the increase was statistically significant only at 12 wk (Fig. 1), the average increase over the 2nd to 20th wk was 18 μmoles/ml ($P < 0.01$).

The relative amount of bile salts increased slightly but significantly ($P < 0.01$) during the study in both control (slope 0.382) and DHA-fed (slope 0.306) mice (Fig. 2). However, a comparison of the slopes of the two groups showed no significant difference, indicating that DHA feeding did not alter the relative composition of the bile salts

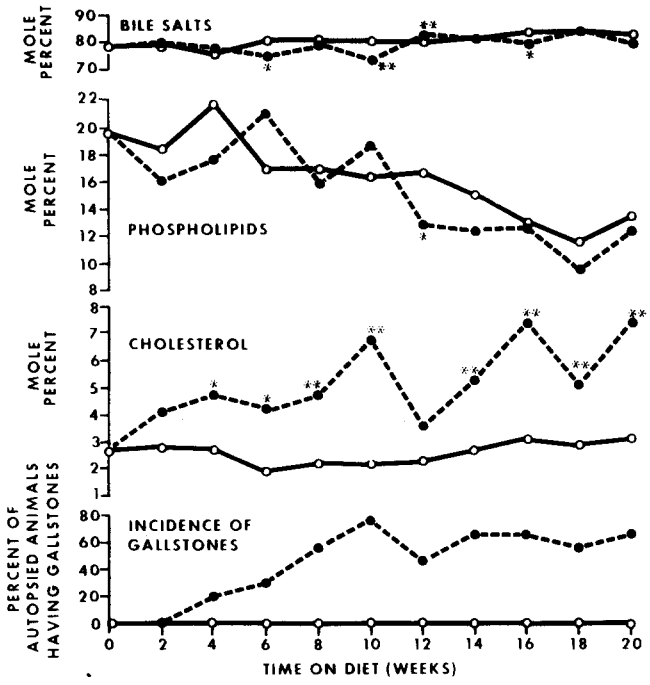

Fig. 2. Relative amounts of gallbladder biliary lipids and incidence of gallstone formation in control and dehydrocholic acid-fed mice. For details of treatment, meaning of asterisks, and slopes of lines, see legend to Fig. 1. The relative amount of each lipid was calculated by summing the concentrations of all three lipids (cumulative lipid value), dividing the concentration of each lipid by the cumulative value, and multiplying by 100.

compared with that in the control mice. At four time periods (6, 10, 12, and 16 wk), there was a significant difference between the two groups, but these did not show any consistent trend (Fig. 2).

Except for a significant increase in the concentration of phospholipids at 6 wk, compared with the controls, there was no significant effect of DHA feeding on this class of lipids (Fig. 1).

The relative amount of phospholipids remained essentially unaltered by DHA feeding except at 12 wk when it was significantly lower than the control value (Fig. 2).

We have also plotted the changes that occur in the cumulative concentration of these three lipids (Fig. 1). DHA feeding resulted in a statistically significant increase in the cumulative lipid concentration only at the 8th and 12th wk (Fig. 1). However, the average difference from the 2nd to the 20th wk was 27 μmoles/ml ($P < 0.01$), indicating that dehydrocholic acid caused an increase in the total output of measured lipids. This increase is the result of elevated bile salt and cholesterol concentrations, not the phospholipid concentration.

Gallstones or crystals began to appear as early as 4 wk after the animals had started eating the lithogenic diet (Fig. 2). The incidence of stones continued to increase until the 10th wk, after which time a maximum level of 60% incidence was maintained. During the entire 20 wk of the experiment, no stones or crystals were found in the control animals.

Body weight gain for each group was calculated. The difference between the two groups was determined at each autopsy period. These differences were then averaged over the duration of the study. The average difference was found to be 1.8 g less in the DHA-fed group ($P < 0.01$). This difference suggests that the DHA-fed group did not ingest any greater amount of food than the controls and thus did not have any greater intake of dietary sterol.

Effects of age

A most interesting observation is that the phospholipid concentration decreased with age in both groups irrespective of diet fed (Fig. 1). The slopes of both lines are negative and statistically significant ($P < 0.01$) from zero (control, -0.8962; DHA, -1.3126). The difference between the two slopes is not statistically significant, indicating that dehydrocholic acid did not alter this age-related, decreased output. The importance of this observation, from the point of view of gallstone formation, is even more obvious when one examines the changes in the relative concentration of the phospholipids (Fig. 2). Again the slopes are negative in both cases (control, -0.420; DHA, -0.455) and significant at $P < 0.01$. There is no statistically significant difference in the slopes of these two curves, indicating that DHA feeding also does not affect the age-related change in the relative amount of phospholipid.

