Kinetic Analysis of Biliary Lipid Excretion in Man and Dog

CHARLES I. WAGNER, BRUCE W. TROTMAN, and ROGER D. SOLOWAY

From the Gastrointestinal Section of the Department of Medicine, Hospital of the University of Pennsylvania, University of Pennsylvania School of Medicine, and the Philadelphia Veterans Administration Hospital, Philadelphia, Pennsylvania 19104, and the Gastrointestinal Unit, Mayo Clinic, Rochester, Minnesota 55901

ABSTRACT To understand better the mechanisms involved in biliary lipid excretion and to evaluate their role in cholesterol gallstone formation, the rates of biliary excretion of bile salts, cholesterol, and phospholipids were measured in two species, man and dog. Seven cholecystectomized patients with balloon-occludable reinfusion T-tubes were studied during intact and interrupted enterohepatic circulation and four cholecystectomized dogs were studied during interrupted enterohepatic circulation. In man and dog both cholesterol and phospholipid outputs were hyperbolically related to bile salt output by the equation $y = x/(a + bx)$. The output curves intersected the origin and showed an initial rapid rise, followed by a slower increase to a maximum, suggesting a rate-limited mechanism. The shape of the curves permitted calculation of the theoretical maximal outputs and the rates of rise to those outputs. Comparison of these values showed that in both man and dog phospholipid output was greater than cholesterol output and that cholesterol and phospholipid were excreted at different rates. These studies (*a*) indicate that cholesterol, phospholipids, and bile salts are not excreted in a fixed relationship and (*b*) demonstrate the usefulness of the derived theoretical maximal lipid output, and the rate of rise of lipid excretion to a maximum, in evaluating the kinetics of biliary lipid excretion.

This work was presented in part at the National Meeting of the American Federation for Clinical Research, Atlantic City, N. J., May 1974, and published in abstract form in 1974, *Clin. Res.* **22**: 370A and 1974, *Gastroenterology.* **66**: 793.

Dr. Trotman is a recipient of a Macy Foundation Faculty Fellowship.

Received for publication 14 November 1974 and in revised form 17 October 1975.

INTRODUCTION

The interrelationships of bile salts, phospholipids, and cholesterol during their excretion into bile are not fully understood. It is known that bile salts are actively secreted by the hepatocyte into the canaliculus (1), and that the excretion of cholesterol and phospholipid are intimately related to bile salt secretion, probably mediated by the formation of micelles (2-4). In addition, it has been shown that with low bile salt secretion, there is little excretion of cholesterol or phospholipid, while with increasing bile salt secretion, there is increasing excretion of cholesterol and phospholipid (5-7).

There has been uncertainty, however, as to whether cholesterol output (ChO)[1] is entirely dependent on bile salt output (BSO) (6), or whether there is a bile salt-independent fraction as well (8, 9). A second area of uncertainty is whether cholesterol and phospholipid excretion are coupled to each other over the full range of bile salt excretion or whether they are excreted by independent mechanisms at low BSO (5, 6, 10).

To define further these interrelationships of biliary lipid excretion, the simultaneous outputs of bile salts, phospholipids, and cholesterol were measured in two species, man and dog.

METHODS

Human studies. After informed consent was obtained, three men (ages 45, 49, and 54) and four women (ages 44, 63, 68, and 86) had a balloon-occludable, reinfusion T-tube (11) inserted at the time of cholecystectomy and common duct exploration for cholesterol gallstones. A T-

[1] *Abbreviations used in this paper:* BSO, bile salt output; ChO, cholesterol output; EHC, enterohepatic circulation; O_{max}, maximal output; PLO, phospholipid output; R_m, rate of rise to maximum.

tube cholangiogram was obtained in each patient 7–10 days after surgery. During that study, the T-tube balloon was inflated and shown to occlude the common duct and to prevent contrast medium from passing around it (11).

All studies were performed in the Clinical Research Centers of the Hospital of the University of Pennsylvania, Philadelphia, Pa., or St. Mary's Hospital, Rochester, Minn., and were begun at least 10 days after surgery. The T-tube was clamped and the enterohepatic circulation (EHC) of bile was intact for at least 48 h before the first study.

Before study, all patients had normal values for serum bilirubin, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, and alkaline phosphatase. They were taking no medication and were eating a standard diet. The total caloric content of the diet was based on each patient's ideal weight by a nomogram (12) and the calories were distributed equally between breakfast, lunch, and dinner. Each meal consisted of 15% protein, 45% carbohydrate, and 40% fat.

