PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 165, 490–495 (1980)

Effect of Diabetes during Pregnancy on Maternal and Neonatal Bile Acid Metabolism in the Rat[1] (41010)

ASLAM S. HASSAN AND M. T. RAVI SUBBIAH

Department of Pathology and Medicine (Lipid Research Center), University of Cincinnati Medical Center, Cincinnati, Ohio 45267

Abstract. The effect of streptozotocin-induced diabetes in pregnant rats, on subsequent maternal and neonatal bile acid metabolism was investigated. Plasma glucose levels (mg%) of diabetic pregnant (DP) rats was significantly greater ($p < 0.05$) in comparison to control pregnant (CP) rats. Total bile acid pool (mg/100 g body wt) in DP was significantly higher ($p < 0.05$) when compared to CP (DP, 29.42 ± 4.44 vs 8.14 ± 1.13 in CP) with a marked increase in cholic acid pool size in DP (DP, 22.65 ± 3.33 vs 3.50 ± 0.48 CP, $p < 0.05$). The fecal excretion of bile acid was significantly lower ($p < 0.05$) in DP rats when compared to CP rats. Thin-layer chromatographic analysis of bile acid conjugates at postpartum revealed a significant increase in glycine conjugates in bile from DP rats. Examination of the cholic acid pool (μg/100 g body wt) in neonates (2 days old) derived from the two groups of rats indicated a strikingly reduced pool of cholic acid in neonates derived from diabetic mothers (neonates from DP 853.45 ± 213.98 vs 3690.86 ± 797.26 in CP, $p < 0.05$). Plasma cholesterol levels (mg%) in neonates of diabetic mothers was also significantly ($p < 0.05$) reduced. This study demonstrates that the diabetic state during pregnancy not only alters maternal bile acid metabolism, but also affects fetal bile acid metabolism in *utero* such that the effects may persist into neonatal life.

Diabetes has been shown to induce profound changes in bile acid metabolism (1–3). Most strikingly, the cholic acid pool is greatly increased accounting for the noted increase in total bile acid pool. The pool of chenodeoxycholic acid is either unchanged or decreased (1, 3). Such an alteration during pregnancy would be expected to have an effect on the bile acid biogenesis in the developing fetus and in early neonatal life as a significant fraction of the fetal bile acid pool may be of maternal origin (4, 5). The mechanism whereby such polar substances are transferred across the placenta and their consequent effects on fetal bile acid metabolism are not known. In continuation of our studies on neonatal bile acid metabolism (6, 7), the present experiment was carried out to determine the effect of diabetes on maternal bile acid and sterol metabolism and to see if the changes in maternal bile acid metabolism have an effect on neonatal bile acid metabolism.

Materials and methods. I. Experimental plan. Timed pregnant Sprague–Dawley rats were obtained from Harlan Animal Supply (Indianapolis, Ind.) and housed individually in the animal care facility. Food and water were available *ad libitum*. On the seventh day of pregnancy the animals were lightly anesthetized with diethyl ether and injected with either 10 mg of streptozotocin (approximately 50 mg/kg body wt) in 0.5 ml of citrate buffer, pH 5.0 (8) or with 0.5 ml of the above buffer alone via the jugular vein. Whether an animal received the drug or buffer alone was determined by a strict random order. For plasma glucose determination, a blood sample was obtained from the tail of each animal at the time of the injection and placed in Vacutainer tubes containing iodoacetic acid. Forty-eight hours after the injection, the animals were again lightly anesthetized with diethyl ether and another blood sample was collected for plasma glucose determination. Plasma glucose was determined by the standard glucose oxidase technique, using a Beckman glucose analyzer.

After establishing that the drug-injected animals were indeed diabetic (as evidenced by the profound hyperglycemia), a number

[1] This is paper No. 5 in the series "Sterol and Bile Acid Metabolism during Development."

0037-9727/80/110490-06$01.00/0

of studies were performed as described below. No insulin was used throughout the duration of the experiment.

