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Radioimmunoassay of MIF-1/Tyr-MIF-1-Like Material in Rat Pineal

ABBA J. KASTIN, SHARON P. LAWRENCE
AND DAVID H. COY

Endocrinology Section of the Medical Service, Veterans Administration Medical Center
and
Department of Medicine, Tulane University School of Medicine, New Orleans, LA

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KASTIN, A. J., S. P. LAWRENCE AND D. H. COY. *Radioimmunoassay of MIF-1/Tyr-MIF-1-like material in rat pineal.* PHARMAC. BIOCHEM. BEHAV. 13(6) 901–905, 1980.—The hypothalamic peptide MIF-1 (Pro-Leu-Gly-NH_2) was coupled to thyroglobulin and injected into rabbits. The resulting antiserum reacted with the tetrapeptide Tyr-MIF-1 to a greater extent than with the tripeptide MIF-1, presumably because of a better conformation for antibody binding. By radioimmunoassay (RIA), immunoreactive MIF-1/Tyr-MIF-1-like material was found in the pineal gland of each of the 100 rats examined. The tendencies for slightly higher levels in pineals obtained from rats kept in constant darkness for two weeks, from rats in a normal light cycle decapitated at noon, or from rats which had been hypophysectomized were not statistically significant. Gel filtration of pineal extracts on a column of Sephadex G-10 revealed that by RIA one immunoreactive peak eluted near MIF-1 and oxytocin, and another peak near Tyr-MIF-1. The results suggest the presence in pineal tissue of an MIF-1-like material as well as a novel peptide containing Tyr-Pro-Leu-Gly-NH_2 or a closely related structure for which oxytocin is unlikely to be the precursor.

Radioimmunoassay MIF-1 Pineal Hypophysectomy Circadian Light cycle Peptide

MIF-1 (Pro-Leu-Gly-NH_2) has been shown to exert effects on the central nervous system (CNS) of laboratory animals and human beings [6]. Methods for its measurement by bioassay, however, are relatively insensitive, non-specific, and unreliable. Therefore, we attempted to develop a radioimmunoassay (RIA) for MIF-1 by immunizing rabbits with the tripeptide coupled to thyroglobulin. The antibody we generated reacted with MIF-1, Tyr-MIF-1, and oxytocin. The tissue we chose for assay, the pineal, contains only negligible amounts of oxytocin [1,3].

The involvement of MIF-1 in the pineal-hypothalamic-pituitary axis is not clear. However, there is evidence that the pineal may interact with the hypothalamic peptide MIF-1. The IV injection of labeled MIF-1 resulted in significant accumulation of radioactivity in the pineal [2,8]. Thin layer electrophoresis of extracted pineals obtained from rats 60 minutes after injection of the MIF-1 revealed that a substantial portion of the injected material had the same mobility as intact MIF-1 [8]. This does not necessarily mean that MIF-1 occurs naturally in the pineal gland. Injection of labeled alpha-melanocyte-stimulating hormone (α-MSH), for example, resulted in its apparent localization in parts of the brain containing relatively low endogenous levels of bioactive MSH [4,9]. It was not certain, therefore, that MIF-1 would be found in pineal tissue obtained from untreated rats.

The pituitary gland also appears to interact with MIF-1 and the pineal. Delayed disappearance of radioactivity was found in hypophysectomized rats after the IV administration of labeled MIF-1 but not after similar administration of labeled inulin [5]. In addition, plasma of hypophysectomized rats apparently contained increased amounts of MIF-1 as determined by bioassay and thin-layer chromatography (TLC) [7]. These results raised the possibility that in the absence of the pituitary gland, increased amounts of MIF-1 might accumulate in the pineal and elsewhere.

METHOD

Animals

Male, albino rats weighing about 200 g were obtained from Zivic-Miller (Allison Park, PA). They were housed for two weeks with free access to food and water in sound-attenuated rooms. A 12 hour light (0600–1800), 12 hour dark (1800–0600) cycle was used for the study of diurnal variation and comparison of hypophysectomized and intact rats. Hypophysectomized rats received special food (ICN Nutritional Biochemicals) but regular tap water. The ten rats in each group were decapitated and their pineals rapidly removed, weighed, frozen, and assayed within a few days.