Patients were studied during intact and interrupted EHC. During intact EHC, the T-tube balloon was inflated and the draining limb of the T-tube was connected to a stream splitter (13), (Suburban Electronics Co., Peabody, Mass.), which diverted 5% of the bile into graduated tubes accurate to 0.1 ml. The remaining bile was reinfused into the T-tube through a separate lumen and entered the common duct distal to the balloon. Bile was collected in 30-min periods and total flow was determined by multiplying the collected volume by 20. Patients were studied for 2–4 days with an intact and 1 day with an interrupted EHC. During interruption of the EHC, the T-tube balloon was inflated and all bile was collected and none reinfused. Studies began at 0700, 1 h before breakfast, and ended at 1500, 3 h after lunch.

Canine studies. Four mongrel dogs, two male and two female, were each studied twice during interruption of the EHC. Each had a cholecystectomy, lesser pancreatic duct ligation, and placement of a Thomas cannula opposite the ampulla of Vater (14). Studies were begun 3 wk after surgery. After an overnight fast, a polyethylene tube, PE 190, (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.) was inserted into the common duct and bile was collected by gravity drainage into graduated tubes in 30-min periods for 7 h.

Analytical methods. Aliquots of human and canine bile specimens were promptly placed in Folch solution (chloroform-methanol 2:1, vol/vol) for phospholipid extraction. Specimens were stored at −20°C and later assayed for total bile acids by a modification of the method of Talalay (15), cholesterol by the method of Abell et al. (16), and phospholipids by the method of Fiske and Subbarow (17).

Curve fitting. All relationships plotted had the shape of a rectangular hyperbola and permitted curve fitting with a PDP-10 computer (Digital Equipment Corp., Marlboro, Mass.) with a program developed by E. L. Forker[2] (University of Iowa, Iowa City, Iowa) for fitting a nonlinear regression of the form $y = x/(a + bx)$. This program, based on the paper by Johansen and Lumry (18), tested for the best weighting factor to fit a curve to the data. The best fit resulted when it was assumed that the errors in the measurement of the variables x and y were proportional to their measured values and that the error in x was twice as much as the error in y.

As noted by Wheeler et al. (10), the equation $y = x/(a + bx)$ is the same form as in Michaelis-Menten enzyme kinetics, where

$$V = \frac{(S)}{\frac{K_m}{V_{max}} + \frac{(S)}{V_{max}}}$$

with $a = K_m/V_{max}$ and $b = 1/V_{max}$.

In Michaelis-Menten analysis, V_{max} represents the maximal velocity of the reaction, and K_m the substrate concentration at half-maximal velocity. Although this data may not represent enzymatic reactions, the shapes of the curves are identical and it is possible to use similar terms to describe them. To emphasize the distinction from enzyme kinetics, a parallel set of terms are used: O_{max}, analogous to V_{max}, and representing the theoretical maximal output of y, and R_m, the value of the BSO at the half maximum for y, analogous to K_m, and giving a measure of the rate of rise of the curve to maximal value. The lower the value of R_m, the more rapid the rise of the curve toward its maximum.

Statistics. Values are expressed as the mean±SE and were derived by the method of Johansen and Lumry (18). Results were compared with the t test for significance between means (19).

RESULTS

Man

Cholesterol and phospholipid output. The relationship between ChO and BSO for all seven patients is seen in Fig. 1 and for phospholipid output (PLO) and BSO in Fig. 2. The 328 data points are depicted to show the fit of the computer-derived curves. Both ChO and PLO were close to zero at very low BSO, increased rapidly with increasing BSO, and leveled off and approached maxima at high BSO. These data suggest that there is no bile salt-independent excretion of cholesterol or phos-

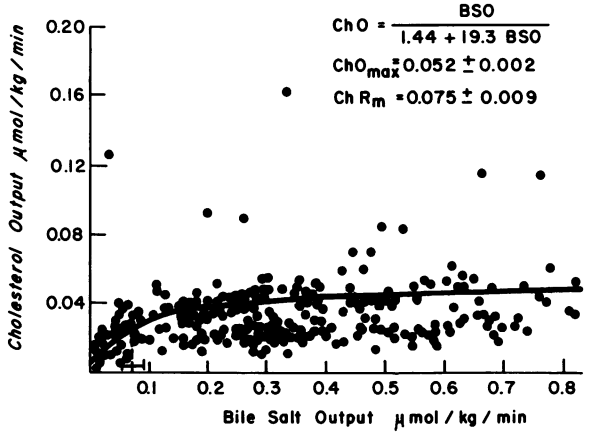

FIGURE 1 Relationship between ChO and BSO in seven patients. All data points are plotted. The solid line represents the equation that best fits the data. The theoretical maximal cholesterol output (ChO_{max}) and BSO at half theoretical maximal cholesterol output (Ch R_m) were calculated. The vertical dashed line (lower left corner) represents Ch R_m ±2 SE.

