PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 161, 123–127 (1979)

Effects of Fasting on Hepatic Bile Acid Clearance[1] (40504)

LARRY R. ENGELKING AND RONALD GRONWALL

College of Veterinary Medicine, University of Florida Gainesville, Florida 32610

Fasting induces a hyperbilirubinemia in normal individuals (1, 2), in patients with liver disease (2), hemolytic jaundice (3) or inherited hepatic dysfunction (2–4), and in normal ponies, where the response is pronounced (5, 6). The hepatic clearance of both plasma bilirubin (5–7) and sulfobromophthalein (BSP) (5) has been shown to decrease during fasting. The reduction in fasting hepatic clearance, which could result from a decreased hepatic extraction efficiency or a decreased hepatic circulation (8), has been related to the entire rise in plasma bilirubin rather than increased total bilirubin production (7). Since hepatic extraction efficiency for bile acid (8) is much greater than that for bilirubin (9), a slight reduction in hepatic blood flow would be expected to have a greater effect on bile acid clearance than on bilirubin clearance (8, 10, 11). The following experiments were designed to study the effect of fasting on bile acid clearance (3 days) in normal ponies with (a) intact and (b) diverted enterohepatic circulation where both hepatic bile acid clearance and excretion could be evaluated.

Materials and methods. Six female ponies weighing from 150 to 236 kg were studied while fed (native hay and water *ad libitum*, and Purina horse chow) and following a 3-day fast (water *ad libitum*). Three ponies had been surgically prepared with chronic external biliary fistulas 2–5 years prior to this study (12). During the course of each study, animals stood in a stanchion under minimal restraint and were allowed movement of head and limbs. Bile was quantitatively collected from the exteriorized ends of the T-tubes by gravity drainage. The external ends of the T-tubes were closed immediately following each study in order to allow for the reestablishment of the normal enterohepatic cycling of bile acids between studies. Each jugular vein was prepared with an indwelling polyethylene catheter to allow for injection of isotope into one vein and removal of heparinized blood samples from the opposite vein.

Experimental design. Two types of studies were conducted. Type I studies were conducted on ponies with chronic biliary fistulas. Bile was allowed to drain for 2 hr preceding the injection of isotope. During that period, the enterohepatic bile acid pool was depleted, and the excretion rate of bile acid through the T-tube was equivalent to the hepatic bile acid synthesis rate (13). After isotope injection, bile was quantitatively collected for 5-, 10- and 15-min intervals during the first, second and third hours, respectively, to prevent intestinal recycling of isotope. Type II studies were conducted on normal ponies without biliary fistulas. For both type I and type II studies, 20–38 μCi of 24-[^{14}C]cholic acid (sp act, 108.7 μCi/mg, New England Nuclear, Boston, MA) were injected into a jugular vein, and blood samples (8 ml) were withdrawn from the opposite vein at 1, 2, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 110, 120, 135, 150, 165 and 180 min in order to determine the plasma isotope retention.

The ^{14}C activity in each 1 ml plasma and 0.2 ml bile samples was determined in 10 ml Insta-Gel (Packard Inst. Co., Downers Grove, IL) by use of a Packard C-2425 scintillation spectrometer. External standard was used to correct for sample quench.

Samples taken at the beginning, middle and end of each of 18 clearance studies were used for determination of endogenous plasma bile acid concentrations.

Analytical methods. Total bile acid concentration in plasma was determined by an enzymatic, fluorometric method using 3-α-hydroxysteroid dehydrogenase (Grade III, Sigma Chemical Co.) and based on modifications of the methods of Murphy (14) and Schwartz (15). One ml of each plasma sample was centrifuged after addition of 50 mg char-

[1] This work has been supported in part by NIH Research Grant No. AM18923.

0037-9727/79/060123-05$01.00/0

coal (washed Norit A) to obtain a bile acid free (BAF) sample for determination of non-bile acid fluorescence. To determine the extraction efficiency of charcoal treatment, [^{14}C]cholic acid was added to plasma samples from both fed and fasted ponies. Samples were incubated at room temperature for at least 12 hr while they were continuously and gently mixed. Charcoal extraction removed over 99% of radioactive bile acid from all samples.

