hypoglycemia is initiated by alterations in cerebral metabolism, whereas the feeding response is not, and a decrease in the utilization of glucose per se does not appear to be the critical stimulus in either case.

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References and Notes

1. W. B. Cannon, M. A. McIver, S. W. Bliss, *Am. J. Physiol.* **69**, 46 (1924); B. A. Houssay, E. A. Molinelli, J. T. Lewis, *Rev. Asoc. Med. Argent.* **37**, 486 (1924); A. Goldfien, M. S. Zileli, R. H. Despointes, J. E. Bethune, *Endocrinology* **62**, 749 (1958).
2. J. La Barre and R. Saric, *C. R. Soc. Biol.* **124**, 287 (1937); H. Duner, *Acta Physiol. Scand. Suppl.* **102**, 7 (1953).
3. E. M. MacKay, J. W. Callaway, R. H. Barnes, *J. Nutr.* **20**, 59 (1940); D. A. Booth and T. Brookover, *Physiol. Behav.* **3**, 439 (1968).
4. J. Mayer, *Ann. N.Y. Acad. Sci.* **63**, 15 (1955); A. B. Steffens, *Physiol. Behav.* **4**, 823 (1969); G. P. Smith and A. N. Epstein, *Am. J. Physiol.* **217**, 1083 (1969).
5. J. P. Flatt, G. L. Blackburn, G. Randers, J. B. Stanbury, *Metabolism* **23**, 151 (1974).
6. O. E. Owen, A. P. Morgan, H. G. Kemp, J. M. Sullivan, M. G. Herrera, G. F. Cahill, Jr., *J. Clin. Invest.* **46**, 1589 (1967); T. Itoh and J. H. Quastel, *Biochem. J.* **116**, 641 (1970); R. A. Hawkins, D. H. Williamson, H. A. Krebs, *ibid.* **122**, 13 (1971).
7. C. R. Park, L. H. Johnson, J. H. Wright, Jr., H. Batsel, *Am. J. Physiol.* **191**, 13 (1957); W. H. Oldendorf, *ibid.* **221**, 1629 (1971).
8. S. Nicolaïdis, N. Rowland, M. Meile, P. Marfaing-Jallet, A. Pesez, *Pharmacol. Biochem. Behav.* **2**, 131 (1974).
9. Two grams of Purina Chow pellets yield 7.2 kcal. For rats weighing 333 g, the estimated caloric loads were approximately 6.6 kcal when β-hydroxybutyrate was infused (of the racemic mixture administered, only the D-isomer can be metabolized) and 5.2 kcal when the sugars were given.
10. Hypertonic NaCl is known to decrease food intake in hungry rats, presumably because of increased thirst [J. S. Schwartzbaum and H. P. Ward, *J. Comp. Physiol. Psychol.* **51**, 555 (1958); T. H. Yin, C. L. Hamilton, J. R. Brobeck, *Am. J. Physiol.* **218**, 1054 (1970)]. In our experiments, rats infused with 1.2*M* NaCl solution drank 18.8 ± 2.2 ml (mean ± standard error of the mean), significantly more than that consumed by rats infused with 0.15*M* NaCl (2.3 ± 0.6 ml; $P < .001$). Water intakes of rats infused with β-hydroxybutyrate or the sugars ranged from 0 to 5.9 ml and were not significantly different than those of rats receiving 0.15*M* NaCl.
11. A. Saifer and S. Gerstenfeld, *J. Lab. Clin. Med.* **51**, 448 (1958).
12. J. Haggendal, *Acta Physiol. Scand.* **59**, 242 (1963); H. C. Campuzano, J. E. Wilkerson, S. M. Horvath, *Anal. Biochem.* **64**, 578 (1975).
13. R. L. Himsworth, *J. Physiol. (London)* **206**, 411 (1970).
14. S. Maddock, J. E. Hawkins, Jr., E. Holmes, *Am. J. Physiol.* **125**, 551 (1939); E. J. Drenick, L. C. Alvarez, G. C. Tamasi, A. S. Brickman, *J. Clin. Invest.* **51**, 2757 (1972).
15. G. Hetenyi, Jr., *Diabetes* **21**, 797 (1972). The absence of this effect may account for the lower blood glucose levels in insulin-treated rats given mannose than in rats given equimolar amounts of fructose (Table 2; $P < .001$).
16. M. Russek, in *Neural Integration of Physiological Mechanisms and Behaviour*, G. J. Mogenson and F. R. Calaresu, Eds. (Univ. of Toronto Press, Toronto, 1975), pp. 128–147; M. I. Friedman and E. M. Stricker, *Psychol. Rev.* **83**, 409 (1976).
17. H. A. Krebs, D. H. Williamson, M. W. Bates, M. A. Page, R. A. Hawkins, *Adv. Enzyme Regul.* **9**, 387 (1971).
18. Consistent with this interpretation are findings that adrenal demedullation, which retards the increase in hepatic glucose production (*1*), attenuates the feeding induced by insulin [D. A. Booth, *Physiol. Behav.* **8**, 1069 (1972)]. Thus, the effectiveness of β-hydroxybutyrate, glucose, and mannose in our experiments may result from their demonstrated capacity to suppress the sympatheticoadrenal response (Fig. 1) (*15*), whereas the effects of fructose may instead result from a direct influence on hepatic metabolism which could not be reversed by this level of sympathetic activation. In this regard, we have recently found that intravenous injection of 4.4 units of insulin per kilogram, which lowers blood glucose to 30 to 40 mg/100 ml, stimulates larger increases in plasma catecholamines than are reported here, and elicits feeding in rats that could be totally suppressed by glucose or mannose but not by equimolar fructose solutions (N. E. Rowland and E. M. Stricker, in preparation).
19. We thank J. Yen for her technical assistance. Supported by NIMH grant MH-25140 (to E.M.S.).