[2] Personal communication.

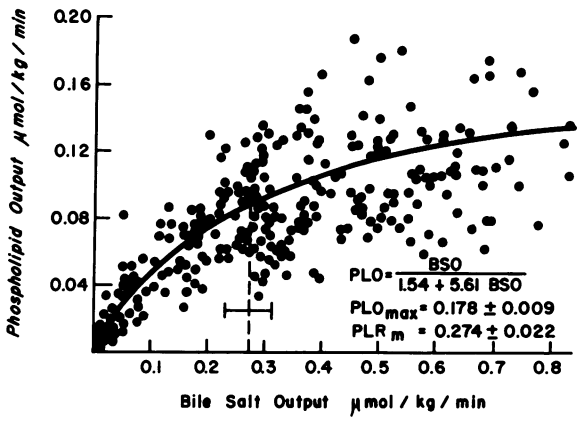

FIGURE 2 Relationship between PLO and BSO in seven patients. All data points are plotted. The solid line represents the equation that best fits the data. The theoretical maximal phospholipid output (PLO_{max}) and the BSO at half theoretical maximal phospholipid output (PL R_m) were calculated. The vertical dashed line represents PL $R_m \pm 2$ SE.

pholipid and indicate that BSO continued to increase after ChO and PLO have approached maxima.

ChO_{max} is significantly lower than PLO_{max} ($P < 0.001$) and cholesterol R_m is significantly lower than phospholipid R_m ($P < 0.001$), indicating that ChO approaches its maximum at a lower BSO than PLO.

Phospholipid-to-cholesterol ratio. Fig. 3 demonstrates the change in the phospholipid-to-cholesterol ratio (PL/Ch) that occurred with increasing BSO for all patients. The ratio was variable at low BSO and was almost constant at BSO above 0.25 μmol/kg/min, with a calculated maximal ratio of 3.53. This variable ratio at low BSO occurred because the rates of excretion of cholesterol and phospholipid were different, as noted previously.

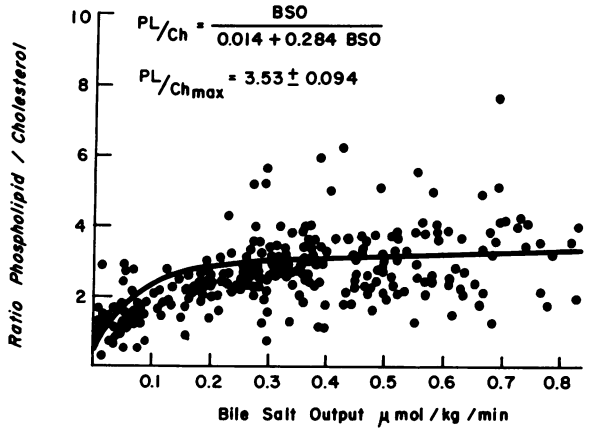

FIGURE 3 Relationship between the ratio of phospholipid to cholesterol versus BSO in seven patients. The solid line represents the equation that best fits the data. The theoretical maximal ratio was calculated.

Lipid ratio. The molar lipid ratio of bile salts plus phospholipids divided by cholesterol, (BS + PL)/Ch, gives a close approximation of the solubility of cholesterol in bile, with a ratio of 10 or less representing bile supersaturated with cholesterol (20). This ratio increased hyperbolically with BSO, with (BS + PL)/Ch = BSO/(0.011 + 0.032 BSO). Thus, a BSO over 0.16 μmol/kg/min was needed to obtain the ratio greater than 10 required for bile to be undersaturated with cholesterol.

Dog

Cholesterol and phospholipid output. Similar hyperbolic relationships in the excretion of biliary lipids were found in dog. The equations that describe the data in dog were ChO = BSO/(41.0 + 34.6 BSO) and PLO = BSO/(2.90 + 1.01 BSO).

