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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# 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

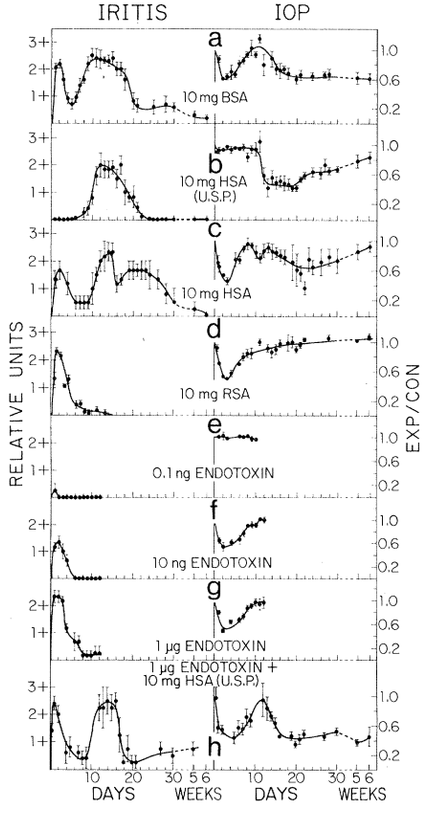


Fig. 1. The inflammatory effects of intravitreous injections of protein preparations and shigella endotoxin, as indicated by iridial hyperemia (iritis) and decreased intraocular pressure (IOP) (mean ± standard error; $N > 4$). An initial inflammatory reaction was observed 1 to 6 days after injection 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.

Table 1. The endotoxin content of various protein preparations, and the ocular inflammatory effects of these preparations and of shigella endotoxin following their intravitreous injections into normal rabbit eyes. The extent of inflammation is indicated by the degree of iridial hyperemia [iritis; see (7)], elevated white cell count in the aqueous humor, decreased intraocular pressure (IOP), and increased protein and decreased ascorbic acid concentrations in the aqueous humor.

| Protein (10 mg per eye) | N | Endotoxin (ng/10 mg)* | Intraocular inflammatory effects | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Initial (1 to 3 days) | | | | | Immunogenic (10 to 18 days) | | | | |
| | | | Iritis | White cells (No./μl) | Percentage change† | | | Iritis | White cells (No./μl) | Percentage change† | | |
| | | | | | IOP | Protein | Ascorbic acid | | | IOP | Protein | Ascorbic acid |
| BSA | 8 | 11 | 2.1 | 613 | −39 ± 3 | 2827 | −39 ± 6 | 1.9 | 385 | −41 ± 3 | 2949 | −80 ± 6 |
| RSA | 5 | 2 | 2.3 | 640 | −50 ± 2 | 2984 | −76 ± 5 | 0 | 8 | −5 ± 4 | 379 | −21 ± 17 |
| HSA | 7 | 1 | 1.8 | | −55 ± 3 | | | 2.5 | | −45 ± 3 | | |
| HSA/USP | 6 | 0 | 0 | 0 | 0 ± 2 | 0 | −8 ± 6 | 2.0 | 2037 | −57 ± 3 | 4489 | −76 ± 4 |
| Ovalbumin | 5 | 1 | 2.4 | | −45 ± 3 | | | 2.5 | | −28 ± 4 | | |
| Endotoxin (10 μg) | 11 | | 2.9 | 2111 | −60 ± 4 | 2938 | −59 ± 7 | 0 | 25 | 0 ± 2 | 172 | 2 ± 7 |

*In nanograms of *S. marcescens* endotoxin equivalent per 10 mg of protein as determined by the limulus test (8). †The percentage change is calculated from [(experimental − control)/control] × 100, where the control is the value obtained on the saline-injected contralateral eye.

vitreous humor resulted in the development of two distinct inflammatory episodes, essentially indistinguishable from those observed after the intravitreous injection of protein preparations obtained from commercial sources.

One eye of New Zealand white rabbits was injected with 100 μl of sterile pyrogen-free saline and served as a control. The contralateral eye was injected with the same volume of sterile pyrogen-free saline containing 100 mg/ml of BSA, RSA, ovalbumin, or human serum albumin (HSA), distributed by commercial suppliers; HSA/USP, distributed by a pharmaceutical company (4); or 0.1 ng to 10 μg of shigella endotoxin (5).

The intraocular pressure (IOP) was measured in both eyes (6) several times before and at 1- to 18-hour intervals after the injection, and the extent of intraocular inflammation was noted (7). In some cases, the rabbits were killed at the peak of the initial or second inflammatory response and the aqueous humor was removed to determine the ascorbic acid, protein, and white cell content (3).