Tissue Extraction

Each pineal was placed in 0.5 ml of 1.0 M acetic acid preheated to 102°C in an oil bath. After 5 minutes in the bath, the sample was cooled in ice, homogenized, and then centrifuged. The pellet was washed with 1 M acetic acid and centrifuged two more times. The pooled supernatant was

lyophilized. For identification, the extract was added to a column (1×30 cm) of Sephadex G-25 fine in a solvent of 0.1 M acetic acid containing 0.1% human serum albumin (HSA). Fractions of 1.5 ml each were collected and measured in the RIA. The void volume was 8.5 ml. A second column (1×150 cm) of Sephadex G-10 was also used with the same solvent. Its void volume was 36 ml, and 1.0 ml fractions were collected and assayed.

Antibody Formation

MIF-1 was conjugated to thyroglobulin with water-soluble dicarbodiimide and stirred for 24 hours at pH 5.5. The mixture was then dialyzed against distilled water for 48 hours and lyophilized. Determination of the weight after lyophilization indicated about 80% conjugation. The material was then dissolved in 0.9% NaCl and Freund's complete adjuvant (Difco) and emulsified. The mixed breed rabbits received multiple intradermal injections and a single IP injection of the material every two weeks. Venous blood was obtained from the ear.

RIA

Tyr-MIF-1 was iodinated by the chloramine-T method. The following mixture was allowed to react for one minute; 25 μl of 0.5 M phosphate buffer (pH 7.5), 5 μg Tyr-MIF-1 in 0.1 M acetic acid, 1 mCi $Na^{125}I$ (Industrial Nuclear), and 1 μg chloramine-T in 0.05 M phosphate buffer. The reaction was stopped with 10 μg sodium metabisulfite. It was then purified on a column (0.5×62 cm) of Sephadex G-25 (fine). After the void volume of 6 ml (as determined with dextran blue) was excluded, 1 ml fractions were collected and counted in a gamma counter. Three peaks of radioactivity were identified and each one checked separately for damage by chromato-electrophoresis on Toyo 514 paper at 500 V, 4mA for 1 hour at 4°C in 0.05 M phosphate buffer.

A five day incubation procedure was used. On the first day, duplicates of the standard curve (doubling dilutions of Tyr-MIF-1 between 8 and 512 pg) were prepared in Tris HCl buffer (pH 8.0) containing 0.1% HSA and 4.0% Trasylol. The pineal samples were reconstituted in the same buffer. Aliquots of 100 μl of standard or pineal sample, 100 μl antibody (1:6000 initial dilution), and 200 μl of the Tris buffer were mixed and placed at 4°C overnight. The next day, 100 μl (9,500 cpm) ^{125}I-Tyr-MIF-1 in buffer was added, mixed, and kept at 4°C for three days. On the fifth day, 0.5 ml of a 1% charcoal-0.1% dextran mixture freshly prepared in the assay buffer was added to all tubes (except those for total counts). The tubes were mixed and 30 minutes later centrifuged at 2400 g for 30 min. The supernatant was removed by careful decanting and counted in a gamma counter (Micromedic 4/200).

Statistical Analysis

A Hewlett-Packard 9815A data reduction device directly reduced the data obtained from the gamma counter. Based on the Rodbard-LeWald system of log-logit transformation and weighted linear regressions, the ratio of bound (B) to original bound (B_o) was automatically calculated and values computed for the range representing 20% to 80% inhibition. Comparisons between values obtained for the hypophysectomized vs intact groups as well as the constant illumination vs constant dark groups were made by a 2-tailed Student's *t*-test. Comparison of values for diurnal variation was made by analysis of variance followed by Duncan's Multiple Range Test. The coefficient of variation (CV), i.e., the standard deviation expressed as a percentage of the mean, was calculated both within and between the assays.

