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The Maternal Pheromone and Bile Acids in the Lactating Rat[1]

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KILPATRICK, S. J., M. BOLT AND M. MOLTZ. *The maternal pheromone and bile acids in the lactating rat.* PHARMAC. BIOCHEM. BEHAV. 12(4) 555–558, 1980.—Bile was drawn from virgin rats and from postpartum rats that were with young for 5, 12, 21, and 30 days, respectively. The bile thus drawn was analyzed enzymatically after chromatographic separation to test an hypothesis relating cholic acid and one of its metabolites, deoxycholic acid, to the appearance of the maternal pheromone. Our finding that cholic acid, but not deoxycholic acid, reached a peak that was tied specifically to the period of pheromonal emission led us to advance a revised hypothesis. We now think that cholic acid alone, or more likely a cholic metabolite other than deoxycholic acid, underlies the appearance of the pheromone.

Maternal pheromone Cholic acid Bile Lactation

NUMEROUS reports [6, 7, 8, 9, 10, 11, 12, 17] have attested to the presence of a pheromone in the feces of lactating rats and in the feces of nulliparous rats that come to behave maternally through continuous association with young. In both, the pheromone first appears 14 days after the start of the maternal episode and disappears at approximately 27 days [11]. During this 14- to 27-day period, and only then, are the mother's feces attractive to young [7,11].

Prolactin is known to be essential for pheromonal release [12], a finding underscored by data showing that prolactin is significantly elevated during the two-week period immediately preceding the appearance of the attractant [1,14]. Cognizant of this fact, and knowing that high concentrations of prolactin in blood result in high uptake of the hormone at the liver [2, 13, 18], Moltz and Leidahl [16] suggested that the release of the pheromone may involve a prolactin-induced change in hepatic functioning, specifically a change in bile-acid content. They thought of this change, in turn, as altering the chemistry of the gut, leading to the excretion of pheromone-containing feces.

The male rat is non-pheromone emitting, even when he is behaving as maternally as his female counterpart [16]. Taking advantage of this fact, Moltz and Leidahl drew bile from pheromone-emitting females for injection into the ceca of intact males. The males came to show full evidence of the pheromone [16].

Based in part on the data just reviewed, Moltz and Kilpatrick [15] advanced an hypothesis relating to the identity of the pheromone. They suggested that maternally-behaving females, as a consequence of their high hepatic binding of prolactin, come to synthesize cholic acid, a primary bile acid, in greater-than-normal amounts, enabling a critical fraction of the cholic metabolite, deoxycholic acid, to appear in their anal excreta. It is this fraction, in combination with other compounds contained in the fecal material, that was thought to constitute the pheromone, attracting young.

Several predictions issue from the Moltz-Kilpatrick hypothesis. Perhaps the most obvious is the expectation of an increase in both biliary cholic- and biliary deoxycholic-acid coincident with the time of pheromonal emission. Thus, females known to be emitting the pheromone, females lactating 21 days for example, would be expected to have a higher concentration of these steroids in their bile than non-pheromone emitting females, such as virgins and primiparae lactating for only a few days. The present experiment was designed to test this expectation, using thin layer chromatography and an enzymatic spectrophotometric assay. To assess the specificity of the results, that is, to assess whether other bile salts might not also be elevated during the time of pheromonal emission, we monitored not only chenodeoxycholic acid which, together with cholic acid, comprise the primary bile-acids of the rat, but total bile-acid concentration as well.

METHOD

Subjects

A total of 20 female Wistar rats was used, each born and reared in the authors' laboratory. At approximately 90 days of age, they were either mated, and allowed subsequently to care for a litter of six young, or left unmated.

In the case of each mated female, bile was drawn on an assigned postpartum day: for some this day corresponded to Day 5 of lactation, for others to Days 12, 21, and 30, respec-

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tively. These postpartum days were chosen to provide a bile-acid profile for animals known to be (a) non-pheromone emitting (5 days), (b) pre-pheromone emitting (12 days), (c) strongly pheromone emitting (21 days), and (d) post-pheromone emitting (30 days). For purposes of comparison, bile was also drawn from nulliparous females.

Bile Collection

Since the rat does not have a gall bladder, bile had to be taken directly from the bile duct. Each female was placed under deep Diabutal anesthesia (0.1 cc/100 g BW) after which she was laparotomized to expose the duct. A small piece of silastic tubing was inserted into the duct and secured with a suture. At the end of exactly one hour, the cannulation was terminated and the volume of bile was recorded. All bile was frozen until analyzed.