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Bile, Prolactin, and the Maternal Pheromone

Abstract. *When bile from females that had been lactating for 21 days was injected into the cecum of male rats it induced release of a maternal pheromone. Males injected with bile drawn from females in which prolactin had been inhibited, or from females that had been lactating for only 5 days, did not emit the pheromone. These data suggest a sex difference in the way prolactin alters the composition of bile so that the female can emit the maternal pheromone while the male normally cannot.*

The maternally behaving lactating rat emits a pheromone that strongly attracts young (*1*). This pheromone, contained in the female's feces, is first released about 16 days after the start of lactation, corresponding to the age at which the young first become responsive to the pheromone. At about 27 days, pheromonal release ceases, which, in turn, coincides with the age at which the young cease to be attracted to the pheromone (*1*).

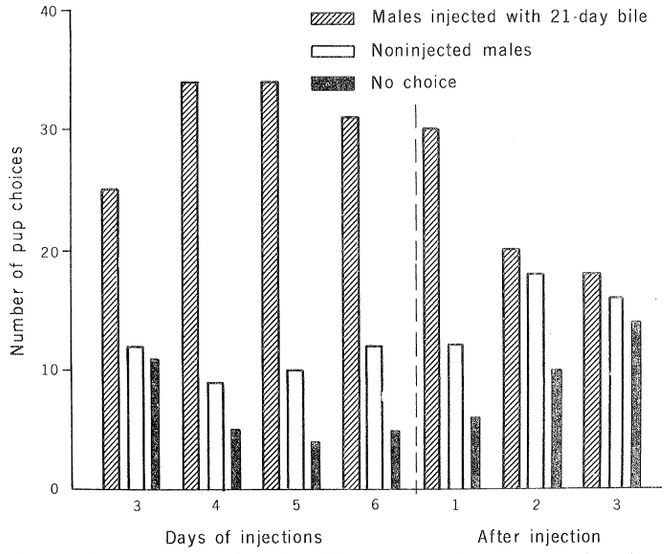
Under certain conditions, nulliparous females and adult males behave maternally. That is, they come to build a nest, lick, retrieve, and even crouch in a nursing posture when housed continuously with young—a procedure known as concaveation (*2*). Leidahl and Moltz (*3*) studied such concaveated animals to determine whether they also emit the pheromone. Briefly, the procedure was to provide each animal with a litter of foster pups approximately 24 hours old. These foster pups were allowed to remain in the cage until the following day, at which time a new litter of the same age was substituted. Replacing one 24-hour-old litter with another continued until the day a given animal started to behave maternally. Thereafter it was proffered fresh litters that advanced commensurately in age, so that on day 2 of maternal behavior it was caring for young 2 days old, on day 3 for young 3 days old, and so on.