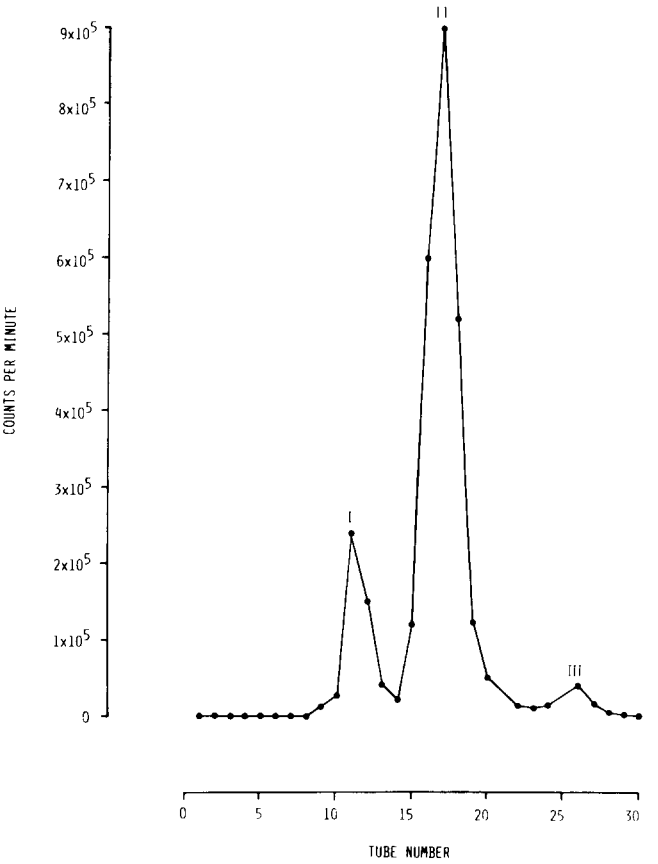

FIG. 1. Elution pattern of iodination mixture after gel filtration on a column of Sephadex G-25.

RESULTS

Molecular sieving of iodinated Tyr-MIF-1 on a Sephadex G-25 column revealed three peaks of radioactivity (Fig. 1). Chromatoelectrophoresis, binding to antibody, and assessment of non-specific binding were used to determine that the peaks represented damaged peptide (peak I), undamaged peptide (peak II), and free labeled iodine (peak III). The chromatoelectrophoretic pattern of the iodination mixture before purification is shown in Fig. 2 (left). After sieving on a Sephadex G-25 column, the chromatoelectrophoretic pattern of the undamaged ^{125}I-Tyr-MIF-1 (peak II) revealed a single peak (Fig. 2—right) and this material was used as the trace. The antiserum was used at a dilution (1:30,000, final) sufficient to give a mean (±SEM) total binding of 28.6 ± 1.0%. The mean non-specific binding of the assays was 0.71 ± 0.11% of total binding.

The RIA performed in this study reliably detected 17.2 ± 1.47 pg/tube of immunoreactive material at the 80%

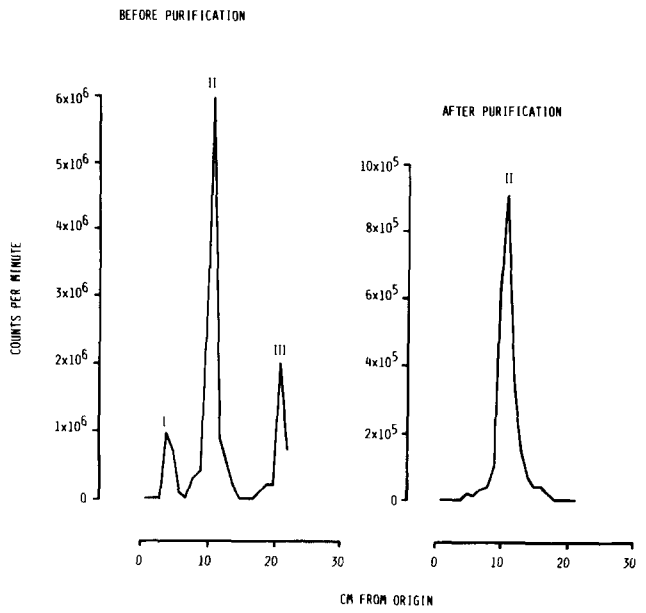

FIG. 2. Chromatoelectrophoretic pattern of the iodination mixture before purification (left) and of the middle peak shown in Fig. 1 after purification by gel filtration on a column of Sephadex G-25 (right).

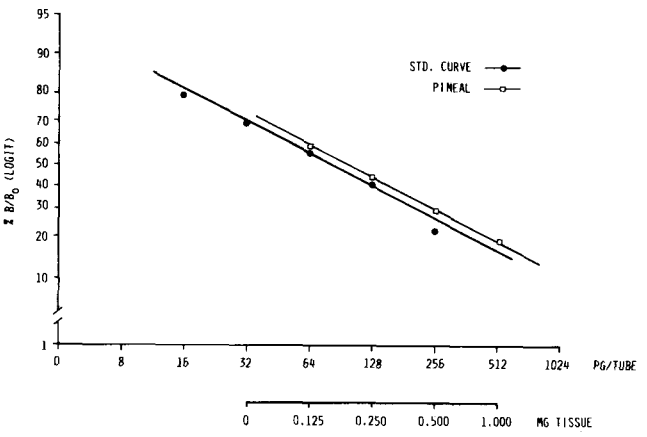

FIG. 3. Logit plot of dilution curves for pineal tissue and standard (Tyr-MIF-1).