Table I indicates that dog had a significantly lower ChO_{max} than PLO_{max}. In addition, the R_m for cholesterol was lower than for phospholipid, although not statistically significant. This lack of significance occurred because the experimental data described only the early part of the theoretical PLO curve in dog and did not allow precise prediction of the latter part of the curve as demonstrated by the large SE for the phospholipid R_m.

DISCUSSION

Curve fitting. When the data for the outputs of cholesterol, phospholipid, and the ratio of phospholipid to cholesterol in man and dog were plotted against BSO, they all had a similar shape. The lowest data points were near the origin, and the curves rose rapidly with increasing BSO and then leveled off and approached a plateau at high BSO. The shape of this data was best described by curves of the family $y = x/(a + bx)$, and

TABLE I
Kinetics of Biliary Lipid Excretion in Man and Dog

	Man	P	Dog	P	Man vs. dog P
			μmol/kg/min ± SE		
ChO_{max}*	0.052 ± 0.002		0.029 ± 0.005		<0.001
		>0.001		>0.01	
PLO_{max}*	0.178 ± 0.009		0.989 ± 0.408		<0.001
Ch R_m‡	0.075 ± 0.009		1.187 ± 0.214		<0.001
		>0.001		>0.2	
PL R_m‡	0.274 ± 0.002		2.870 ± 1.229		<0.001

* Theoretical maximal outputs of cholesterol or phospholipid.
‡ BSO at half maximal output of cholesterol or phospholipid. R_m is a measure of the rate of output to maximum: the lower the R_m, the more rapid the approach to maximal output.

the best equation for a particular set of data points was chosen with the computer program designed by Dr. E. L. Forker.

Curve fitting its risky, since the data may not fit a natural function and erroneous conclusions could be drawn if the wrong curve is chosen. Here, the likelihood of error is lessened since there are multiple data points over the entire range of observations and the shape of the curve is well defined. The hyperbolic function is the best curve to fit the data by inspection, and the exact curve of that family of curves is chosen by statistical means.

Once the equation $y = x/(a + bx)$ is chosen, it is possible to use analogues of the Michaelis-Menten relationship to describe them. Thus, instead of using a and b to describe the curves, it is possible to use their algebraic equivalents O_{max} and R_m. The advantage of these terms is that they provide concrete indices for how fast the rate of rise (R_m) and how high the theoretical outputs (O_{max}) of lipids are with increasing BSO. With these terms it is possible to use quantitative and statistical values to compare the kinetics of biliary lipid output in different species and in the same species in response to drug and dietary manipulations.

Cholesterol and phospholipid output. The shape of the plots of ChO and PLO versus BSO suggest that there is no ChO or PLO at zero BSO. This has been observed before for PLO (5–7) but with ChO, the question has been raised as to whether ChO continues without BSO: a BSO-independent ChO (8, 9). Our observations include multiple measurements at very low BSO produced by complete interruption of the EHC with the balloon-occludable T-tube and prolonged fasting. Although it is impossible to obtain zero BSO because of continued hepatic synthesis of bile salts, these observations approached zero BSO and suggest that neither phospholipid nor cholesterol are excreted independently of BSO.

Another implication of the shape of the data is that since the excretion of each lipid approached a maximum with increasing BSO, there is some rate-limiting step to the excretion of that lipid. These studies, however, do not provide information as to the site or mechanism of that limitation.

It has been suggested that the excretion of cholesterol and phospholipid are coupled at high BSO, but that an independent mechanism for ChO exists at low BSO (5, 6, 10). With the data obtained here, it is seen that cholesterol and phospholipid have different rates of excretion (R_m). This difference in excretion is clearly seen when the ratio PL/Ch is plotted against BSO. Again, the data have a hyperbolic shape with an almost constant ratio at high BSO, but a decreasing ratio with decreasing BSO. This demonstrates that at low BSO relatively more cholesterol and less phospholipid was excreted. This difference in excretion rates could be explained by the existence of completely separate excretion pathways for cholesterol and phospholipid, or by a coupled excretion process at high BSO, altered in some way at low BSO.

Previous studies (21–28) have shown that alterations in the excretion of either cholesterol or phospholipid can be produced without affecting the excretion of the other lipid. This too suggests the possibility of an uncoupled mechanism or of a varying relationship in the excretion of cholesterol and phospholipid. Kinetic analysis should be able to quantitate those changes in lipid excretion and might provide insight into the mechanism of those changes.