II. Cholesterol excretion study. Randomly selected animals from the diabetic pregnant (DP) and control pregnant (CP) groups were placed in metabolic cages. After allowing 48 hr for adjustment to the cages, feces were collected (free of urine and food) for 3 days, pooled, and frozen until analyzed. Food intake during the 3 days was also measured. An aliquot of the diet was used for the determination of β-sitosterol and cholesterol content.

Feces were analyzed for neutral sterols and bile acids by the methods of Miettinen *et al.* (9) and Subbiah *et al.* (10) as described previously. The fecal samples were saponified with 2.5 *N* NaOH for 1 hr at 110° and the neutral sterols were extracted with petroleum ether. The aqueous extract was further saponified for 3 hr, acidified to pH 3 with concentrated HCl, and the bile acids were extracted with chloroform/methanol (2:1) and chloroform as previously described (10). Bile acids were methylated and quantitated as their methyl ester trifluoroacetates by gas–liquid chromatography (glc) (5, 10) using hyocholic acid as an internal standard. A F&M Model 402 gas–liquid chromatograph with 1% QF-1 (Gas-Chrom Q, 100/120 mesh) was used. The operating temperatures were: column 220°, injector, 235°, and detector 250°. Nitrogen was used as the carrier gas at a flow rate of 50 ml/min.

The neutral sterol fraction was analyzed by glc on 3% OV-17 (Gas-Chrom Q, 100/120 mesh) columns using 5α-cholestane as the internal standard. The operating temperatures were: column 250°, injector 290°, and detector 300°. Nitrogen was used as the carrier gas at a flow rate of 50 ml/min.

Fecal excretion of cholesterol and bile acids was corrected for fecal flow and sterol losses during intestinal transit, by the fecal recovery of dietary β-sitosterol as described previously (6, 10).

III. Bile acid pool analysis. On the 17th day of pregnancy, randomly selected animals from both DP and CP groups were anesthetized with diethyl ether. The entire gastrointestinal tract and liver were dissected out and frozen until analyzed. After obtaining a blood sample by cardiac puncture, the animals were killed by an overdose of sodium pentobarbital. The maternal tissues were thawed, homogenized in saline, and a weighed aliquot was saponified as described earlier for feces. The bile acids were methylated with freshly prepared diazomethane and purified from fatty acids by thin-layer chromatography (tlc) as described by Grundy *et al.* (11). The glc analysis of the purified bile acids was performed as described for fecal bile acids. Plasma from these animals was analyzed for glucose, cholesterol, and triglycerides. Plasma glucose was determined as before using Beckman glucose analyzer. Plasma cholesterol and triglycerides were determined by the standard LRC method (12). Fetal tissues were homogenized and analyzed for total cholesterol and bile acids in a manner similar to the maternal tissues.

IV. Neonatal plasma cholesterol and bile acid pool analysis. Two-day-old neonates derived from control and diabetic mothers were anesthetized with diethyl ether and exsanguinated by drawing blood via cardiac puncture. About 200–300 μl of blood was obtained from individual neonates. Blood from two or three neonates was usually pooled to give sufficient plasma for analysis. Plasma (100 μl) was saponified and the nonsaponifiable neutral sterols were extracted with petroleum ether. Cholesterol was determined by glc analysis of the extract using 5α-cholestane as the internal standard as described earlier.

For the analysis of bile acid pool, the entire gastrointestinal tract and liver were dissected out and subjected to the procedure described earlier for maternal bile acid pool analysis.

V. Biliary bile acid analysis. Postpartum biliary bile acid composition and conjugation pattern were determined on animals from both control and diabetic groups. The animals were anesthetized with sodium pentobarbital (35 mg/kg body wt) and the bile duct was cannulated with a polyethylene 10 cannula. Bile was collected for the first 30–60 min and frozen until analyzed. Biliary bile acid concentration was deter-

TABLE I. PLASMA GLUCOSE, CHOLESTEROL AND TRIGLYCERIDE LEVELS ON 17th DAY OF PREGNANCY IN CONTROL AND DIABETIC RATS (MEAN ± SEM)

Parameters	Control (N = 4)	Diabetic (N = 4)
Body weight (g)	244.53 ± 14.18	225.25 ± 14.42
Plasma glucose (mg/100 ml)	106.50 ± 7.33	445.0 ± 11.23*
Plasma cholesterol (mg/100 ml)	61.50 ± 1.94	77.25 ± 4.77*
Plasma triglyceride (mg/100 ml)	83.25 ± 20.38	313.50 ± 42.69*

* Significantly different compared to controls; $p < 0.05$.

mined by glc as described earlier. Biliary bile acid conjugate patterns were examined using an alcohol extract of the bile as described by Hofmann (13).