Age did not affect the control cholesterol concentration because the slope (0.062) is not significantly different from a slope of zero (Fig. 1). Feeding DHA caused a slight increase in cholesterol concentration with time (slope 0.190, $P < 0.01$). Although the curves are at different concentration levels, there is no significant difference between the two slopes.

In the control group, the slope of the relative amount of cholesterol was not significantly different from zero (slope 0.038), but that of the DHA-fed mice was (slope 0.149, $P < 0.01$). The difference between the slopes was statistically significant ($P < 0.05$), suggesting that there is a significant alteration of the relative amount of cholesterol with time as a result of DHA feeding.

There is no age-related change in the concentration of bile salts with time in either group. The slopes of the relative amounts of the bile salts do show a slight increase with time in both the control mice (slope 0.382) and the DHA-fed mice (slope 0.306), but there is no significant difference between the slopes of the two groups.

Over the 2–20-week period, the slope of the cumulative lipid concentration curve is not significantly different from zero in the control mice (slope -0.716), but that of the DHA-fed mice is (slope -2.157, $P < 0.05$). There is no significant difference between the two slopes.

DISCUSSION

The objectives of this study were (*1*) to quantitate the time-dependent changes in biliary lipids that occur with dehydrocholic acid feeding, and (*2*) to confirm the rate and extent of gallstone formation. Two alterations in biliary lipid concentrations merit discussion in light of current concepts of biliary lipid secretion.

First, there was an approximately twofold increase in cholesterol concentration of bile in the DHA-fed animals compared with the control; this was also observed by Gerolami et al. (7). Recently, Hardison and Apter (14) reviewed the role of bile salts in solubilizing phospholipid and cholesterol in the hepatocyte as a micellar aggregate prior to secretion into bile. Considering the concepts discussed, it is difficult to explain a twofold increase in cholesterol concentration when similar bile salt or phospholipid concentrations exist in each group. The fact that there is a twofold increase in the relative amount of cholesterol suggests that it is not simply the secretion of a greater number of micelles in the DHA-fed mice, for one would then expect the relative lipid composition to be the same. The results also raise a question as to the source of the cholesterol secreted, because the DHA-fed group had a similar or lower dietary cholesterol intake than the controls, based on the observation of a reduced body weight gain.

Based on the rat liver perfusion studies of Entenman et al. (15), one might postulate that DHA feeding per se stimulated cholesterol secretion. However, Hardison and colleagues (14, 16) were unable to stimulate biliary lipid secretion using DHA. Furthermore, it has been shown in rabbits (17) and mice (7) that DHA is totally converted to metabolites assayable by 3-hydroxysteroid dehydrogenase, suggesting that DHA is hydroxylated at position 3.

An alternative explanation is that DHA may be metabolized to micelle-forming bile salts. Gerolami et al. (7) recently showed cholic acid to be the major metabolite of DHA in mice. In addition, they cite unpublished data showing that the two minor metabolites are capable of forming micelles. In contrast, Hardison (18) has stated that metabolites of DHA in the rat have only 10–17% of the micelle-forming capacity of sodium taurocholate, and most of this was due to cholic acid. Although Danielsson and Kazuno (19) have suggested that bile acid metabolism is similar in mouse and rat, it is unclear whether or not this applies to the metabolism of DHA. The differences between the work of Gerolami et al. (7) and Hardison (18) could be due to species differences or to the effect of chronic vs. acute administration of DHA. In any case, the slightly enhanced bile salt concentration in DHA-fed mice does not seem adequate to account for the amount of cholesterol found.

