specific role for progesterone in the reproductive physiology of snakes must await conclusive identification of progesterone in peripheral blood and establishment of a relationship between the steroid and the reproductive process.

Summary. Ovaries and adrenals of both *Natrix sipedon pictiventris* and *Coluber c. constrictor* synthesized progesterone *in vitro* from ^{3}H-16-pregnenolone. Progesterone synthesis by the ovaries and adrenals of Natrix was 3 times that of Coluber.

1. Clausen, H. J., *Endocrinol.,* 1940, v27, 700.
2. Bragdon, D. E., *J. Exp. Zool.,* 1951, v118, 419.
3. Porto, A., *Mem. Inst. Butatan,* 1942, v15, 27.
4. Bragdon, D. E., Lazo-Wesem, E. A., Zarrow, M. X., Hisaw, F. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 477.
5. Betz, T. W., *Copeia,* 1962, 284.
6. Short, R. V., *J. Endocrinol.,* 1960, v20, 147.
7. Bush, I. E., *Biochem. J.,* 1952, v50, 370.
8. Eberlein, W. R., Bongiovanni, A. M., *Arch. Biochem. Biophys.,* 1955, v59, 90.
9. Warren, J. C., Salhanick, H. A., *J. Clin. Endocrinol.,* 1961, v21, 128.
10. Bush, I. E., *The Chromatography of Steroids,* Pergamon Press, 1961.

Received November 8, 1963. P.S.E.B.M., 1964, v115.

Dietary Bile Acids and Lipid Metabolism. IV. Dietary Level of Lithocholic Acid for Chicks. (28972)

G. A. LEVEILLE, R. D. HUNT* AND H. E. SAUBERLICH
U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colo.

Several recent reports have demonstrated the hypercholesterolemic effect of dietary lithocholic acid in the chick(1-4). Previously, we have demonstrated that dietary lithocholic acid also markedly increases plasma lipid phosphorus levels and liver size(4,5). The increase in liver size has been shown to be the result of a bile duct proliferation(6) or the "ductular cell reaction"(7). These observations have recently been confirmed(3).

The effect of various levels of dietary lithocholic acid has not been studied. The data presented here were obtained in an effort to determine the minimal level of lithocholic acid required to produce the effects previously reported. In addition, the influence of dietary lithocholic acid on plasma β-lipoprotein levels has been studied.

Experimental. Male Hy-line White Leghorn chicks were employed in 2 experiments. In Exp. 1, the chicks were fed a stock diet for 7 days and in Exp. 2 for 10 days. At the end of this time, the chicks were allocated to the various experimental groups in such a way as to have almost identical initial mean body weights for all groups. In Exp. 1, 8 chicks were used per group with an average group weight of 65 or 66 g; 14 chicks were allocated to each group in Exp. 2 with an average group weight of 82 to 86 g.

The basal diet employed was identical to that used in previous studies(4). Lithocholic acid was added to the basal diet at the expense of glucose at the levels indicated in the Tables. The chicks were housed in heated cages having raised wire floors. Food and water were supplied *ad libitum.* Body weight and food consumption were determined at weekly intervals.

The experimental period in Exp. 1 was three weeks. In Exp. 2, half of each group was sacrificed after having received the experimental diets for 4 weeks; the remaining 7 chicks in each group were fed the diets for an additional 4 weeks. At termination of the study periods, blood was obtained by cardiac puncture with a heparinized syringe. The animals were sacrificed and the livers excised. After blotting to remove excess blood, the livers were weighed and a slice was fixed in 10% neutral buffered formalin. The remainder of the liver was frozen and stored at —20°C for chemical analysis.

* Animal Research Center, Harvard Med. School, Boston, Mass.

TABLE I. Body Weight, Liver Size, Plasma and Liver Lipids of Chicks Fed Different Livels of Lithocholic Acid.
Exp 1

Lithocholic acid (% of diet)	Body weight (g)	Plasma		Liver*			
		Cholesterol (mg/100 ml)	Lipid phosphorus (mg/100 ml)	Size (% body wt)	Fat (%)	Cholesterol (mg/g)	Lipid phosphorus (mg/g)
0	258 ± 25†	122 ± 28	14.9 ± 2.4	3.1 ± .4	4.9 ± .7	3.7 ± .2	.96 ± .09
.025	268 ± 25	134 ± 25	12.8 ± 2.1	3.0 ± .6	4.7 ± .4	3.4 ± .3	.97 ± .13
.050	304 ± 35	127 ± 23	12.5 ± 1.2	3.0 ± .4	4.4 ± .4	3.4 ± .4	1.00 ± .10
.100	287 ± 21	191 ± 53	20.2 ± 4.8	3.9 ± 1.0	4.0 ± .5	3.4 ± .3	.90 ± .09
.200	232 ± 28	440 ± 61	37.9 ± 5.7	6.7 ± .6	3.7 ± .2	3.8 ± .3	.88 ± .03

* Liver values expressed on a wet weight basis.

† Mean for 8 chicks ± standard deviation.