Typical signs of severe uveitis were observed within the first day after the intravitreous injection of 10 mg of BSA; a pronounced iritis developed in all eyes, and after a very transient increase (not shown here) the IOP gradually decreased (Fig. 1a). The aqueous humor contained numerous white cells 1 to 3 days after BSA injection, and its protein concentration was increased almost 30-fold, whereas its ascorbic acid concentration was less than one-half that of the control eyes (Table 1). Iritis subsided and normal IOP recovered partially between days 3 and 5 (Fig. 1a), but this partial recovery was invariably followed by a second wave of immunogenic inflammation evidenced by iritis, a decrease in the IOP (Fig. 1a), appearance of cells, and changes in concentrations of protein and ascorbic acid in the aqueous humor (Table 1). Similar results were obtained after intravitreous injections of three other protein preparations obtained from various commercial sources (Table 1).

In contrast, intravitreal injection of the same amount of pyrogen-free HSA/USP did not produce signs of early iritis and did not measurably alter the IOP during the first 7 days (Fig. 1b). This lack of initial inflammatory effect is further substantiated by the fact that 2 days after HSA/USP was injected, the aqueous humor contained normal levels of protein and ascorbic acid, and no cells (Table 1). The HSA/USP was clearly antigenic since it caused a typical immunogenic inflammatory response between days 8 to 24. At the peak of this reaction (10 to 18 days), the iritis and the decrease in IOP, as well as the changes in aqueous humor, were comparable to those observed after the injection of the commercial albumin preparations (Fig. 1, panel b compared to panels a or c; Table 1). Injection of a homologous RSA preparation, on the other hand, produced only an initial inflammatory response, with no evidence of immunogenic inflammation between days 10 and 18 (Fig. 1d). In fact, the concentrations of protein and ascorbic acid in the aqueous humor were returning toward normal during this time period (Table 1). These experiments show that the initial inflammatory effects of protein preparations are independent of their antigenic properties.

A biological assay in which the limulus lysate test (8) for endotoxins was used showed that the four commercial protein preparations (BSA, RSA, ovalbumin, and HSA) contained endotoxin-like activity equivalent to 1 ng to 0.8 μg of *Serratia marcescens* endotoxin in the amount of protein (10 mg) injected per eye (Table 1). The only preparation that had no measurable endotoxin activity was the HSA/USP, which had no initial inflammatory effects on the eye (Fig. 1b and Table 1).

Endotoxins from different sources show different biological activities, and the activity of any given endotoxin can be modified by a number of factors (2). Thus, an exact quantitative correlation between the assayed endotoxin activity and the inflammatory effects of these protein preparations cannot be expected. It is clear, however, that the dose of shigella endotoxin that causes a submaximal ocular inflammatory response (10 ng per eye) is within the range of the assayed endotoxin content of these protein preparations (1 to 11 ng/10 mg of injected protein). On the other hand, we can rule out the possibility that this initial inflammatory effect was due to the trauma of the injection or the presence of a protein since eyes which were injected with HSA/USP or the control eyes of over a hundred rabbits which were injected with 100 μl of pyrogen-free saline did not show such an effect.

It may be concluded, therefore, that the observed initial inflammatory effects of protein preparations which are distributed by commercial suppliers (9) are due to endotoxin-like contaminants. The rabbit eye seems to be particularly sensitive to these endotoxins and, therefore, may serve as a good test system for the inflammatory effects of biological preparations. It should be noted, however, that endotoxins are known to affect a variety of other biological processes, apparently through the activation of the prostaglandin and the cyclic nucleotide systems (10), and may influence the course of cellular responses even when toxic or inflammatory signs are not overtly apparent. These considerations indicate that

the use of endotoxin-contaminated protein preparations should be avoided in any kind of biomedical research unless it can be shown that the particular system is insensitive to endotoxins. The endotoxin content of processed biological materials should be stated by the supplier, or should be tested by the investigator before they are used in biological research.

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4. Protein preparations were made up in pyrogen-free saline to a concentration of 100 mg/ml, and filtered through a Millipore GS 0.22 membrane; 1-ml portions were kept at 2°C until injection. The following protein preparations were used: sterile bovine albumin (BSA), 30 percent solution (Gallard-Schlesinger); ovalbumin, 2× crystallized (Worthington); rabbit and human albumin (RSA and HSA, respectively) crystallized (Pentex, Miles Laboratories); and normal serum albumin (human; HSA/USP), 25 percent solution (Abbott).
5. Five tenfold serial dilutions (0.1 ng to 10 μg/100 μl of pyrogen-free saline) of shigella endotoxin (lipopolysaccharide B, *S. flexneri*; Difco) were filtered as described above and stored at −10°C.
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8. The endotoxin assay was kindly performed for us by J. Fine of Harvard University Medical School according to the limulus technique; see R. B. Reinhold and J. Fine, *Proc. Soc. Exp. Biol. Med.* **137**, 334 (1971).
9. The fact that the crystallized HSA, which is prepared from sterile human blood, also contains endotoxins suggests that processing is an important source of this contamination. Thus processed proteins in general, including enzymes of both animal and plant origin, should be suspected of endotoxin contamination.
10. There is accumulating evidence that such diverse effects of endotoxins as induction of fever [C. J. Woolf, G. H. Willis, H. Laburn, C. Rosendorff, *Neuropharmacology* **14**, 397 (1975)], pulmonary hypertension [F. L. Anderson, D. J. Tsagavis, W. Jubiz, H. Kudia, *Am. J. Physiol.* **228**, 1479 (1975)], renal hypotension [A. G. Herman and J. R. Vane, *Arch. Int. Pharmacol.* **208**, 365 (1974)], or the sensitization of fat cells to norepinephrine in vitro [J. A. Spitzer, *Proc. Soc. Exp. Biol. Med.* **145**, 186 (1974)] are mediated by stimulation of prostaglandin synthesis or the production of 3′,5′-adenosine monophosphate (cyclic AMP). Since both the prostaglandin and the cyclic AMP systems are ubiquitous and play a basic role in the control of cellular processes, the apparent ability of endotoxins to stimulate these systems further emphasizes their broad potential to affect biological processes.
11. I thank J. Fine for the endotoxin assays; and R. A. Baroody, E. V. Salvador, and D. W. Garnick for assistance. Supported by NIH grant EY 00402-09.