Analysis of Bile Acids in Bile

The bile acids contained in the bile were deconjugated by alkaline hydrolysis. After a 0.1 ml or a 0.2 ml aliquot of bile was brought to 5N by adding 10N NaOH and water, each sample was hydrolysed for 3 hr at 10 lb of pressure. The hydrolysed samples were acidified to a pH of 1.0 with concentrated HCl, extracted three times with twice their volume of ethyl ether, and washed three times with 5 ml of water. Prior to the hydrolysis, trace amounts (20,000 dpm) of cholic- (40.0 mCi/mmol) and deoxycholic acid C^{14} (52.0 mCi/mmol) were added to each sample to assess recovery.

The pooled ether extracts were dried on a rotary evaporator, quantitatively transferred with small volumes of methyl alcohol to 15 ml test tubes and then dried with forced air. The sides of the test tubes were rinsed with additional methyl alcohol and dried. Methyl alcohol was again added and the sample quantitatively applied to a pre-coated silica gel chromatographic plate (EM Labs). Standards for cholic-, chenodeoxycholic- and deoxycholic acid were applied to the plates to locate the individual samples. The plates were then developed for 2 hr, using solvent-system No. 11 of Eneroth (trimethylpentane-ethyl acetate-acetic acid) [3]. It should be noted that although this particular solvent-system separates the dihydroxy bile acids (chenodeoxycholic- and deoxycholic-acid), it does not separate the trihydroxy acids, of which cholic acid is one. Unfortunately, there is no single solvent-system that does separate both the dihydroxy- and the trihydroxy-bile acids [3]. Moreover, it is doubtful that a clear separation among the trihydroxy bile acids can be achieved on silica gel plates to permit precise quantitation [20]. However, procedures using gas-liquid chromatography [4, 19, 20, 21] have revealed that cholic acid comprises approximately 84% of the total trihydroxy acids in rat bile, and so we can be confident that it is largely cholic acid that we are isolating in the present system.

Visualization of the developed plates was achieved with I_2 vapor. Areas on the plates corresponding to the three standards were scraped into test tubes, eluted with methyl alcohol, vortexed and centrifuged. The supernatant was retained and a second extraction performed on the remaining pellet. The combined supernatants were dried on a rotary evaporator and 1 to 5 ml of methyl alcohol were added to each residue. Aliquots totalling one-tenth of the entire sample volume were drawn for scintillation counting and 0.1 ml aliquots were drawn for enzymatic-spectrophotometric assay. The recovery figures were used as correction factors in the final calculation of concentration values (μ moles/ml).

An enzymatic kit (Sterognost -3, Nyegaard Co.) was used to prepare individual bile acids for spectrophotometric analysis. This analysis enabled us to obtain concentration values for cholic, chenodeoxycholic- and deoxycholic-acid. In addition, total bile-acid concentration in whole bile was measured for each group following the procedure of Iwata and Yamasaki [5]. The hydroxysteroid dehydrogenase (STDHP) used for this assay was obtained from Worthington Biochemical Corp.

RESULTS

Data for the individual bile acids were expressed in μ moles/hr, which is a measure of *output* and in μ moles/ml, which is a measure of *concentration*. Output was calculated by multiplying each μ mole/ml value of every animal by the mean volume (ml/hr) of the group to which it belonged. The output of a bile acid reflects the amount of that acid delivered to the gut per unit time.

The mean volume of bile excreted during the hour-long cannulation varied systematically across groups, with nulliparous females yielding the lowest volume (0.62 ml) and with females lactating for 12 days the highest volume (1.4 ml). The mean output of each group, for each of the three bile-acids analyzed, is presented in Table 1.

Bile-Acid Output

Analysis of variance revealed a significant difference between groups with respect to the output of cholic acid ($F=17.04$; $p\leq0.001$). The Newman-Keuls test indicated that pheromone-emitting females, that is, those lactating for 21 days, had a significantly higher ($p\leq0.05$) mean cholic-acid output than nulliparous females and those lactating for 5-, 12-, and 30-days, respectively. The mean cholic-acid output for those females designated as pre-pheromone emitting (12 days) also differed significantly from that of the nulliparous females and from that of the 5- and 30-day groups, respectively. The last two groups did not differ significantly from each other nor did they differ significantly from nulliparous females.