The higher relative amount of cholesterol might also be attributed to an abnormal absorption of bile salts by the gallbladder, since gallbladder lesions have been reported to occur with DHA feeding (4). Hall et al. (20) proposed such a mechanism to explain the lithogenic potential of DHA, although the nonmicellar nature of DHA was considered to be of greater significance. Several lines of evidence suggest that this increased reabsorption mechanism is not responsible. First, Gerolami et al. (21) have shown that feeding cholic acid to mice produces both gallstones and alterations in the gallbladder mucosa resembling those described by Besancon (4) for the DHA model. However, mucosal changes were independent of the frequency or time of stone appearance. Second, we found no significant difference in the concentration of phospholipids between control and DHA-fed mice. An increased secretion of bile acids, which might have been reabsorbed, should have resulted in increased phospholipid secretion as well as cholesterol. Thus, one would need to invoke significant phospholipid absorption or decomposition, as suggested by Hall et al. (20) for stasis, in the DHA-fed animal to explain why phospholipid concentration does not differ from controls at points of equal or higher bile salt concentrations. Finally, the data of Gerolami et al. (7, Table I), dealing with the relative composition of freshly secreted bile, suggest that reabsorption of bile acids is not significant. If selective reabsorption were occurring, the relative amount of bile salts should be lower in bile from mice fasted overnight when compared with freshly secreted bile. In both control and DHA-fed groups there is good agreement between our data and those of Gerolami et al. (7) in regard to the relative amount of bile salts, suggesting that absorption is not a major mechanism.

Recently, two reports (14, 22) suggested a component of cholesterol secretion that is independent of bile salt and phospholipid secretion. Our data in mice tend to support these authors and suggest that DHA may stimulate this component.

The second interesting alteration in lipid output is the significant decrease in phospholipid concentration that occurs with age (2–7 months) in both control and DHA-fed mice. This decrease is significant, whether expressed as concentration or relative amount.

Several investigators (15, 16, 22, 23) have studied the role of bile salts in the hepatic secretion of phospholipids. Based on these reports, one would expect phospholipid concentrations to remain constant or to increase when accompanied by equivalent or slightly higher bile salt concentration. Our data suggest that biliary phospholipid secretion decreases with age independently of bile salt secretion. The age-related decrease in phospholipid secretion might be an alternative explanation for the spontaneous development of gallstones observed by Besancon and Marche (24) in three of six multiparous control mice at 24 months. Further studies are required to determine wheth-

er the age-related changes in the relative amounts of fatty acids in hepatic phospholipids and neutral lipids observed by Turchetto et al. (25) have any effect on biliary phospholipid secretion.

The relative amounts of each of the three lipid classes were plotted (data not shown) using the method of Admirand and Small (9). The data were compared for fit in the micellar zones defined by these authors or by Danzinger et al. (26) using the data of Dam and Hegardt (27). All control values were within either micellar area, while plots of DHA-fed mice from the 8th to 20th wk were outside the micellar zone of Danzinger et al. (26) but within the micellar zone of Admirand and Small (9). Further discussion would seem to be inappropriate until resolution of the discrepancy between the data of Hardison (18) and Gerolami et al. (7) relative to the micelle-forming capability of DHA metabolites.

In agreement with the results of Duteil et al. (5), but in contrast to Besancon et al. (4), we were unable to produce gallstones in 100% of the mice fed DHA. The discrepancy may be due to the more sensitive method used by Besancon et al. to detect crystal formation.