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## An Analog of Enkephalin Having Prolonged Opiate-Like Effects in vivo

Abstract. *Intraventricular administration of the enkephalin analog, [D-Ala²]-Met-enkephalin, induces profound and long-lasting analgesia, as well as other opiate-like behavioral effects in the rat. This analgesia was highly dose dependent, of much greater magnitude, and about 30 times longer lasting than that produced by the naturally occurring peptide, methionine-enkephalin. The behavioral effects of the [D-Ala²] analog could be completely reversed by the opiate antagonist, naloxone, suggesting that these effects were mediated by opiate receptors. Systemic administration of naloxone alone resulted in a significant increase in pain sensitivity. These findings support the view that endogenous opiate systems may play an important role in modulating pain sensitivity.*

Endogenous peptides with strong opiate agonist properties have been isolated from the mammalian central nervous system and identified by Hughes as the pentapeptides methionine-enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) and leucine-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) (*1*). The enkephalins were shown to have potent opiate-like activity in a number of assay systems (*2*). Similarly, behavioral tests have confirmed the opiate-like properties of enkephalin. A very short-lasting but significant analgesia was demonstrated with the D'Amour-Smith tail-flick test (*3*) after intraventricular or localized midbrain injections of methionine-enkephalin (100 to 200 μg) in the rat and mouse (*4*). The relative selectivity of the tail-flick test for opiate agonists (*5*) and the reports that enkephalin-induced analgesia can be antagonized by naloxone (*4*) suggest that the analgesic effects of this peptide are due to its actions on opiate receptors. These results, in conjunction with reports that electrical stimulation of localized regions in the brainstem can induce profound analgesia which appears to be mediated by opiate receptors (*6*), have prompted suggestions that endogenous enkephalins may act to modulate pain systems.

The brief analgesia produced by the enkephalins, however, stands in marked contrast to the much more prolonged effects of similar administrations of morphine (*7*). Structurally, methionine-enkephalin resembles morphine and morphine-related narcotics most closely when a type I β-bend conformation is assigned to the H-Tyr-Gly-Gly sequence of the pentapeptide (*8*). Additional evidence (*9*) suggests the possibility that the preferred type I β-bend conformation may be stabilized by the substitution of a D-amino acid for the second glycine in this sequence.

We now report a profound and long-lasting analgesia induced by intracerebroventricular administration of [D-Ala²]-methionine-enkephalin, a finding consistent with a recent report on a similar analog published since the submission of this report (*10*). Further, we found that naloxone, a blocker of opiate receptors, produced the opposite effect of increased sensitivity in otherwise untreated animals. These findings provide evidence for a functional role of endogenous opiate systems in the modulation of pain sensitivity.

Permanent intraventricular cannulas were surgically implanted in six male albino (Holtzman) rats 120 to 180 days old (*11*). Examination of cannula placements after injection of a marker dye revealed the presence of dye in the ventricles of all animals. After 1 week for postoperative recovery, each rat was tested for analgesia by the tail-flick test both before and after intraventricular administration of 200 μg of methionine-enkephalin, 200 μg [D-Ala²]-methionine-enkephalin, or the injection vehicle. Peptide synthesis has been described (*12*). Peptides were dissolved in Ringer's solution just before injection and were then administered in 10-μl volumes over a period of 1 minute by means of a microsyringe. Control injections consisted of the vehicle alone adjusted to a *p*H of 4.0 to approximate the *p*H of the drug solution. Each rat received all treatments with tests separated by at least 48 hours. Control tests with the vehicle alone were given first and last, with the two drug treatments intervening in counterbalanced order. Each test consisted of a baseline period during which the latency to withdraw the tail from a radiant heat source (*3*) was recorded every 2 minutes. A maximum latency of 10 seconds was used since the intense heat created by this procedure could cause lasting tissue damage. After the establishment of stable tail-flick latencies (3.5 to 5.5 seconds), baseline latencies were recorded for a period of 10 minutes. The drug or control was then administered, and tail-flick latencies were recorded every 2 minutes for a minimum of 10 minutes or until latencies had returned to within 30 percent of baseline values.

Both methionine-enkephalin and the