Results. As early as 2 days after injecting the drug, plasma glucose level (mg%) in streptozotocin-treated rats (381 ± 12) was significantly greater than the values noted in control pregnant rats (129 ± 2). On the 17th day of pregnancy plasma glucose level was four times higher in the streptozotocin-treated animals (Table I). Plasma cholesterol and triglyceride in streptozotocin-treated animals were significantly higher ($p < 0.05$) than those in control pregnant animals. No difference in body weights was noted between the two groups of animals.

Table II shows the bile acid pool size in control pregnant and diabetic pregnant rats. While the pool size of deoxycholic acid, 3β,12α-dihydroxycholanic acid and cholic acid was significantly higher ($p < 0.05$) in diabetic pregnant rats, the pool size of β-muricholic acid was significantly lower in this group of animals. In addition to an increased bile acid pool size, the diabetic pregnant rats showed a decrease in fecal excretion of bile acids (Table III). As can be seen from the table, both dietary intake and fecal weight of diabetic pregnant rats was significantly higher ($p < 0.05$) when compared to those normal pregnant rats. Fecal excretion of cholesterol and its degradation product in both groups of animals were similar despite the significantly greater intake of cholesterol by the diabetic pregnant animals. Total excretion of endogenous cholesterol in diabetic pregnant rats was significantly lower ($p < 0.05$) than that of control pregnant rats.

Table IV shows the plasma cholesterol levels and bile acid (cholic acid) pool size in

TABLE II. BILE ACID POOL SIZE IN CONTROL AND DIABETIC PREGNANT RATS

Bile acid	Bile acid pool size (μg/100 g body wt.) (mean ± SEM)		
	Diabetic (N = 4)	Controls (N = 4)	P value
Lithocholic acid	234.73 ± 35.47	405.66 ± 69.58	N.S.
3β,12α-Dihydroxycholanoic acid	1,207.50 ± 415.43	108.63 ± 19.12	$p < 0.05$
Deoxycholic acid	1,915.76 ± 281.18	722.32 ± 152.83	$p < 0.05$
Chenodeoxycholic acid	2,542.34 ± 382.93	2667.04 ± 388.54	N.S.
Hyodeoxycholic acid	120.65 ± 55.75	506.74 ± 149.17	N.S.
Cholic acid	22,650.10 ± 3331.32	3501.41 ± 479.69	$p < 0.05$
β-Muricholic acid	13.68 ± 13.68	192.08 ± 20.37	$p < 0.05$
Others[a]	738.85 ± 258.71	144.68 ± 53.12	N.S.
Total	29,423.62 ± 4444.57	8248.53 ± 1147.88	$p < 0.05$

[a] Others include bile acid peaks with retention times corresponding to 7-ketolithocholic and 3-keto-7α-hydroxycholanoic acids.

TABLE III. FECAL EXCRETION OF STEROLS AND BILE ACIDS IN CONTROL AND STREPTOZOTOCIN DIABETIC RATS

Parameters	Control	Diabetic
No. of Rats	4	4
Dietary intake (g/day/rat)	19.73 ± 0.77	28.13 ± 2.49[c]
Fecal output (g/day/rat)	8.05 ± 0.87	13.87 ± 0.76[c]
Cholesterol[a] + coprostanol (mg/day/rat)	11.58 ± 1.15	8.95 ± 0.54
Bile acids[a] (mg/day/rat)	30.86 ± 6.26	12.90 ± 2.09[c]
Cholesterol intake (mg/day/rat)	4.60 ± 0.18	6.57 ± 0.58[c]
Endogenous sterol Excretion[b] (mg/day/rat)	37.84 ± 6.75	15.28 ± 1.48[c]

[a] Corrected for fecal flow by using the fecal recovery of dietary β-sitosterol.