Liver sections were prepared as previously described for microscopic examination(6). Liver fat, cholesterol and lipid phosphorus, and plasma cholesterol and lipid phosphorus were determined as previously described(4). Plasma β-lipoproteins were determined by a polyanion precipitation procedure(8). To one volume of plasma were added a half volume each of a 1% solution of Mepesulfate† and a 12% solution of calcium acetate. After standing 30 minutes, the suspension was mixed and drawn into a capillary tube; the tube was flame sealed and centrifuged in a microhematocrit centrifuge for 5 minutes. The length of the serum mixture and the precipitate column were measured and the per cent β-lipoprotein calculated as follows:

$$\beta\text{-lipoprotein } \% = \frac{\text{precipitate mm}}{\text{serum mixture mm}} \times 200$$

The data from Exp. 1 were analyzed statistically by means of the "t" test and from Exp. 2 using a sequential method and the "t" test.

Results. Exp. 1 (Table I). The 0.1 and 0.2% dietary levels of lithocholic acid increased plasma cholesterol and lipid phosphorus levels and liver size. Lithocholic acid levels below 0.1% were ineffective. Body weight was depressed only at the 0.2% level. Liver fat was decreased and liver cholesterol increased in chicks fed 0.2% lithocholic acid. Microscopic examination of the livers revealed no significant lesions in chicks fed the diet devoid of or containing 0.025% lithocholic acid. The livers of 4 of the 8 chicks receiving the 0.05% level of the bile acid had no lesions; the remaining 4 showed a slight proliferation of bile ductules. At the 0.1% level, the livers of 2 chicks had no lesion, 4 showed a slight and 2 a moderate degree of bile ductule proliferation. Marked ductular proliferation was observed in the livers of all chicks fed the 0.2% level of lithocholic acid. The data obtained in this experiment indicate that a lithocholic acid level of from 0.1 to 0.2% of the diet is necessary to induce the increased plasma and liver lipid levels and liver size.

Exp. 2. (Table II). In Exp. 2, chicks were sacrificed after receiving the experimental diets for 4 or 8 weeks. In general, the results after 4 or 8 weeks are in good agreement. Body weight was depressed by lithocholic acid levels of 0.15% or higher, but not by the 0.1% level. Liver size was significantly increased by lithocholic acid levels of 0.1% and higher after feeding the diets for 4 weeks and by levels of 0.15% and higher after 8 weeks. The fact that the 0.1% level caused an increase in liver size after 4 weeks, but not after 8 weeks might be indicative of the chick's ability to adapt to this level of the bile acid. Plasma cholesterol levels were significantly higher in chicks fed 0.1% or higher levels of lithocholic acid. Plasma lipid phosphorus levels generally paralleled plasma cholesterol changes. Plasma β-lipoproteins were significantly elevated by levels of lithocholic acid in excess of 0.1%. At the 0.1% level, the determined values were higher than values for chicks fed lower levels of lithocholic acid, but the difference was not statistically significant.

† Mepesulfate—sodium salt of sulfated polygalacturonic acid, methyl ester methyl glycoside. Purchased from Hoffmann-LaRoche, Inc., Nutley, N. J.

TABLE II. Body Weight, Liver Size, Plasma and Liver Lipids and Plasma β-Lipoproteins of Chicks Fed Different Livels of Lithocholic Acid.

Exp 2

Lithocholic acid (% of diet)	Body weight (g)	Plasma			Liver*			
		Cholesterol (mg/100 ml)	Lipid phosphorus (mg/100 ml)	β-lipoprotein (%)	Size (% body wt)	Fat (%)	Cholesterol (mg/g)	Lipid phosphorus (mg/g)
4 weeks:								
0	427 ± 19†	125 ± 17	9.8 ± 1.9	2.56 ± .74	2.6 ± .2	5.6 ± .2	4.0 ± .2	.81 ± .10
.025	422 ± 29	148 ± 16	14.3 ± 4.4	1.90 ± .30	2.6 ± .2	5.8 ± .7	3.9 ± .2	.82 ± .07
.050	423 ± 39	144 ± 14	12.2 ± 1.7	2.13 ± .41	2.5 ± .2	5.7 ± .5	4.1 ± .2	.85 ± .09
.100	431 ± 49	190 ± 31	15.6 ± 2.5	2.95 ± 1.43	3.6 ± .6	5.4 ± .3	4.2 ± .3	.77 ± .08
.150	354 ± 60	324 ± 89	29.0 ± 3.6	5.48 ± 1.54	5.9 ± 1.8	5.6 ± .4	4.6 ± .4	.81 ± .07
.200	266 ± 49	703 ± 102	50.4 ± 8.2	8.25 ± 1.22	9.1 ± 2.5	5.1 ± .2	5.0 ± .2	.83 ± .13
.250	225 ± 43	737 ± 169	47.8 ± 10.3	11.12 ± 1.44	11.9 ± 2.5	5.0 ± .5	5.4 ± .4	.83 ± .06
8 weeks:								
0	917 ± 50	122 ± 13	11.4 ± 1.0	2.52 ± .90	2.4 ± .3	4.7 ± .4	4.4 ± .3	.94 ± .06
.025	919 ± 94	123 ± 9	11.2 ± 1.1	2.57 ± .60	2.0 ± .2	4.7 ± .5	4.3 ± .4	.99 ± .06
.050	947 ± 59	127 ± 10	12.1 ± 1.3	2.25 ± .67	2.2 ± .2	4.8 ± .4	4.5 ± .5	.98 ± .12
.100	897 ± 91	203 ± 35	15.0 ± 1.5	2.93 ± 1.25	2.4 ± .3	4.9 ± .4	5.6 ± .4	1.00 ± .10
.150	840 ± 108	486 ± 237	31.4 ± 13.0	6.65 ± 4.26	5.1 ± 1.8	4.3 ± .7	5.3 ± .9	.97 ± .08
.200	641 ± 134	705 ± 171	41.2 ± 9.0	12.51 ± 3.27	9.5 ± 3.6	4.9 ± .7	6.3 ± .9	.90 ± .19
.250	427 ± 113	460 ± 110	23.8 ± 6.4	11.61 ± 3.24	10.0 ± 2.5	4.7 ± .6	5.8 ± .8	1.03 ± .11