B/B_o level. The competitive binding seemed to be relatively specific since there was no cross-reactivity at doses up to 10,000 pg with the following substances: Met-enkephalin, Leu-enkephalin, β_h-lipotropin, β_h-endorphin, Leu^5-β-endorphin, dynorphin, α-MSH, β_h MSH (LPH 37–58), MSH/ACTH 4–10, ACTH 1–24, DSIP, somatostatin, TRH, T_3, T_4, LH-RH, VIP, HPP, motilin, gastrin, neurotensin, vasotocin, pGlu-Leu-Gly-NH_2, tyrosine, proline, leucine, glycine, or glycine amide. Oxytocin cross-reacted about 5%, MIF-1 about 4%, Lys-vasopressin about 0.6%, and isotocin about 0.3%. Since MIF-1 was much less reactive than the Tyr-MIF-1 used for iodination, the latter was used as standard in all assays.

MIF-1 and Tyr-MIF-1 were checked by TLC in butanol:acetic acid:water:ethanol (1:1:1:1) during each day of a 5-day incubation at both 4°C and −20°C. A single spot with an Rf identical to that of the unincubated peptide was found in each case. The immunological integrity of the ^{125}I-Tyr-MIF-1 was checked in the following manner. Tubes containing the labeled peptide both with and without extracted pineal tissue and antibody diluted 1:6000 were incubated for 24 hr. The binding in both cases was 29%. Tubes containing only the labeled peptide both with and without extracted pineal tissue were incubated for 5 days before addition of antibody (1:6000). These tubes were reincubated for 24 hr and the binding was also about 29% for both cases. This procedure assures the immunological stability of the trace throughout the 5-day assay period.

Inter- and intra-assay variability was checked with the standard at 50 pg/tube and 100 pg/tube. For both doses, the CV was about 3.5% within assay and about 16.5% between assays. Another set of these two standards was run through the extraction procedure in the same way as the pineal tissue. An 89% recovery of immunoreactivity was determined by comparison of the values with those of the same standards assayed directly.

The dilution curve for pineal tissue was parallel to that of the standard curve (Fig. 3). Treatment of the pineal extract with charcoal/dextran removed all measurable immunoreactive material. Addition of this extract to tubes containing the concentrations of peptide used to generate the standard curve resulted in a line superimposable upon that generated by the standard curve. This indicated that large proteins not extracted by the charcoal-dextran did not interfere with measurement of antibody binding of the immunoreactive material.

MIF-1/Tyr-MIF-1-like immunoreactivity was found in the pineal from each of the 100 rats tested, although the variability was very large. Gel filtration revealed that the major part of immunoreactivity in rat pineal tissue co-eluted with MIF-1 and Tyr-MIF-1 on Sephadex G-25 (Fig. 4). However, since this column did not separate the two forms of the peptide, we used Sephadex G-10 in a longer column, which resolved three peaks of immunoreactivity in the pineal extract (Fig. 5). The second peak eluted with MIF-1 and the third peak near Tyr-MIF-1. The identity of the first peak is not known, but it may be a larger molecular weight protein that could represent a precursor for the immunoreactive MIF-1/Tyr-MIF-1 material. Oxytocin eluted slightly later than MIF-1, but far from Tyr-MIF-1.

The mean levels of MIF-1/Tyr-MIF-1-like immunoreactivity in the pineal glands of groups of 10 intact rats decapitated every 4 hours throughout a 24 hour cycle are shown in Fig. 6. Although the highest level seemed to occur at noon, no statistically significant differences were found throughout the cycle. Pineals from rats kept for two weeks in the dark (281.3 ± 63.3 pg/pineal, 311.5 ± 70.6 pg/mg) seemed to contain more MIF-1/Tyr-MIF-1-like immunoreactivity than a comparable group of 10 rats kept in constant illumination (155.7 ± 29.9 pg/pineal, 178.8 ± 34.0 pg/mg). These differences were also not significant; when expressed per whole pineal, the probability was $p<0.1$ by the 2-tail test, $p<0.05$ by the 1-tail test. Similarly, the slight tendency for the pineals from hypophysectomized rats to contain more MIF-1/Tyr-MIF-1-like immunoreactivity than those from intact rats was not statistically significant.

