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Fasting Hyperbilirubinemia and Its Relationship to Free Fatty Acids and Triglycerides in the Horse (40938)[1]

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Abstract. Bilirubinemia was studied in eight horses fasted for 5 days and then refed for 5 days. In six healthy horses bilirubin rose from 1.3 ± 0.4 mg/dl (mean ± SD) in fed horses to reach a plateau of 5.3 ± 1.4 mg/dl between 64 and 136 hr of starvation. This was almost entirely due to an increase in the unconjugated bilirubin fraction. On refeeding, serum bilirubin fell to prestarvation concentrations within 2 days. Scleral icterus lagged behind changes in serum bilirubin. There was a strong positive correlation between serum bilirubin and free fatty acids, $R^2 = 0.79$. This correlation was not improved when differences in serum albumin were considered, so changes in the affinity of albumin for bilirubin are probably not responsible for fasting hyperbilirubinemia. Including serum triglycerides in the regression equation improved its predictive value, $R^2 = 0.91$. Free fatty acids may influence bilirubin metabolism as a result of competition between free fatty acids and bilirubin for binding to ligandin.

Hyperbilirubinemia during fasting occurs in man and many animals (1–5), the response being greatest in horses. Fasting hyperbilirubinemia can be confused with hepatic disease. The response is exaggerated in fasted patients with Gilbert's syndrome, including subclinical cases (6).

Biochemical changes responsible for fasting hyperbilirubinemia have not been elucidated, but there may be some relationships with free fatty acids. Free fatty acids rise in fasted horses (7) and humans (8–10). Free fatty acids can displace bilirubin from albumin (11–13). There are also interactions between free fatty acids and ligandin (14), the hepatocyte bilirubin binding protein. This study investigates the relationship between fasting hyperbilirubinemia and free fatty acids in serum of fasted horses.

Materials and methods. The management of horses is described in detail elsewhere (7) and is summarized here. Horses were accommodated to a maintenance diet of a complete pelleted feed, two feeds daily for 10 days prior to the study. "Fed" serum samples were collected 16 hr postprandial on 3 consecutive days. The horses were deprived of food but not water for 5 days and blood was collected at 40, 64, 88, 112, and 136 hr postprandial. Refeeding was commenced at half maintenance for 1 day and then maintenance for 4 days; blood was collected 16 hr after meals on all except the fourth day of refeeding.

General health and scleral icterus were monitored daily. Six horses remained healthy throughout the study and had normal temperature, complete blood count, and attitude. One horse died of peracute hemorrhagic colitis on the first day of refeeding. One had a profuse watery diarrhea with weakness for 3 of the 5 days of starvation. This had no effect on hydration and was not accompanied by fever or changes in blood leukocyte counts. Healthy horses were used to compare the bilirubinemic response to fasting and refeeding. Data from all horses were used in statistical tests of relationships between bilirubin, free fatty acids, and albumin.

Estimation of free fatty acids was performed using the technique of Dole (15); all other assays were performed on an autochemist.[2] The method for albumin utilized

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[2] Metpath, 1 Belmont Avenue, A.S.B. Building, Suite 820, Bala Cynwyd, Penn. 19004.

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binding to bromocresol green (16); bilirubin estimation involves diazotization with sulfanilic acid (17). γ-Glutamyl transpeptidase activity was estimated using hydrolysis of L-γ-glutamyl-P-nitroanalide (18), alkaline phosphatase estimation utilized a thymolphthalein method (19, 20), and aspartate aminotransferase a hydrazone method (21, 22). Triglyceride estimation (23) involved hydrolysis with *Rhizopus delemar* lipase to release glycerol. This was subsequently phosphorylated and oxidized to dihydroxyacetone phosphate with conversion of NAD to NADH. Triplicate analysis of a laboratory control solution[3] yielded a coefficient of variation of 12% for total bilirubin, 0% for direct bilirubin, 2% for aspartate aminotransferase, 6% for alkaline phosphatase, 5% for γ-glutamyl transpeptidase, and 0% for albumin.

Statistical analysis was performed on a calculator[4] with a statistics module. Analysis of variance revealed no significant variation between days during the 3 fed days for any of the items studied. The mean fed value for each horse was compared with fasted and refed observations using analysis of variance and Duncan's new multiple range test (24).