For each plasma sample, both unaltered and BAF aliquots were assayed in duplicate with the addition of 0, 2.5 and 5 nmoles of cholic acid to standardize the detected fluorescence. The standard curves for unaltered and BAF aliquots of each sample were compared by paired comparison of the slopes and intercepts of the fed and fasted samples. The fluorescence of the reaction solution without enzyme was subtracted from the fluorescence of the final combined reaction solutions. Each reaction mixture contained 0.1 ml plasma (unaltered or BAF), cholic acid standard (0, 2.5 or 5 nmole), 1.0 ml 1.0 *M* hydrazine hydrate (J. T. Baker Co.), 0.2 ml 5 m*M* NAD solution (grade III, Sigma), 0.1 ml 3-α-hydroxy steroid dehydrogenase (2.5 units/ml in Tris–EDTA, pH 7.2, grade III, Sigma), and 0.2 *M* phosphate buffer (pH 9.9) to bring each solution to 3 ml total volume. Before adding enzyme, the solution was heated in a 65° water bath for 5 min to denature endogenous plasma enzymes. The mixture was cooled to room temperature, enzyme was added, and the solution allowed to incubate at room temperature for 1 hr. The fluorescence for each sample was determined in a spectrofluorometer at room temperature using a mercury–xenon lamp with an exitation input of 350 nm and an emission wavelength of 460 nm.

No significant difference was found ($p > 0.05$, paired t test) between the slopes of the bile acid free and unaltered sample standard curves ($r = 0.86$, $n = 36$, $p < 0.001$). However, there was a significant difference between fed and fasted sample standard curves ($p < 0.05$, Student's t test). Therefore, separate standard curves were included within each sample in order to adjust for the variability between concentration/fluorescence slopes of various samples. The final bile acid concentration in each of three samples per clearance study was determined according to the following relationship:

$$\text{Conc.} = (\text{SAM}_o - \text{BAF}_o)\cdot R\cdot K \text{--(A)}$$

where:

Conc. = Plasma Bile Acid Concentration (micromolar).
BAF_o = Bile Acid Free sample fluorescence without added standard.
SAM_o = Unaltered sample fluorescence without added standard.
R = Ratio of standard bile acid concentrations to fluorescences (slope) in unaltered sample.
K = Dilution constant to correct to 1 liter.

Data treatment. Assumptions used to assess the properties of the physiological [^{14}C]bile acid distribution were (a) the tracer behaved in a manner indistinguishable from the endogenous bile acids; (b) mixing within the plasma compartment was rapid prior to significant hepatic removal; (c) recycling of [^{14}C]bile acid from intestine and distribution to extrahepatic, extravascular spaces was negligible (see results); and (d) hepatic removal from plasma conformed to the kinetics of a first order process.

For type I studies, the plasma disappearance curves of injected 24-[^{14}C]cholic acid (expressed as percent of injected dose per liter plasma) were fitted to the sums of three exponential functions. Data from the full 180 min collection period was used. For type II studies where intestinal recycling of isotope was observed after 20 min, two exponential functions were necessary to fit the curves and only data from the first 20 min were used. A digital computer program was used to fit the data after estimates and limits of the intercepts and slopes of the exponential components were determined by integration peeling (16, 17). The equations for the 3-exponential non-linear curves took the form:

$$P(t) = A\cdot\epsilon^{-k_a\cdot t} + B\cdot\epsilon^{-k_b\cdot t} + C\cdot\epsilon^{-k_c\cdot t} \text{--(B)}$$

where P(t), A, B and C had units of percent injected dose per liter plasma, k_a, k_b and k_c had units of minutes^{-1}, and t was time in minutes.

From this mathematical expression, the

following physiological parameters were determined (9, 18).

$$MP = 1 \cdot 10^5/(A + B + C)---(C)$$

where mixing pool volume (MP) was the initial distribution volume of injected [^{14}C]-bile acid (milliliters).

$$Cl = 1 \cdot 10^5 \Big/ \int_o^\infty P(t)dt---(D)$$

where hepatic bile acid clearance (Cl) was the volume of plasma cleared by the liver each minute (milliliters/minute).