[b] Endogenous sterol excretion = (fecal cholesterol + coprostanol + bile acids − dietary cholesterol intake).

[c] Significantly different from control at $p < 0.05$.

neonates derived from normal and diabetic pregnant rats. Plasma cholesterol in neonates derived from diabetic mothers was significantly lower ($p < 0.05$) than the values in neonates derived from normal pregnant animals. Cholic acid pool size in neonates derived from diabetic mothers was also significantly lower ($p < 0.05$) when compared to that of neonates derived from normal pregnant rats. Apart from cholic acid, a number of secondary bile acids were also identified in the neonates and their concentration (μg/100 gm) was higher in the neonates of diabetic mothers (control neonates: 169.07 ± 46.4 vs 838.62 ± 542.9 in neonates from diabetic mothers). On the basis of percentage composition these were deoxycholic acid (1.2%), 3β,12α-dihydroxycholanic acid (3.4%), 3α,12β-dihydroxycholanic acid (34.4%), and a small amount of chenodeoxycholic acid.

Biliary concentration of bile acids was analyzed in fistula bile obtained from postpartum control and diabetic rats (Table V). The concentration of cholic acid in diabetic rats was significantly higher in diabetic postpartum rats. Examination of biliary bile acid conjugation pattern by thin-layer chromatography (Fig. 1) indicated an increase in glycine conjugated bile acids in diabetic postpartum rats.

Discussion. Previous studies of bile acid metabolism in spontaneously diabetic rats in our laboratory (3) and in alloxan-induced diabetic rats (1, 2) have shown a marked increase in bile acid pool under this condition. Our present studies using pregnant rats have shown a specific increase in cholic acid pool and a decrease in β-muricholic acid pool during diabetic pregnancy. In addition, our studies have clearly shown a decrease in the fecal excretion of bile acids in the diabetic state, a finding hitherto unreported. The findings that the fecal excretion of cholesterol and its metabolite (coprostanol) were similar in both groups despite significantly greater cholesterol intake by the diabetic rats, suggests an enhancement of cholesterol absorption in the diabetic state. This is in agreement with Nervi *et al.* (1), who showed that diabetic rats absorbed more cholesterol than normal controls. The noted enlargement of rat

TABLE IV. BODY WEIGHT, PLASMA CHOLESTEROL AND CHOLIC ACID POOL SIZE IN NEONATES OF NORMAL AND DIABETIC MOTHERS (MEAN ± SEM)[a]

Parameters	Neonates from controls	Neonates from diabetics
Body weight (g)	8.17 ± 0.20 (8)	5.60 ± 1.00[c] (4)
Plasma cholesterol (mg/100 ml)	68.38 ± 4.37 (6)[b]	13.84 ± 3.30[c] (5)
Cholic acid pool size (μg/100 gm body wt)	3690.86 ± 797.26 (8)	853.45 ± 213.98[c] (4)

[a] Neonates were 2 days old.

[b] Numbers in parentheses indicate the number of samples or animals studied.

[c] Significantly different, $p < 0.05$, compared to normal values.

TABLE V. BILIARY BILE ACID CONCENTRATION IN POSTPARTUM CONTROL AND DIABETIC RATS

Bile acid	Bile acid concentration (mg/ml)		
	Control (3)	Diabetic (3)	*P* value
Lithocholic acid	0.16 ± 0.03	0.15 ± 0.11	N.S.
3β,12α-Dihydroxycholanoic Acid	0.21 ± 0.03	0.86 ± 0.30	N.S.
Deoxycholic acid	0.17 ± 0.05	0.27 ± 0.05	N.S.
Chenodeoxycholic acid	1.30 ± 0.31	0.43 ± 0.19	N.S.
Hyodeoxycholic acid	0.48 ± 0.21	0.06 ± 0.03	N.S.
Cholic acid	4.22 ± 0.19	12.69 ± 2.17	$p < 0.05$
β-Muricholic acid	0.04 ± 0.03	0.04 ± 0.04	N.S.
Others[a]	0.13 ± 0.06	0.27 ± 0.03	N.S.
Total	6.71 ± 0.34	14.76 ± 1.86	$p < 0.05$