Results. Total bilirubin concentration rose rapidly from 1.28 ± 0.38 mg/dl (mean ± SD) in fed horses to reach a plateau of 5.35 ± 1.38 mg/dl between 64 and 136 hr of food deprivation (Fig. 1). Unconjugated bilirubin accounted for 98% of this increase. Conjugated bilirubin rose in parallel from 0.13 ± 0.04 mg/dl in fed horses to 0.22 ± 0.06 mg/dl between 64 and 136 hr of caloric deprivation.

Upon refeeding, unconjugated bilirubin fell to prestarvation concentrations within 48 hr. Conjugated bilirubin fell to 0.16 ± 0.03 mg/dl at 24 hr of refeeding and then progressively rose to 0.19 ± 0.04 mg/dl at 120 hr; these values are not significantly different from prestarvation concentrations.

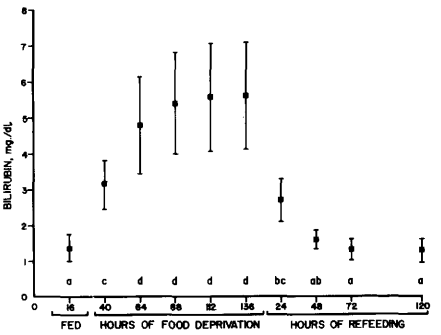

FIG. 1. Effects of caloric deprivation on total bilirubin in six horses. Values are means ± 1 standard deviation. Letters denote significant differences at the $P < 0.05$ level using Duncan's multiple range test.

Five of eight horses became icteric. Yellowing of sclera and oral mucosae occurred in parallel. Icterus developed at least 24 hr after total bilirubin was elevated above 5 mg/dl. In icteric horses, total bilirubin was 5.9 ± 0.6 mg/dl on the first day of icterus and 5.8 ± 0.97 mg/dl 24 hr earlier. Total bilirubin fell rapidly on refeeding, and three of four healthy horses were no longer icteric 24 hr after refeeding. One horse which had the highest fasting bilirubinemia, 7.56 mg/dl, remained icteric for the first 72 hr of the refeeding period. Total bilirubin concentration was 2.83, 1.87, and 1.07 mg/dl after 24, 48, and 72 hr, respectively.

A linear regression of peak fasting total bilirubin on fed total bilirubin was not significant ($P > 0.1$; correlation coefficient, $r = 0.36$). The only significant relationship between bilirubin concentrations (B, mg/dl) was for fed (B_{16}) and 40 hr food-deprived (B_{40}) samples: $B_{40} = 1.56 + 1.23\ B_{16}$; number of observations, $n = 8$; $r = 0.73$; $P < 0.05$. Serum concentrations of total bilirubin were related significantly to free fatty acids (F, mg/dl). These observations are depicted graphically in Fig. 2; the regression equation is: $B = 1.48 + 0.13F$; sample standard error of estimates = 0.90; $n = 75$; $r = 0.89$; $P < 0.005$. There was no improvement in the coefficient of determination, $R^2 = 0.785$, when a 24-hr time lag of bilirubin behind free fatty acids was introduced or when either serum albumin or the mean fed bilirubin concentrations were introduced into multiple-regression equations. The

[3] Ortho normal control, serum-assayed, Lot 6R018. Ortho Diagnostics, Inc., Raritan, N.J. 08869.

[4] T159 with PC100A printer; Texas Instruments, Inc., Dallas, Tex., 75222.

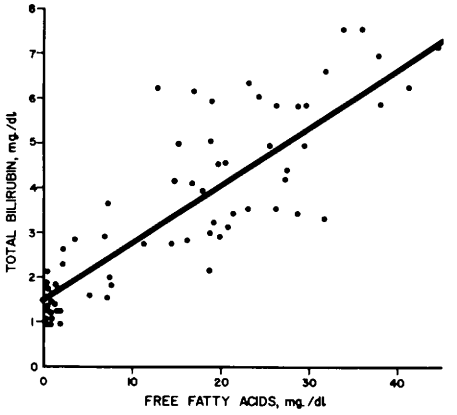

FIG. 2. Graph of total bilirubin against free fatty acids.

coefficient of determination was improved, $R^2 = 0.91$, by addition of triglyceride (TG, mg/dl): B = 1.1 + 0.086F + 0.011TG; sample standard error of estimate = 0.59; $n = 75$; $r = 0.95$; $P < 0.001$. There were no changes in serum enzymes with fasting. Significant between horse effects were found for all items studied.