The concaveated females began to emit the pheromone when their foster young reached 16 days of age, a time coincident with the onset of emission in the lactating female. We repeatedly tested the concaveated males, but in contrast to the concaveated females, they did not emit the pheromone. The question arises as to why the male, when behaving maternally in the same manner as the female, fails to emit the pheromone.

Knowing that high titers of prolactin are essential for pheromonal emission (*4*), we thought that perhaps the failure of the male to release the maternal pheromone was due to a failure to synthesize such high titers. Therefore, we took both intact and castrated males and injected them daily with prolactin [either 25 or 50 international units (I.U.)], starting on the first day that they began to behave maternally. The injections continued for a full 24 days, during which time they were tested repeatedly for the pheromone, according to the procedure previously described by Leon and Moltz (*1*). Not a single male showed evidence of the pheromone (*5*).

We then thought that perhaps the failure of these males to release the pheromone was due to a lack of estrogen. Accordingly, we undertook daily injections of both estradiol benzoate (5 μg) and prolactin, beginning, once again, on the first day of maternal behavior. Again, not a single male gave evidence of the pheromone (*6*). After this we gave up the idea of a simple endocrine insufficiency and sought instead to explain the failure of pheromonal emission by reference to events within the liver. What led us to focus on the liver can be described briefly as follows.

We knew from the work of Leon (*1*) that the pheromone is not the product of some anal gland but instead is synthesized within the cecum. We knew also from the work of Posner and his colleagues (*7*) and from that of Costlow *et al.* (*8*) that prolactin induces its own hepatic receptors and that the male characteristically shows a lower level of such



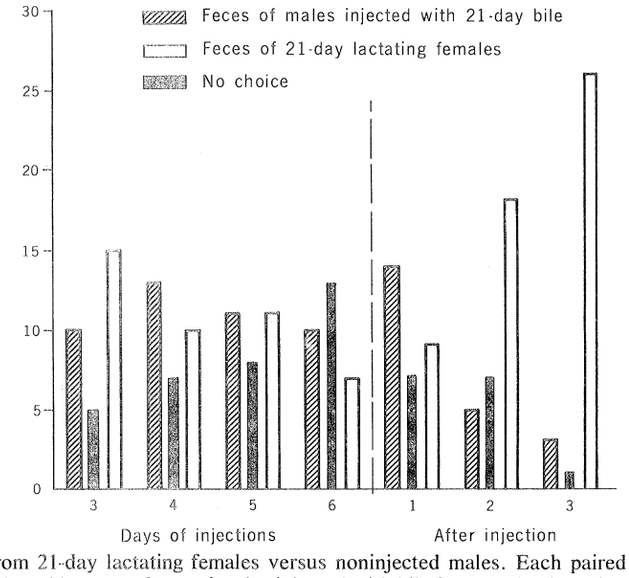


Fig. 1 (left). Response of 16-day-old pups to males injected with bile from 21-day lactating females versus noninjected males. Each paired combination was tested with six young. Fig. 2 (right). Response of 16-day-old pups to feces of males injected with bile from 21-day lactating females versus feces of 21-day lactating females. Each paired combination was tested with six young.

receptor-induction than the female. Thinking of some prolactin-hepatic interaction as underlying synthesis of the pheromone, we decided to collect bile from pheromone-emitting females for injection into the ceca of adult males. If our speculations regarding the liver are correct, then such males might be expected to show evidence of the pheromone.