[a] Others include bile acid peaks with retention times corresponding to 7-ketolithocholic and 3-keto-7α-hydroxycholanoic acids.

small intestine (14) and the enhanced absorption of hexoses (15) and amino acids (16) in diabetes suggest a general change in intestinal absorption in this disease state.

Our study is the first to show a decreased bile acid pool size in neonates derived from diabetic mothers when compared to neonates derived from normoglycemic mothers. Although many factors could be responsible for this effect, a possibility that alteration in maternal bile acid pool (through its effects on fetal bile acid metabolism) may be involved deserves serious consideration.

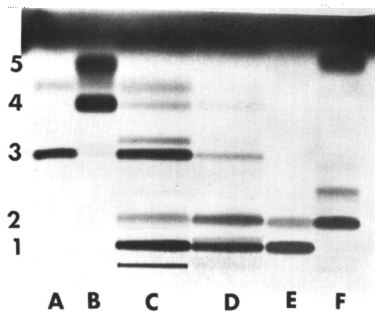

FIG. 1. Thin-layer chromatography of conjugated bile acids. Lanes A, B, E, and F represent the authentic standards of bile acids. C and D represent biliary extracts from diabetic and control rats, respectively. Bile acid identification: 1, taurocholic; 2, taurochenodeoxycholic; 3, glycocholic; 4, glycochenoxycholic; and 5, free cholic acid. Absorbent: silica gel g; solvent system; isoamyl acetate; propionic acid; *n*-propanol:water (4:3:2:1, v/v/v/v). Plates were stained with 10% phosphomolybdic acid in ethanol and heated for 15 min at 110°.

If an increase in maternal bile acids (presumably via placental transfer) can suppress the synthesis of bile acids in the fetus due to feed back inhibition (17), these effects may persist into early neonatal life. A higher concentration of the secondary bile acids was noted in neonates derived from diabetic mothers. Whether these bile acids can suppress the synthesis of fetal bile acids remains to be investigated. The transfer of cholic acid itself across the placenta is considered minimal (18). Because of the extremely low concentration of bile acids in the fetus it could not be conclusively shown that increased maternal bile acid pool itself could effect fetal bile acid metabolism. It might be pointed out here that the measurement of neonatal bile acid should provide a valid indication of the effects of maternal factors on fetal bile acid metabolism since bile acids appear in the rat fetus only a day or two before birth (19). Since, (a) many of the neonates derived from diabetic mothers are delivered premature (20) and (b) the pool size of premature infants is lower than that of normal infants (21), diabetic state during pregnancy might further decrease the pool size in these infants. The possibility that some of the effects noted in the neonatal rats could be due to the direct action of streptozotocin or its metabolite is unlikely as studies have shown (22) that administration of streptozotocin to pregnant rats does not decrease the pancreatic insulin content in the fetus.

Another possibility, that a deficient nutritional status of the fetus in diabetic pregnancy might cause overall retardation of the development of hepatic function, needs serious consideration. This is evident in the lower body weights of neonates from diabetic mothers. The neonates derived from diabetic mothers also showed a decrease in plasma cholesterol. This finding cannot be explained at the present time. A preliminary study indicating a decrease in liver cholesterol content of neonates derived from diabetic rats has been reported recently (23).

A study of biliary bile acids in postpartum rats indicated a persistent increase in the concentration of cholic acid and a decrease in chenodeoxycholic acid. Furthermore, thin-layer chromatographic examination of biliary-conjugated bile acids indicated an increase in glycine-conjugated bile acids. This could be due to the noted decrease in liver taurine content of diabetic rats (24) or to the tremendous increase in bile acid pool necessitating increased conjugation with glycine.