The plasma volume (PV) was calculated from constants derived in studies using ^{131}I-labeled human serum albumin in ponies (6).

$$\text{Fed PV (milliliters)} = 38.8 \cdot BW---(E)$$

$$\text{Fasted PV (milliliters)} = 37.0 \cdot BW---(F)$$

where body weight (BW) is in kg.

Statistical methods. Non-linear regression analysis (17), Students *t* test, paired *t* test and least squares linear regression analysis (19) were used for data analysis.

Results. The results of [^{14}C]bile acid clearance studies in both fed and fasted ponies are listed in Table I. Hepatic bile acid clearance (equation D) was significantly ($p < 0.01$) reduced from normal fed levels following a 3-day fast. The reduction in bile acid clearance was apparent in both type I (Fig. 1) and type II (Fig. 2) studies, even though the clearance curves in type II studies were restricted to 20-min interpretations because of recycling of isotopic cholic acid. When the clearance curves from type I studies were fit to the 2 exponential functions (using only data collected during the first 20 min), the calculated clearance increased 20.2% ± 5.7 (SE) above that calculated by the 3-exponential function (using all data). The clearance calculated for the type II studies were 20.0% greater than those from the type I studies (using all 180 min of data) during feeding and 17.4% greater when fasted. There was no significant difference in clearance between the type II and type I studies when 2-exponential functions (using first 20 min of data) were used for calculating the type I values. Thus the 20-min

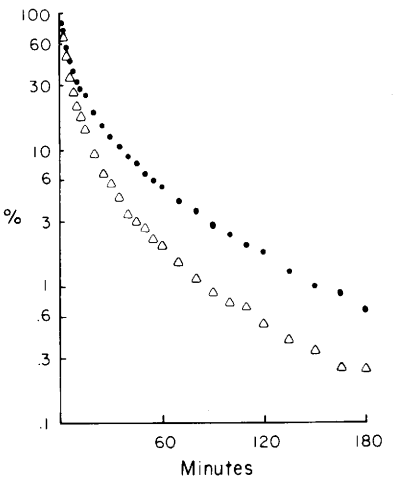

FIG. 1. Plasma levels of injected 24-[^{14}C]cholic acid (as percent of dose in mixing pool) from a typical pony (B) with an open external biliary fistula (Type I study). (Δ) Represents values in the fed state (•) values following a 3-day fast.

TABLE I. CLEARANCE OF [^{14}C]CHOLIC ACID IN FED AND FASTED (3 DAYS) PONIES.

		Mean ± SE		
	No. studies	Fed	Fasted	% Change
Clearance (ml/min/Kg)	6	4.94 ± 0.37	3.13 ± 0.18[a]	−35.9 ± 3.6
MP/PV (Type I studies)	3	0.99 ± 0.04[b]	0.98 ± 0.04[b]	
MP/PV (Type II studies)	3	0.77 ± 0.06[c]	0.85 ± 0.04[c]	
Biliary isotope excretion (% Injected dose in 180 min type I studies)	3	90.6 ± 2.4	86.6 ± 1.5[b]	−4.4 ± 2.7
Plasma bile acid (μM)	6	12.9 ± 1.2	22.2 ± 3.6[d]	+72.1 ± 22.1
Body weight (Kg)	6	194.3 ± 11.4	172.3 ± 10.6[a]	−11.4 ± 0.4

MP = Mixing pool volume. PV = Plasma volume.

[a] Significantly different between fed and fasted ($P < 0.01$, paired *t* test).

[b] No significant difference between MP and PV ($P > 0.05$, paired *t* test) or between fed and fasted.

[c] Significant difference between MP and PV ($P < 0.05$, paired *t* test).

[d] Significantly different between fed and fasted ($P < 0.05$, paired *t* test).