Bile was collected by cannulating the bile duct while the females were under deep Nembutal anesthesia. For each female, the period of collection lasted approximately 8 hours, during which a total of about 6 ml of bile was accumulated. All bile was frozen until just prior to use and none was kept longer than 1 week.

We collected bile from three groups of females: (i) those that had been lactating for 21 days and consequently were actively emitting the pheromone (21-day bile); (ii) those that had also been with their litters for 21 days, but who, since day 10, had been receiving daily injections of ergocornine hydrogen maleate (0.5 mg in 0.15 ml of 70 percent ethanol), a potent prolactin inhibitor (*9*), and so were not emitting the pheromone (21-day bile + Ergo); and (iii) those that had been lactating for only 5 days and thus had not yet begun to emit the pheromone (5-day bile).

Intact adult males of the Wistar strain served as subjects. One week prior to the start of the experiment, each male was placed under Nembutal anesthesia and a 1-inch incision (1 inch = 2.54 cm) was made to expose the cecum. A small piece of muscle directly overlying the cecum was removed, after which the cecum was sutured to the exposed skin. The sutures were placed to form a square which, in positioning the cecum, facilitated the subsequent injection of bile.

Operated males ($N = 24$), never before in contact with young, were divided evenly into three groups to receive cecal injections of 2 ml of bile twice daily for a period of 6 days. Beginning on day 3, and continuing until 3 days after the last injection, each male was tested in our olfactory discrimination apparatus. This apparatus, described previously by Leon and Moltz (*1*), was designed to permit approach from a start box, across a triangular open field, to either of two goal boxes each faced with an opaque material. From a single overhead valve, forced air was made to flow through the goal compartments across the open field to the start box. On every trial, one of the compartments contained a male that had been injected with bile and the other a noninjected male that had undergone the same cecal operation.

Testing consisted of placing a single pup in the start box and allowing 15 minutes for it to make a choice. After each pup had registered a choice, or after 15 minutes had elapsed, it was removed from the apparatus and the absorbent paper covering the open field and the start box was replaced. After each of three pups had been run, the goal-box positions of the stimulus animals were reversed. Six pups were run against each stimulus pair; a total of 1008 pups were tested.

The pups themselves were 16 days old; this age was selected because it is known that 16-day-old pups respond strongly to the maternal pheromone (*1*). Each pup was run only once and in no case had a pup been in previous association with a stimulus animal.

Beginning on the fourth day after the start of the injection procedure, those males receiving 21-day bile were overwhelmingly selected in preference to noninjected males ($P < .001$; chi-square). They continued to be preferred until 1 day after the injections ceased; after this the choices were random (see Fig. 1).

In order to obtain additional evidence that males receiving cecal injections of 21-day bile were actively emitting the pheromone, we tested their feces against the feces of females that had been lactating for 21 days. Such females emit the pheromone and so their feces strongly attracts test young (*1*). However, when placed in our olfactory discrimination apparatus, the feces of the lactating female proved no more attractive than that of the male injected with 21-day bile. It was only after the bile injections ceased that the feces of the lactating female came to be preferred overwhelmingly (Fig. 2).