Bile salt excretion. Present results show that at high BSO, BSO increased without further increases in ChO or PLO, and are in agreement with Apter and Hardison's model (29), which proposes a mechanism of excreting bile salts unaccompanied by cholesterol or phospholipid.

Lipid ratio. The data for the lipid ratio versus BSO also had a hyperbolic shape. Thus an equation could be derived to describe the data, and the BSO below which bile became supersaturated with cholesterol could be calculated. This was 0.16 μmol/kg/min, a level frequently observed after an overnight fast. Lower BSO occurred during interruption of the EHC, and higher BSO and undersaturation of bile with cholesterol was seen after meals. This corresponds to previously published data (8, 20). The calculated requirement of 0.16 μmol/kg/min BSO to keep bile unsaturated with cholesterol is similar to the value estimated by Northfield and Hofmann from perfusion studies (30).

Dog. The data obtained from dog studies had the same shape as the human data and the same kinetic analysis could be used. The observations were similar to those made by Wheeler and King (6). Compared to man (Table I), dog excreted significantly less cholesterol and more phospholipid. In addition, dog had a higher R_m for cholesterol and phospholipid and had a less rapid increase in lipid excretion with increasing BSO. As a result, canine bile was never supersaturated with cholesterol, even at the lowest BSO measured, 0.02 μmol/kg/min.

These studies in man and dog demonstrate that cholesterol and phospholipid are not excreted in a fixed relationship, and that increases in BSO occur after ChO and PLO have approached maxima. In addition, the studies show that kinetic analysis is useful in quantitating biliary lipid excretion.

ACKNOWLEDGMENTS

We wish to acknowledge the cooperation of Dr. Ernest F. Rosato, Hospital of the University of Pennsylvania, and

Drs. Donald C. McIlrath and Keith A. Kelley, Mayo Clinic, who performed the surgery and managed the patients before study. We are indebted to Miss Cordelia Shute and the staff at the Clinical Research Center of the Hospital of the University of Pennsylvania, and to Miss Lee Fast and the staff at the Clinical Research Center of Saint Mary's Hospital, Rochester, Minn. Miss Eleanor Cheong, Mrs. Judy Duenas, and Mrs. Linda Moskalik provided expert technical assistance. Dr. E. L. Forker of the University of Iowa, Iowa City, kindly permitted use of his computer program and Mr. C. S. Lee and Dr. Martin Pring of the School of Medicine Computer Facility of the University of Pennsylvania provided statistical and computer assistance.

This work was supported in part by research, training, and facilities grants from the U. S. Public Health Service, National Institutes of Health AM05462, AM16549, RR1512, and FR-40, and the U. S. Veterans Administration TR15.