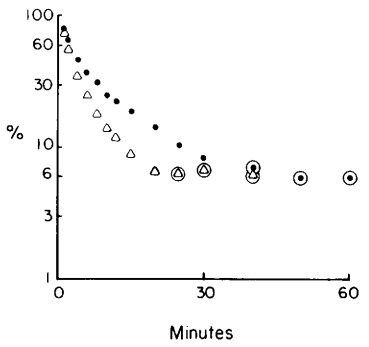

FIG. 2. Plasma levels of injected 24-[^{14}C]cholic acid (as percent of dose in mixing pool) from a typical pony (M) with an intact enterohepatic circulation (Type II study). (Δ) Indicates values obtained in the fed condition, (•) values following a 3-day fast. Circled values represent values elevated due to return of isotope from the intestine.

clearance curves were sufficient to determine the relative difference between the fed and fasted values even though they overestimated the actual hepatic clearance by approximately 20%.

Mixing pool volumes (equation C) from type I studies were not significantly different ($p > 0.05$) from plasma volumes (equations E and F). The mixing pool volumes determined from the type II studies were significantly lower than the plasma volumes ($p < 0.05$).

Approximately 90% of the administered isotope was recovered in bile during the 180-min type I studies (Table I). Plasma bile acid concentrations (equation A) following a 3-day fast (22.2 $\mu M \pm 3.6$ SE) were significantly greater ($p < 0.05$) than the concentrations during the fed state (12.9 $\mu M \pm 1.2$ SE) for both study types. The 72.1 ± 22.1% mean increased in the fasting bile acid concentration was twice the mean percent reduction (35.9 ± 3.6%) in hepatic bile acid clearance following the 3-day fast.

Discussion. Steady-state clearance represents the ratio between production or excretion and plasma concentration. A 36% decrease in clearance, as found in this study, would be expected to cause a 60% increase in plasma concentration. Since plasma bile acid levels may not precisely reflect combined hepatic arterial and portal concentrations, the predicted 60% increase in plasma level was not greatly different from the determined 72% increase. Similar studies of the effect of fasting on bilirubin transfer under the same conditions in ponies indicated 75–80% decreases in bilirubin clearance with 400–500% increases in plasma bilirubin concentration (6). The effect of fasting on bilirubin transfer was thus much greater than the effect on bile acid transfer.

For the modeling and calculation of clearance it was assumed that labeled bile acid distribution to extravascular, extrahepatic spaces was negligible. In the type I studies, which allowed analysis of the disappearance curve without interference of intestinal recycling of bile acid, the initial volume of distribution (MP of equation C) did not differ statistically from the calculated plasma volume (PV). Thus the assumption appears justified. In the shortened type II studies the difference was significant. The advantages of using a 3-exponential function have been reported by Klapor, *et al.* (20). Since bile acids are bound extensively to albumin (21–23) over the normal and pathological range of concentrations observed in blood (22), the identity between the mixing pool and plasma volumes as found in this study might be expected. In studies with dogs, other investigators (24) have reported MP/PV ratios of 1.22 ± 0.12 (SD), values somewhat greater than the results of our type I studies.

Clearance of [^{14}C]bile acid determined in the type I studies reflected the overall process of hepatic clearance of bile acid from mixed systemic–portal blood with approximately 90% of the isotope being recovered in the bile during 180 min studies. The similarity of the clearance reduction ratios of the type II studies (35.7 ± 5.2 SE) to those of the type I studies (35.7 ± 6.4) indicates the utility of the type II method in intact animals for detection of relative changes in clearance. However, the type II method underestimates the area under the plasma isotope retention curve (projected to infinity), thereby overestimating the actual clearance.

The hepatic uptake of bile acid has been found to correspond to an extraction efficiency of 60–86% in normal man (Engelking, unpublished observations), 44–99% in monkeys (25), 79–92% in dogs (8) and approximately 95% in rats (26); yet the hepatic extraction efficiency of bilirubin in man has

been found to be only 6.9 ± 1.7% (9). Extraction efficiency is an exponential function of hepatic blood flow. A decrease in flow results in an increase in extraction efficiency. Compounds with high extraction efficiencies are increased the least (11). Since hepatic clearance is the product of extraction efficiency and hepatic blood flow, a decreased flow would reduce clearance and would have the greatest effect on the clearance of compounds with the highest extraction efficiencies (11). If reduced blood flow through the liver during fasting was the principal cause of the decreased hepatic transport of bile acid and bilirubin, the compound with the greatest extraction efficiency (bile acid) should have been affected to the greatest degree (11). However, bilirubin transfer was affected to a greater degree; thus, a reduction in hepatic perfusion could not be the sole change during fasting.