Discussion. In the present study there is a high correlation between serum total bilirubin and free fatty acids in horses; this was further improved by the addition of triglycerides to the regression equation. An association between plasma triglycerides and bilirubin is not found in Gunn rats (25). However, plasma free fatty acid concentrations are related to the degree of fasting bilirubinemia in Gunn rats (26) and man (27). The correlation between free fatty acids and bilirubinemia in this variety of species suggests a common physiologic mechanism underlying these changes. Furthermore, manipulation of free fatty acid concentrations in man with heparin, epinephrine, caffeine, and insulin administration produce changes in serum free fatty acid concentrations that can be related to the degree of bilirubinemia (27). Similarly, there is an association between free fatty acids and hyperbilirubinemia in myocardial infarction (28). In Gunn rats, alteration of lipolysis by administration of nicotinic acid or insulin causes parallel changes in serum free fatty and bilirubin concentrations (26).

Some studies of the effects of diet are also consistent with a mechanism linking fatty acid and bilirubin metabolism. Low-calorie diets (300 kcal/day) aggravate hyperbilirubinemia in Gilbert's syndrome but maintenance energy diets (2400/day) do not (29). Extrapolation from animal studies (30) suggests that high rates of lipolysis with elevated serum free fatty acids would be expected on the low-calorie diet but not on maintenance energy diets. Serum bilirubin falls in fasted humans fed small quantities (400 kcal) of glucose orally (31); glucose release of insulin depresses plasma free fatty acids (8). Oral saline or 400-kcal quantities of mannitol or amino acids do not affect serum bilirubin (31) or free fatty acid concentrations (32, 33). One aspect of dietary manipulation which is not readily explicable by alterations in plasma free fatty acid concentrations is the effect of dietary triglyceride in individuals deficient in bilirubin glucuronyl transferase. Dietary lipid diminishes hyperbilirubinemia in Gunn rats (34, 35) and in some studies of individuals with Gilbert's syndrome (36). A recent study compared the effects of food deprivation and lipid withdrawal and suggested that these factors may influence bilirubinemia through different mechanisms (25).

The mechanism of fasting bilirubinemia is obscure. Although studies have documented an increase in carbon monoxide formation during fasting (4, 37), increased heme catabolism is unlikely to be important in the genesis of bilirubinemia for two reasons. The increment in carbon monoxide is small compared to that of bilirubin (4, 37); and carbon monoxide production returns towards baseline values after 72 hr of fasting, while bilirubinemia is maintained (37). Furthermore, studies of plasma bilirubin turnover in horses are not consistent with increased production of bilirubin during fasting (1). Individuals with Gilbert's syndrome have a more marked increase in carbon monoxide production (37), suggesting that increased heme degradation may contribute to the unusually marked fasting hyperbilirubinemia seen in these individuals (6).

Free fatty acids can displace bilirubin from albumin (11–13). This is unlikely to be

responsible for fasting hyperbilirubinemia since free, not bound, bilirubin is taken up by the hepatocyte (14). Our data support this contention—there was no correlation between serum albumin values and the degree of fasting hyperbilirubinemia. Another site at which free fatty acids could influence bilirubin kinetics is at ligandin. This molecule traps bilirubin within the hepatocyte (38, 39), speeds microsomal transformations, and may act as an "intracellular albumin" facilitating transfer of bilirubin across the cytosol to the microsomal conjugating enzymes. Ligandin binds a number of other organic anions, including fatty acids (14), and competition between bilirubin and some of these organic anions for ligandin binding has been shown (14). If free fatty acids competitively inhibit bilirubin binding to ligandin this would restrict accumulation of bilirubin within the hepatocyte and could limit transfer of bilirubin to the microsomal conjugating enzymes. Bilirubin would accumulate in plasma and give rise to an unconjugated fasting hyperbilirubinemia.