In contrast to males receiving 21-day bile, those receiving either 5-day bile or 21-day bile + Ergo were never chosen significantly over their noninjected test partners ($P > .05$; chi-square). It is obvious that not all bile injected into the cecum results in pheromonal release. However, the groups just mentioned were designed to serve as more than controls for the effects of bile injection. In fact, they support the following additional conclusions: (i) when prolactin is inhibited, bile loses the capacity to induce release of the pheromone (21-day bile + Ergo) and (ii) 5 days of lactation are not suf-

ficient to alter bile so as to make it pheromone-inducing (5-day bile). The latter conclusion is particularly interesting, since the female that has been lactating for 5 days has high levels of prolactin; in fact, she has had such levels since the time of parturition (*10*). How long prolactin must remain elevated before bile becomes pheromone-inducing can be inferred from the fact that the lactating female, as well as the concaveated female, does not begin to emit the pheromone until she has been with young for about 16 days (*1*, *3*). What may well be occurring during this 16-day period is the formation of new prolactin receptor sites in the liver (*7*, *8*). We can conceive of the resultant increase in prolactin binding as bringing about either an increase in concentration of total bile acids or a change in the ratio of one primary bile acid to another. Through either avenue, the chemistry of the cecum may be altered so that fecal material comes to contain the pheromone. That the male, as mentioned earlier, characteristically forms fewer hepatic prolactin receptors than the female (*7*) would explain why the male, although capable of releasing the pheromone in response to injected bile, cannot do so endogenously. What changes actually occur in bile to support pheromonal release and what the identity of the pheromone is are both topics for future research.

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References and Notes

1. M. Leon and H. Moltz, *Physiol. Behav.* **7**, 265 (1971); *ibid.* **8**, 683 (1972); H. Moltz and M. Leon, *ibid.* **10**, 69 (1973); M. Leon, *ibid.* **13**, 44 (1974).
2. B. P. Wiesner and N. M. Sheard, *Maternal Behaviour in the Rat* (Oliver & Boyd, Edinburgh, 1933); J. Cosnier and C. Couturier, *C.R. Soc. Biol.* **160**, 789 (1966); J. S. Rosenblatt, *Science* **156**, 1512 (1967).
3. L. C. Leidahl and H. Moltz, *Physiol. Behav.* **14**, 421 (1975).
4. M. Leon and H. Moltz, *ibid.* **10**, 65 (1973).
5. H. Moltz and L. C. Leidahl, unpublished data.
6. ———, unpublished data.
7. B. J. Posner, P. A. Kelly, H. G. Friesen, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2407 (1974); *Science* **188**, 57 (1975); B. J. Posner, P. A. Kelly, R. P. Shiu, H. G. Friesen, *Endocrinology* **95**, 521 (1974).
8. M. E. Costlow, R. A. Buschow, W. L. McGuire, *Life Sci.* **17**, 1457 (1975).
9. M. D. Shelesnyak, *Am. J. Physiol.* **179**, 301 (1954); *Recent Prog. Horm. Res.* **13**, 269 (1957); R. H. Lu, F. Koch, J. Meites, *Endocrinology* **89**, 229 (1971); C. J. Shaar and J. A. Clemens, *ibid.* **90**, 285 (1972).
10. Y. Amenomori, C. L. Chen, J. Meites, *Endocrinology* **70**, 506 (1970).
11. Supported in part by NIH grant HD 06872 and by Biomedical Center for Population Research grant PHS HD 07110. The ergocornine hydrogen maleate was kindly contributed by Sandoz Pharmaceuticals. The prolactin was a gift of NIH-NIAMD. L.C.L. was supported by PHS experimental and physiological psychology training grant.

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Inflammatory Effects of Endotoxin-Like Contaminants in Commonly Used Protein Preparations

Abstract. *Protein preparations from commercial suppliers are contaminated with bacterial endotoxins. The continued use of these preparations indicates that many researchers are unaware of this, and they may attribute all observed effects to the proteins themselves. Intravitreous injection of bovine serum albumin has an initial inflammatory effect on the rabbit eye which occurs before an immune reaction to the antigen itself can develop. This direct inflammatory effect can be fully accounted for by endotoxin-like contaminants which are present in protein preparations obtained from commercial suppliers. A pharmaceutical (U.S. Pharmacopeia) serum albumin preparation contains no detectable endotoxin, and has no initial inflammatory effect on the eye. Since endotoxins, even in minute amounts, have a variety of effects, the use of such contaminated protein preparations in biological research can lead to erroneous conclusions and should, therefore, be avoided.*