REFERENCES

1. Schanker, L. S. 1968. Secretion of organic compounds in bile. *Handb. Physiol.* Section 6: Alimentary canal. **5**: 2433–2449.
2. Kay, R. E., and C. Entenman. 1961. Stimulation of taurocholic acid synthesis and biliary excretion of lipids. *Am. J. Physiol.* **200**: 855–859.
3. 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.
4. Swell, L., C. Entenman, G. F. Leong, and R. J. Holloway. 1968. Bile acids and lipid metabolism. IV. Influence of bile acids on biliary and liver organelle phospholipids and cholesterol. *Am. J. Physiol.* **215**: 1390–1396.
5. Hardison, W. G. M., and J. T. Apter. 1972. Micellar theory of biliary cholesterol excretion. *Am. J. Physiol.* **222**: 61–67.
6. Wheeler, H. O., and K. K. King. 1972. Biliary excretion of lecithin and cholesterol in the dog. *J. Clin. Invest.* **51**: 1337–1350.
7. Scherstén, T., S. Nilsson, E. Cahlin, M. Filipson, and G. Brodin-Persson. 1971. Relationship between the biliary excretion of bile acids and the excretion of water, lecithin, and cholesterol in man. *Eur. J. Clin. Invest.* **1**: 242–247.
8. Metzger, A. L., R. Adler, S. Heymsfield, and S. M. Grundy. 1973. Diurnal variation in biliary lipid composition. Possible role in cholesterol gallstone formation. *N. Engl. J. Med.* **288**: 333–336.
9. Grundy, S. M., A. L. Metzger, and R. D. Adler. 1972. Mechanisms of lithogenic bile formation in American Indian women with cholesterol gallstones. *J. Clin. Invest.* **51**: 3026–3043.
10. Wheeler, H. O., R. J. May, and P. M. Loeb. 1973. Determinants of biliary lipid excretion. *In* The Liver. Quantitative Aspects of Structure and Function. G. Paumgartner and R. Preisig, editors. S. Karger AG, Basel. 368–375.
11. Soloway, R. D., H. C. Carlson, and L. J. Schoenfield. 1972. A balloon-occludable T-tube for cholangiography and quantitative collection and reinfusion of bile in man. *J. Lab. Clin. Med.* **79**: 500–504.
12. Committee on Dietetics of the Mayo Clinic. 1971. Mayo Clinic Diet Manual. W. B. Saunders Company, Philadelphia. 4th edition.
13. Lynn, J., L. Williams, J. O'Brien, J. Wittenberg, and R. Egdahl. 1973. Effects of estrogen upon bile: implications with respect to gallstone formation. *Ann. Surg.* **178**: 514–524.
14. Soloway, R. D., K. M. Powell, J. R. Senior, and F. P. Brooks. 1973. Interrelationship of bile salts, phospholipids and cholesterol in bile during manipulation of the enterohepatic circulation in the conscious dog. *Gastroenterology.* **64**: 1156–1162.
15. Talalay, P. 1960. Enzymatic analysis of steroid hormones. *Methods Biochem. Anal.* **8**: 119–143.
16. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. 1952. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* **195**: 357–366.
17. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**: 375–400.
18. Johansen, G., and R. Lumry. 1960. Statistical analysis of enzymic steady-state rate data. *C. R. Trav. Lab. Carlsberg.* **32**: 185–214.
19. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Iowa State College Press, Ames, Iowa. 6th edition. 593 pp.
20. Soloway, R. D., and L. J. Schoenfield. 1975. Effects of meals and interruption of enterohepatic circulation on flow, lipid composition, and cholesterol saturation of bile in man after cholecystectomy. *Am. J. Dig. Dis.* **20**: 99–109.
21. Linscheer, W. G., K. L. Raheja, and E. C. Regensburger. 1974. Increased phospholipid concentrations in bile by β-glycerophosphate administration. *Gastroenterology.* **66**: 732. (Abstr.)
22. Wheeler, H. O. 1973. Biliary excretion of bile acids, lecithin, and cholesterol in hamsters with gallstones. *Gastroenterology.* **65**: 92–103.
23. Sarfeh, I. J., D. A. Beeler, D. H. Treble, and J. Balint. 1974. Studies of the hepatic excretory defects in essential fatty acid deficiency. Their possible relationship to the genesis of cholesterol gallstones. *J. Clin. Invest.* **53**: 423–430.
24. DenBesten, L., S. Safaie-Shirazi, W. E. Connor, and S. Bell. 1974. Early changes in bile composition and gallstone formation induced by a high cholesterol diet in prairie dogs. *Gastroenterology.* **66**: 1036–1045.
25. Davis, R. A., and F. Kern, Jr. 1974. Effect of ethinyl estradiol (EE) and bile salt (BS) in cholesterol (CH) excretion: A possible mechanism of gallbladder disease. *Clin. Res.* **22**: 356A. (Abstr.)
26. Bennion, L. J., and S. M. Grundy. 1975. Effects of obesity and caloric intake on biliary lipid metabolism in man. *J. Clin. Invest.* **56**: 996–1011.
27. DenBesten, L., W. E. Connor, and S. Bell. 1973. The effect of dietary cholesterol on the composition of human bile. *Surgery (St. Louis).* **73**: 266–273.
28. Northfield, T. C., N. F. LaRusso, A. F. Hofmann, and J. L. Thistle. 1975. Biliary lipid output during three meals and an overnight fast. II. Effect of chenodeoxycholic acid treatment in gallstone subjects. *Gut.* **16**: 12–17.
29. Apter, J. T., and W. G. M. Hardison. 1970. Parameter estimation in phospholipid regulation of bile salt stimulated cholesterol appearance in bile. *Proc. Natl. Electron. Conf.* **26**: 2–6.
30. Northfield, T. C., and A. F. Hofmann. 1975. Biliary lipid output during three meals and an overnight fast. I. Relationship to bile acid pool size and cholesterol saturation of bile in gallstone and control subjects. *Gut.* **16**: 1–17.