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REFERENCES

1. Tepperman, J., F. T. Caldwell, and H. M. Tepperman. 1964. Induction of gallstones in mice by feeding a cholesterol–cholic acid containing diet. *Amer. J. Physiol.* **206:** 628–634.
2. Besancon, F., C. Marche, and J. Parrot. 1970. Experimental lithiasis due to excess cholic acid in mice. *Biol. Gastroenterol.* **2:** 147–160.
3. Gerolami, A., C. Crotte, A. Mule, Y. Varette, and H. Sarles. 1971. Lithiase biliaire expérimentale de la souris. Action comparée de l'acide cholique et de l'acide taurocholique associés ou non à du cholestérol. *Biol. Gastroenterol.* **1:** 23–32.
4. Besancon, F., C. Marche, M. Souchard, and C. Debray. 1965. Lithiase vésiculaire déterminée par le déhydrocholate chez la souris. *C. R. Soc. Biol.* **159:** 2333–2337.
5. Duteil, J., F. Rambert, and A. M. Desgranges. 1968. Les effets du phloroglucinol sur la lithiase biliaire de la souris. *Thérapie.* **23:** 433–440.
6. Besancon, F., C. Marche, C. Barret, and J. Parrot. 1970. Pharmacologie de la lithiase vésiculaire. Effets préventif et curatif de diverses médications chez la souris. *Thérapie.* **25:** 463–485.
7. Gerolami, A., C. Crotte, A. Mule, and H. L. Vigne. 1972. Experimental gallstones in the mouse. Mechanism of action of dehydrocholic acid. *Rev. Eur. Etud. Clin. Biol.* **17:** 500–502.
8. Nakayama, F. 1967. Quantitative microanalysis of bile. *J. Lab. Clin. Med.* **69:** 594–609.
9. Admirand, W. H., and D. M. Small. 1968. The physicochemical basis of cholesterol gallstone formation in man. *J. Clin. Invest.* **47:** 1043–1052.
10. Talalay, P. 1960. Enzymic analysis of steroid hormones. *Methods Biochem. Anal.* **8:** 119–143.
11. Rosenthal, H. L., M. L. Pfluke, and S. Buscaglia. 1957. A stable iron reagent for determination of cholesterol. *J. Lab. Clin. Med.* **50:** 318–322.
12. Zlatkis, A., B. Zak, and A. J. Boyle. 1953. A new method for the direct determination of serum cholesterol. *J. Lab. Clin. Med.* **41:** 486–492.
13. Bartlett, G. R. 1959. Phosphorous assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
14. Hardison, W. G. M., and J. T. Apter. 1972. Micellar theory of biliary cholesterol excretion. *Amer. J. Physiol.* **222:** 61–67.
15. Entenman, C., R. J. Holloway, M. L. Albright, and G. F. Leong. 1968. Bile acids and lipid metabolism. I. Stimulation of bile lipid excretion by various bile acids. *Proc. Soc. Exp. Biol. Med.* **127:** 1003–1006.
16. Hardison, W. G. M., and T. I. Francis. 1969. The mechanism of cholesterol and phospholipid excretion in bile. *Gastroenterology.* **56:** 1164.
17. Erlinger, S., M. Dumont, and J. P. Benhamou. 1970. Étude quantitative de l'effet cholérétique du déhydrocholate chez la lapin. *Biol. Gastroenterol.* **2:** 192.
18. Hardison, W. G. M. 1971. Metabolism of sodium dehydrocholate by the rat liver: its effect on micelle formation in bile. *J. Lab. Clin. Med.* **77:** 811–820.
19. Danielsson, H., and T. Kazuno. 1959. *Acta Chem. Scand.* **13:** 1141–1144. Cited in "Cholesterol, Bile Acid and Atherosclerosis." H. Van Belle, editor. 1965. North Holland Publishing Co., Amsterdam. 101.
20. Hall, R. C., J. T. Crosby, D. L. Fast, and J. Tepperman. 1971. Gallbladder distention and ischemia causing "absorption" of bile acids. *Curr. Top. Surg. Res.* **3:** 439–451.
21. Gerolami, A., C. Crotte, A. Mule, I. Varette, and H. Sarles. 1969. Lithiase biliaire expérimentale de la souris. Action de l'acide taurocholique et d'un régime dépourvu de cholestérol. *Biol. Gastroenterol.* **2:** 215–216.
22. Nilsson, S., and T. Scherstén. 1969. Importance of bile acids for phospholipid secretion into human hepatic bile. *Gastroenterology.* **57:** 525–532.
23. Swell, L., C. C. Bell, Jr., and C. Entenman. 1968. Bile acids and lipid metabolism. III. Influence of bile acids on phospholipids in liver and bile of the isolated perfused dog liver. *Biochim. Biophys. Acta.* **164:** 278–284.
24. Besancon, F., and C. Marche. 1970. Lithiase de cause médicamenteuse chez la souris: comparaison entre le déhydrocholate et d'autres cholérétiques. *Thérapie.* **25:** 487–501.
25. Turchetto, E., H. Weiss, E. Formiggini, and G. Bocchini. 1969. Die Fettsaürezusammensetzung der Lipoide von Rattenorganen in verschiedenen Altersstufen. *Nutr. Dieta.* **11:** 23–33.
26. Danzinger, R. G., A. F. Hofmann, L. J. Schoenfield, and J. L. Thistle. 1972. Dissolution of cholesterol gallstones by chenodeoxycholic acid. *N. Engl. J. Med.* **286:** 1–8.
27. Dam, H., and F. G. Hegardt. 1971. The relation between formation of gallstones rich in cholesterol and the solubility of cholesterol in aqueous solutions of bile salts and lecithin. *Z. Ernaehrungswiss.* **10:** 239–252.