1. Gronwall, R., and Mia, A. S., Amer. J. Dig. Dis. **17**, 473 (1972).
2. Reid, I. M., Harrison, R. D., and Collins, R. A., J. Comp. Pathol. **87**, 253 (1977).
3. Tennant, B., Baldwin, B., Evans, C. D., and Kaneko, J. J., Proc. Ann. Convention Amer. Assoc. Eq. Pract. **21**, 410 (1975).
4. Bloomer, J. R., Barrett, P. V., Rodkey, F. L., and Berlin, N. I., Gastroenterology **61**, 479 (1971).
5. Cowan, R. E., Thompson, R. P. H., Kaye, J. P., and Clark, G. M., Clin. Sci. Mol. Med. **53**, 155 (1977).
6. Felsher, B. F., and Carpio, N. M., Gastroenterology **69**, 42 (1975).
7. Naylor, J. M., Kronfeld, D. S., and Acland, H., Amer. J. Vet. Res. **41**, 899 (1980).
8. Doar, J. W. H., Stamp. T. C. B., Wynn, V., and Andhya, T. K., Diabetes **18**, 633 (1969).
9. Morse, W. I., and Mahabir, R., Diabetes **13**, 286 (1964).
10. Mueller, P. S., and Evans, W. H., J. Lab. Clin. Med. **61**, 953 (1963).
11. Odell, G. B., Cukier, J. O., Ostrea, E. M., Maglalang, A. C., and Poland, R. L., J. Lab. Clin. Med. **89**, 295 (1977).
12. Starinsky, R., and Shafrir, E., Clin. Chim. Acta **29**, 311 (1970).
13. Yong, F. C., and Cheah, S. S., Res. Commun. Chem. Pathol. Pharmacol. **17**, 679 (1977).
14. Arias, I. M., Fleischner, G., Listowsky, I., Kamisaka, K., Mishkin, S., and Gatmaitan, Z., *in* "The Hepatobiliary System: Fundamental and Pathological Mechanisms," p. 81, NATO Advanced Study Institute on the Biliary System, 2nd, Aalborg, Denmark. Plenum Press, New York/London (1975).
15. Dole, V. P., J. Clin. Invest. **35**, 150 (1956)
16. Rodkey, F. L., Clin. Chem. **11**, 478 (1968).
17. Gambino, S. R., Golden, W. R. C., Goodwin, J. F., and Smith, A. W., Standards Meth. Clin. Chem. **5**, 55 (1965).
18. Szasz, G., Clin. Chem. **15**, 124 (1969).
19. Proksch, G. J., Bonderman, D. P., and Griep, J. A., Clin. Chem. **19**, 103 (1973).
20. Roy, A. V., Clin. Chem. **16**, 431 (1970).
21. Reitman, S., and Frankel, S., Amer. J. Clin. Pathol. **28**, 56 (1957).
22. Fingerhut, B., Ferzola, R., Marsh, W. H., and Levine, J. B., Ann. N.Y. Acad. Sci. **102**, 137 (1962).
23. Bucolo, G., and David, H., Clin. Chem. **19**, 476 (1973).
24. Steele, R. G. D., and Torrie, J. H., *in* "Principles and Procedures of Statistics," p. 107. McGraw–Hill, New York (1960).
25. Gollan, J. L., Hole, D. R., and Billing, B. H., Clin. Sci. **57**, 327 (1979).
26. Rosenthal, E., and Thaler, M. M., Gastroenterology **72**, A-99/1122 (1977).
27. Cowan, R. E., Thompson, R. P. H., Kaye, J. P., and Clark, G. M., Clin. Sci. Mol. Med. **53**, 155 (1977).
28. Cowan, R. E., Kaye, J. P., and Thompson, R. P. H., Gut **16**, 399 (1975).
29. Felsher, B. F., Amer. J. Clin. Nutr. **29**, 705 (1976).
30. Holmes, J. H. G., and Lambourne, L. J., Res. Vet. Sci. **11**, 27 (1970).
31. Barrett, P. V. D., Gastroenterology **68**, 361 (1975).
32. Blackburn, G. L., Flatt, J. P., Clowes, G. H. A., O'Donnell, T. E., and Hensle, T. E., Ann. Surg. **177**, 588 (1973).
33. Blackburn, G. L., Flatt, J. P., Clowes, G. H. A., and O'Donnell, T. E., Amer. J. Surg. **125**, 447 (1973).
34. Rosenthal, E., and Thaler, M. M., Gastroenterology **71**, 927/A34 (1976).
35. Gollan, J. L., Hatt, K. J., and Billing, B. H., Clin Sci. Mol. Med. **49**, 229 (1975).
36. Gollan, J. L., Bateman, C., and Billing, B. H., Gut **17**, 335 (1976).

37. Kutz, K., Egger, G., Bachofen, H., and Preisig, R., *in* "Chemistry and Physiology of Bile Pigments," p. 156. DHEW Publication No. NIH 77-1100 (1977).
38. Listowsky, I., Gatmaitan, Z., and Arias, I. M., Proc. Nat. Acad. Sci. USA **75**, 1213 (1978).
39. Wolkoff, A. W., Goresky, C. A., Sellin, J., Gatmaitan, Z., and Arias, I. M., Amer. J. Physiol. **236**, E638 (1979).

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