Summary. Hepatic bile acid clearance, as determined with [^{14}C]cholic acid, was reduced 36% and plasma bile acid concentration was increased 72% in ponies following a 3-day fast. Eighty-seven to 91% of the isotopic bile acid was recovered in bile in 3 hours following tracer injection. The initial volume of distribution of injected tracer was not significantly different from the plasma volume. Reduced hepatic blood flow alone could not explain the changes in hepatic organic anion transport observed during fasting, since previous similar studies on the effects of fasting on hepatic bilirubin clearance showed a much greater fasting effect than the reduction of bile acid clearance in this study.

1. Gronwall, R., and Cornelius, C. E., N. Engl. J. Med. **283,** 204 (1970).
2. Barrett, P. V. D., J.A.M.A. **317,** 1349 (1971).
3. Felsher, B. F., and Carpio, N. M., Gastroenterology **69,** 42 (1975).
4. Felsher, B. F., Rickard, D., Redeker, A. G., N. Engl. J. Med. **283,** 170 (1970).
5. Gronwall, R., Amer. J. Vet. Res. **36,** 145 (1975).
6. Gronwall, R. and Mia, A. S., Amer. J. Dig. Dis. **17,** 473 (1972).
7. Kirshenbaum, G., Shames, D. F., and Schmid, R., J. Pharmacokinet. Biopharm. **4,** 115 (1976).
8. O'Maille, E. R. L., Richards, T. G., and Short, A. H., J. Physiol. **189,** 337 (1967).
9. Barrett, P. V. D., Berk, P. D., Menken, M., and Berlin, N. I., Ann. Intern. Med. **68,** 355 (1968).
10. Keiding, S., Scand. J. Clin. Lab. Invest. **36,** 113 (1976).
11. Wilkinson, G. R. and Shand, D. G., Clin. Pharmacol. Ther. **18,** 377 (1975).
12. Gronwall, R., Engelking, L. R., Anwer, M. S., Erichsen, D. F., and Klentz, R. D., Amer. J. Vet. Res. **36,** 653 (1975).
13. Anwer, M. S., Gronwall, R. R., Engelking, L. R., and Klentz, R. D., Amer. J. Physiol. **229,** 592 (1975).
14. Murphy, G. M., Billing, G. H., and Baron, D. N., J. Clin. Pathol. **23,** 594 (1970).
15. Schwartz, H. P., Bergmann, K. V., and Paumgartner, G., Cilica Chim. Acta **50,** 197 (1974).
16. Engelking, L. R., Gronwall, R., and Anwer, M. S., Amer. J. Vet. Res. **37,** 47 (1976).
17. Metzler, C. M., in "Proceedings," p. 380 5th Nat. Meeting, Amer. Pharmaceut. A. Acad. Pharmaceut. Sci., Amer. Pharmaceut. Assoc., Washington D. C. (1970).
18. Berk, P. D., *in* "Jaundice" (C. A. Goresky and M. M. Fisher, eds.) p. 134. Plenum Press, New York (1975).
19. Snedecor, G. W., and Cochran, W. G., "Statistical Methods", Iowa State University Press, Ames (1967).
20. Klapor, R., Bolte, J., and Schlosser, G. A., Res. Exp. Med. **166,** 209 (1975).
21. Cowen, A. E., Korman, M. G., Hofmann, A. F., and Thomas, P. J. Gastroenterology **68,** 1567 (1975).
22. Burke, C. W., Lewis, B., Panveliwalla, D., and Tabaqchali, S., Clin. Chem. Acta, **32,** 207 (1971).
23. Rudman, D., and Kendall, F. E., J. Clin. Invest. **36,** 538.
24. Glasinovic, J. C., Dumont, M., Duval, M., and Erlinger, S., J. Clin. Invest. **55,** 419 (1975).
25. Portman, O. W., and Shah, S., Archs. Biochem. Biophys. **96,** 516 (1962).
26. Olivecrona, J. and Sjövall, J. Acta. Physiol. Scand. **46,** 284 (1959).

Received September 1, 1978. P.S.E.B.M. 1979, Vol. 161.