Analysis of variance also yielded a significant difference between groups for the output of deoxycholic acid ($F=7.14$, $p<0.001$). As was true in the case of cholic acid, the highest mean value of deoxycholic acid was shown by pheromone-emitting females (21 days). The Newman-Keuls test revealed that this value differed significantly from that of the nulliparous group and from that of the 5- and 30-day groups, respectively. However, the output of deoxycholic acid from the pheromone-emitting, 21-day group did not differ significantly from that of the pre-pheromone emitting, 12-day group.

The output of chenodeoxycholic acid presented a picture sharply at variance with the picture presented by cholic acid and, to a more limited extent, with that presented by deoxycholic acid. Although the analysis of variance was significant ($F=8.10$, $p\leq0.001$), the mean output of chenodeoxycholic acid failed to increase significantly not only from 12 to 21 days but from 5 to 21 days—not only from the pre-pheromone emitting to the pheromone-emitting condition but from the non-pheromone emitting condition as well.

Bile Acid Concentration

When the concentration rather than the output of each bile acid was compared across groups, analysis of variance

TABLE 1

MEAN OUTPUT (μ moles/hr) OF CHOLIC-, DEOXYCHOLIC- AND CHENODEOXYCHOLIC-ACID FOR EACH GROUP

Group*	Cholic acid	Deoxycholic acid	Chenodeoxycholic acid
Nulliparous (0.62 ml)	14.67	0.82	2.34
Day 5 postpartum (1.15 ml)	20.22	1.50	4.01
Day 12 postpartum (1.40 ml)	30.30†	2.09	5.71§
Day 21 postpartum (1.29 ml)	37.38†	2.48‡	5.43§
Day 30 postpartum (0.92 ml)	19.12	1.18	3.12

*Number in parentheses indicates mean volume of bile/hr.
†Differs significantly from all other groups.
‡Does not differ from Day 12 postpartum, but does differ significantly from remaining groups.
§Differs significantly from nulliparous and Day 30 postpartum groups.

TABLE 2

MEAN TOTAL BILE ACID CONCENTRATION (μ moles/ml)

Group	μ moles/ml
Nulliparous	35.52
Day 5 postpartum	26.22*
Day 12 postpartum	29.45
Day 21 postpartum	44.05†
Day 30 postpartum	29.80

*Differs significantly both from the nulliparous group and the Day 21 postpartum group.
†Differs significantly from all other groups.

revealed only cholic acid to be significant ($F=4.07, p\leq 0.05$). Additionally, the Newman-Keuls test showed that the concentration of cholic acid was higher on Day 21 postpartum than on Days 5, 12, and 30 postpartum. An analysis of variance comparing *total* bile acid concentration in the five groups was also significant ($F=13.67, p\leq 0.001$). As shown in Table 2, the Newman-Keuls test indicated that total bile-acid concentration was significantly higher in the bile of females lactating for 21 days than in bile drawn from any other group.

DISCUSSION

The hypothesis of Moltz and Kilpatrick, namely that the pheromone has deoxycholic acid as an essential moiety, demands that both cholic- and deoxycholic-acid be significantly elevated during the period of pheromonal emission. Only cholic acid, the precursor of deoxycholic acid, confirmed the prediction, in that only cholic acid showed a peak output as well as a peak concentration at 21 days postpartum.

Of course it may be that Moltz and Kilpatrick are correct, that deoxycholic acid is an essential moiety of the peromone. The output, if not the concentration, of deoxycholic acid was elevated at 21 days postpartum relative, at least, to its level at 5- and 30-days. However, the fact remains that, of the individual bile acids we monitored, only cholic acid reached a height in the bile that was tied specifically to the period of pheromonal emission. Perhaps it is cholic acid, then, that is critical to the pheromone, or perhaps what is critical is not cholic acid but some metabolite of cholic acid not yet considered in relation to the pheromone. However, the choice between these alternatives is confounded by the fact that an elevation in total bile-acid concentration was also tied to the period of pheromonal emission. This raises the possibility that it may not be cholic acid at all that underlies the appearance of the attractant; rather, what may be necessary for its appearance is simply an increase in the overall bile-acid content of bile. The question has been partly resolved by preliminary results obtained recently in the authors' laboratory: nulliparous females fed cholic acid came to emit the pheromone, while those fed either deoxycholic- or chenodeoxycholic-acid failed to do so. If, under these conditions, we were to find that all three bile acids promoted the same total bile-acid concentration, then we would have strong evidence that a specific biliary agent, namely cholic acid or one of its metabolites, is critical for pheromonal emission. The relevant assay is currently underway in the authors' laboratory.