It was shown almost a decade ago that commercial albumin preparations contain significant amounts of endotoxins (*1*), and it is well known that endotoxins have profound biological effects (*2*). However, such commercial protein preparations continue to be used in biological research. It was recently noted (*3*) that a severe uveitis, lasting 7 to 10 days, develops within a few hours after the intravitreous injection of a sterile bovine serum albumin (BSA). The rapidity of the reaction suggested contamination because the animals were not presensitized to the antigen.

The present experiments show that several different commercially available protein preparations, including homologous (rabbit) serum albumin (RSA), cause a rapid inflammatory response. In contrast, a pyrogen-free pharmaceutical (U.S. Pharmacopeia) preparation of human serum albumin (HSA/USP) had no immediate effects even though it exhibited strong antigenic properties, as evidenced by the development of typical immunologically mediated inflammation 10 to 16 days after its intravitreous injection.

A dose response study showed that intravitreous injection of 1 to 10 ng of shigella endotoxin per eye is sufficient to cause ocular inflammation similar in extent and duration to the initial inflammatory response caused by the intravitreal injection of 10 mg of any one of four commercial protein preparations tested. Endotoxin-like activity of this order of magnitude, representing a contamination of 0.1 to 1 part per million, was indeed found by a biological assay technique in these proteins. Furthermore, addition of shigella endotoxin to the pyrogen-free HSA/USP before it was injected into the

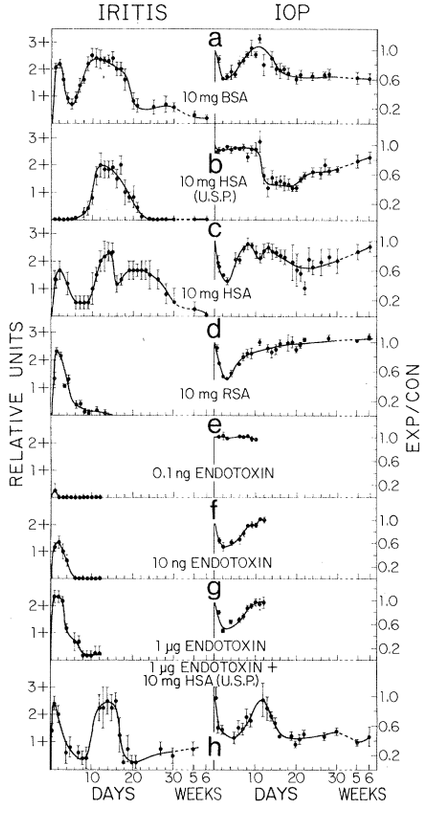

Fig. 1. The inflammatory effects of intravitreous injections of protein preparations and shigella endotoxin, as indicated by iridial hyperemia (iritis) and decreased intraocular pressure (IOP) (mean ± standard error; $N > 4$). An initial inflammatory reaction was observed 1 to 6 days after injection of 10 mg of commercial bovine (a), human (c), or rabbit (d) serum albumin; or 10 ng to 1 µg of shigella endotoxin (f and g), but not after the injection of pyrogen-free HSA (b). Such initial inflammatory reaction was observed with HSA/USP only when endotoxin was added to it prior to its injection (h). Development of an inflammatory response 10 to 18 days after injection, that is, at the time when an immune response to the antigen can be expected to occur, was observed after the injection of all protein preparations except the homologous (rabbit) serum albumin (d) which yielded only an initial but not a second, immunogenic, inflammatory response. The initial inflammatory effects must, therefore, be due to contaminants such as bacterial endotoxins rather than the antigenic properties of the injected protein. *Exp/con*, ratio of experimental to control data.