In conclusion, the present studies suggest that an uncontrolled diabetic state during pregnancy may affect neonatal bile acid pool. Further studies of the mechanism involved are currently underway in our laboratory. Our studies bring a hitherto unexplored area of bile acid metabolism (i.e., influence of maternal factors on bile acid synthesis during development) into focus for further investigation during physiological and pathophysiological states.

These studies were supported in part by Grant HL-24263 from National Heart, Lung and Blood Institute. The expert typing of the manuscript by Helen Haverland is gratefully acknowledged.

1. Nervi, F. O., Gonzalez, A., and Valdivieso, V. D., Metabolism **23**, 495 (1976).
2. Nervi, F. O., Severin, C. H., and Valdivieso, V. D., Biochim. Biophys. Acta **529**, 212 (1978).
3. Hassan, A. S., Subbiah, M. T. R., and Thiebert, P., Proc. Soc. Exp. Biol. Med. **164**, 449 (1980).
4. Smallwood, R. A., Jablonski, P., and McKwatts, J., Clin. Sci. Mol. Med. **45**, 403 (1973).
5. Subbiah, M. T. R., Marai, L., Dinh, D. M. and Penner, J. W., Steroids **29**, 83, (1977).
6. Li, J. R., Bale, L. K., and Subbiah, M. T. R., Atherosclerosis **32**, 93, (1979).
7. Li, J. R., Dinh, D. M., Ellefsson, R. D., and Subbiah, M. T. R., Metabolism **28**, 151 (1979).
8. Rakieten, N., Rakieten, M. L. and Nadkarni, M. V. (NSE-37917), Cancer Chem. Rep. **29**, 91 (1963).
9. Miettinen, T. A., Ahrens, E. H., Jr., and Grundy, S. M., J. Lipid Res. **17**, 78 (1976).
10. Subbiah, M. T. R., Tyler, N. E., Buscaglia, M. D., and Marai, L., J. Lipid Res. **17**, 78 (1976).
11. Grundy, S. M., Ahrens, E. H., Jr., and Miettinen, T. A., J. Lipid Res. **6**, 397, (1965).
12. L. R. C. Manual of Labs Operation Vol. 1. Lipid and Lipoproteins Analysis, NHLI-DHEW publication No. 75-028, U.S. Printing Office, Washington D.C., (1979).
13. Hofmann, A. F., J. Lipid Res. **3**, 127 (1962).
14. Jervis, E. L. and Levin, R. J., Nature (London) **210**, 391, (1966).
15. Schede, H. P., and Wilson, H. D., Amer. J. Physiol **220**, 1739, (1971).
16. Olsen, W. A. and Rosenberg, I. H., J. Clin. Invest. **49**, 90, (1970).
17. Shefer, S., Hauser, S., Berkersky, E., and Mosbach, E. H., J. Lipid Res. **10**, 646 (1969).
18. Lester, R., Little, J. M., Greco, R., Piasecki, G. J., Jackson, B. T., Ped. Res. **6**, 375, (1972).
19. Croizat, B., and Lambiotte, M., Arch. Sci. Physiol **25**, 303 (1971).
20. Gabbe, S. G., *in* "Diabetic Pregnancy: A perinatal perspective" (I. R. Markez and P. A. J. Adams, eds.), p. 45. Grunne & Stratton, New York, (1979).
21. Watkins, J. B., Ingall, D., Szezepanik, P., Dlein, D. D., and Lester, R., New Engl. J. Med. **288**, 431 (1973).
22. Golob, E. K., Rishi, S., Becker, K. L., Moore, C., and Shah. N., Diabetes **19**, 610 (1970).
23. Kupke, I. R., Meyer-Meinen M., and Herbert, L. *in* "Proceedings of Fifth International Symposium on Atherosclerosis." Houston, Tex., November 6–9, Abstract 198 (1979).
24. Reibel, D. K., Shaffer, J. E., Kocsis, J. J., and Neely, J. R., J. Mol. Cell. Card. **11**, 827 (1979).

Received May 1, 1980. P.S.E.B.M. 1980, Vol. 165.