* Liver values are expressed on a wet weight basis.

† Mean for 7 chicks ± standard deviation.

The effects of dietary lithocholic acid on liver lipids were variable. Liver cholesterol was significantly elevated by lithocholic acid levels of 0.15% and higher when the diets were fed for 4 weeks; after 8 weeks of feeding, the 0.1% level also increased liver cholesterol significantly. Liver fat was decreased by the 0.2 and 0.25% levels of dietary lithocholic acid; however, this difference was not evident after feeding the diets for 8 weeks. Liver lipid phosphorus levels were not significantly altered by lithocholic acid feeding.

Microscopic examination of the livers revealed no lesions in the groups fed the 0 or 0.025% levels of lithocholic acid. A very slight or moderate degree of bile ductule proliferation was observed in the livers of chicks fed the 0.05 and 0.10% levels of lithocholic acid, respectively. The livers of chicks fed 0.15% or higher levels of lithocholic acid showed a marked ductular proliferation. The degree of this proliferation was more severe with increasing lithocholic acid levels. At the 0.20 and 0.25% levels, some necrosis and fibrosis were observed.

Discussion. The results obtained with the higher levels of lithocholic acid fed are in agreement with previous reports(1-6). From the data reported, it appears that a dietary lithocholic acid level of 0.1% of the diet is adequate to induce the proliferation of bile ductules and the associated plasma lipid changes. Increasing lithocholic acid levels are shown to be associated with a greater severity of ductular cell proliferation and lipid alterations.

The data reported do not shed light on the mechanism(s) of action of lithocholic acid in producing the observed changes. It would appear from the elevated plasma lipid levels that the increase in liver size and bile ductule proliferation are associated with an increase in lipid synthesis. This would be in accord with the report of Beher *et al*(9) showing that dietary lithocholic acid effected a marked increase in hepatic cholesterol synthesis in the mouse. The relationship between these results in the mouse and our findings in the chick are strengthened by our observation (unpublished) that the dietary level of lithocholic acid which increased hepatic cholesterol synthesis in the mouse(9) will also produce bile duct proliferation in this species.

Further study is required to elucidate the mechanism of action of lithocholic acid and to delineate the possible relationship of this mechanism to other anomalies in which the ductular cell reaction is observed.

Summary. Data are presented demonstrating that a dietary lithocholic acid level of 0.1% is sufficient to induce bile ductule proliferation in the livers of chicks and associated elevations in plasma cholesterol, lipid phosphorus and β-lipoprotein levels. The effects of dietary lithocholic acid were more severe with dietary levels above 0.10%. Body weight was decreased in chicks fed 0.15% or higher levels of lithocholic acid.

The authors express their gratitude to Mr. Gerhard Isaac for statistical evaluation of the data and to Messrs. Wayne Goad, Harold Schneider and Leonard Schiff for technical assistance.

1. Edwards, H. M., Jr., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 742.

2. Edwards, H. M., Jr., Boyd, F. M., *ibid.*, 1963, v113, 294.

3. Eyssen, H., Desomer, P., *Poultry Sci.*, 1963, v42, 1020.

4. Leveille, G. A., Hunt, R. D., Sauberlich, H. E., PROC. SOC. EXP. BIOL. AND MED., 1963, v114, 334.

5. Leveille, G. A., Sauberlich, H. E., Hunt, R. D., *Poultry Sci.*, 1962, v41, 1991.

6. Hunt, R. D., Leveille, G. A., Sauberlich, H. E., PROC. SOC. EXP. BIOL. AND MED., 1963, v113, 139.

7. Popper, H., Kent, G., Stein, R., *J. Mt. Sinai Hosp.*, 1957, v24, 551.

8. Leveille, G. A., Sauberlich, H. E., *U. S. Army Med. Rsch. & Nutrition Lab. Rep.* 272, Jan. 1963, 17.

9. Beher, W. T., Anthony, W. L., Baker, G. D., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 317.

Received November 15, 1963. P.S.E.B.M., 1964, v115.