Although the present study used only females, the data speak to the results of Moltz and Leidahl [16] using males. It will be recalled that these investigators promoted pheromonal emission in the male by intracecal injection of bile drawn from pheromone-emitting females, specifically, from females lactating for 21 days. In addition, they showed that the same volume of bile drawn from non-pheromone emitting females, those lactating for only 5 days, was entirely ineffective. It is now apparent that a substantial difference exists in the bile-acid content of what, succinctly, may be called 5- and 21-day bile.

REFERENCES

1. Amenomori, V., C. L. Chen and J. Meites. Serum prolactin levels in rats during different reproductive states. *Endocrinology* **86:** 506–510, 1970.
2. Costlow, M. E., R. A. Buschow and W. L. McGuire. Prolactin stimulation of prolactin receptors in rat liver. *Life Sci.* **17:** 1457–1466, 1975.
3. Eneroth, P. Thin layer chromatography of bile acids. *J. Lipid Res.* **4:** 11–16, 1963.
4. Haslewood, G. A. D. The biological significance of chemical differences in bile salts. *Biol. Rev.* **39:** 537–574, 1964.
5. Iwata, T. and K. Yamasaki. Enzymatic determination and thin layer chromatography of bile acids in blood. *J. Biochem., Tokyo* **56:** 424–431, 1964.
6. Leidahl, L. C. and H. Moltz. Emission of the maternal pheromone in the nulliparous female and failure of emission in the adult male. *Physiol. Behav.* **14:** 421–424, 1975.
7. Leidahl, L. C. and H. Moltz. Emission of the maternal pheromone in nulliparous and lactating females. *Physiol. Behav.* **18:** 399–402, 1977.
8. Leon, M. Maternal pheromone. *Physiol. Behav.* **13:** 441–453, 1974.
9. Leon, M. Emission of maternal pheromone. *Science* **201:** 938–939, 1978.
10. Leon, M. and H. Moltz. Maternal pheromone: discrimination by pre-weanling albino rats. *Physiol. Behav.* **7:** 265–267, 1971.
11. Leon, M. and H. Moltz. The development of the pheromonal bond in the albino rat. *Physiol. Behav.* **8:** 683–686, 1972.
12. Leon, M. and H. Moltz. Endocrine control of the maternal pheromone in the postpartum female rat. *Physiol. Behav.* **10:** 65–67, 1973.
13. Manni, A., M. J. Chambers and O. H. Pearson. Prolactin induces its own receptors in rat liver. *Endocrinology* **103:** 2168–2171, 1978.
14. Marinari, K. T. and H. Moltz. Serum prolactin levels and vaginal cyclicity in concaveated and lactating female rats. *Physiol. Behav.* **21:** 575–578, 1978.
15. Moltz, H. and S. J. Kilpatrick. Response to the maternal pheromone in the rat as protection against necrotizing enterocolitis. *Neurosci. Biobehav. Rev.* **2:** 277–280, 1978.
16. Moltz, H. and L. Leidahl. Bile, prolactin and the maternal pheromone. *Science* **196:** 81–83, 1977.
17. Moltz, H. and M. Leon. Stimulus control of the maternal pheromone in the lactating rat. *Physiol. Behav.* **10:** 69–71, 1973.
18. Posner, B. I., P. A. Kelly and H. G. Friesen. Prolactin receptors in rat liver: possible induction by prolactin. *Science* **188:** 57–59, 1975.
19. Siegfried, C. M., E. A. Doisy, Jr. and W. H. Elliott. Bile acids XLIV. Quantitation of bile acids from the bile fistula rat given [4^{14}C] cholesterol. *Biochem. biophys. Acta.* **380:** 66–75, 1975.
20. Uchida, K., I. Okuno, H. Takase, Y. Nomura, M. Kadowaki and N. Takeuchi. Distribution of bile acids in rats. *Lipids* **13:** 42–48, 1978.
21. Yousef, I. M., G. Kakis and M. M. Fisher. Bile acid metabolism in mammals. III. Sex differences in the bile acid composition of rat bile. *Can. J. Biochem.* **50:** 402–408, 1972.