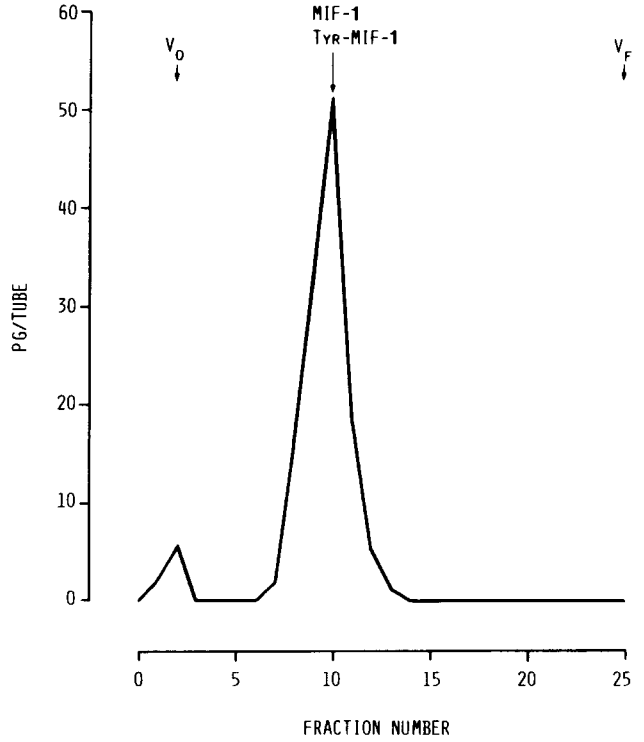

FIG. 4. Concentration of immunoreactivity in eluates obtained by gel filtration of extracted pineal tissue on a column (1×30 cm) of Sephadex G-25. The fractions in which MIF-1 and Tyr-MIF-1 eluted are also shown.

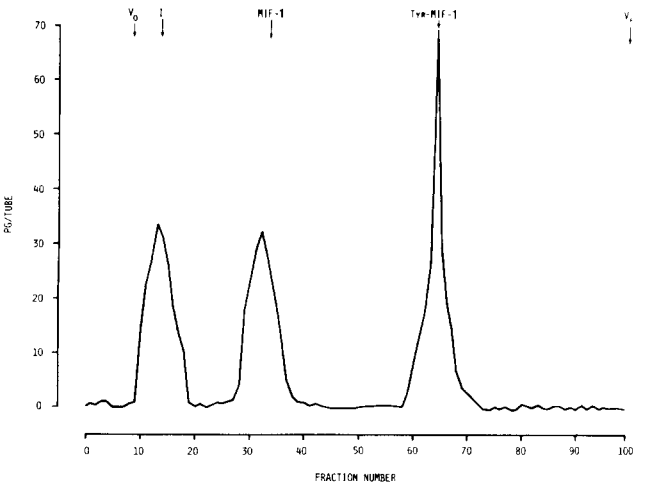

FIG. 5. Concentration of immunoreactivity in eluates obtained by gel filtration of extracted pineal tissue on a column (1×150 cm) of Sephadex G-10. The fractions in which MIF-1 and Tyr-MIF-1 eluted are also shown.

DISCUSSION

Since the MIF-1 tripeptide lacks an amino acid that can be readily iodinated, Tyr-MIF-1 was synthesized and used for that purpose. MIF-1 and Tyr-MIF-1 reacted similarly to the immunoreactive material found in the pineal as ascertained by parallel dose-response curves and gel filtration. Because Tyr-MIF-1 had a much higher affinity for the antibody, it was also used as the standard.

Among the possibilities to explain the greater reactivity of Tyr-MIF-1 than MIF-1 are an increased breakdown of MIF-1 during the incubation procedure and the assumption by Tyr-MIF-1 of a preferred structural conformation. Chromatography (TLC) ruled out the possibility of peptide breakdown. However, the addition of tyrosine to the N-terminus of MIF-1 might have resulted in an improved conformation for binding since MIF-1 was conjugated to thyroglobulin through its amino terminus. This could mean that some of the naturally occurring material measured in rat pineal is also extended at the N-terminus.

Since our antibody reacted more weakly with MIF-1 than Tyr-MIF-1, the presence of the smaller peak of immunoreactive pineal material which eluted with MIF-1 could represent a relatively large amount of an MIF-1-like substance. The larger peak of immunoreactivity which eluted later in the vicinity of Tyr-MIF-1 suggests the presence of Tyr-Pro-Leu-Gly-NH_2 in pineal tissue. This tetrapeptide, despite its increased molecular weight, eluted later than MIF-1

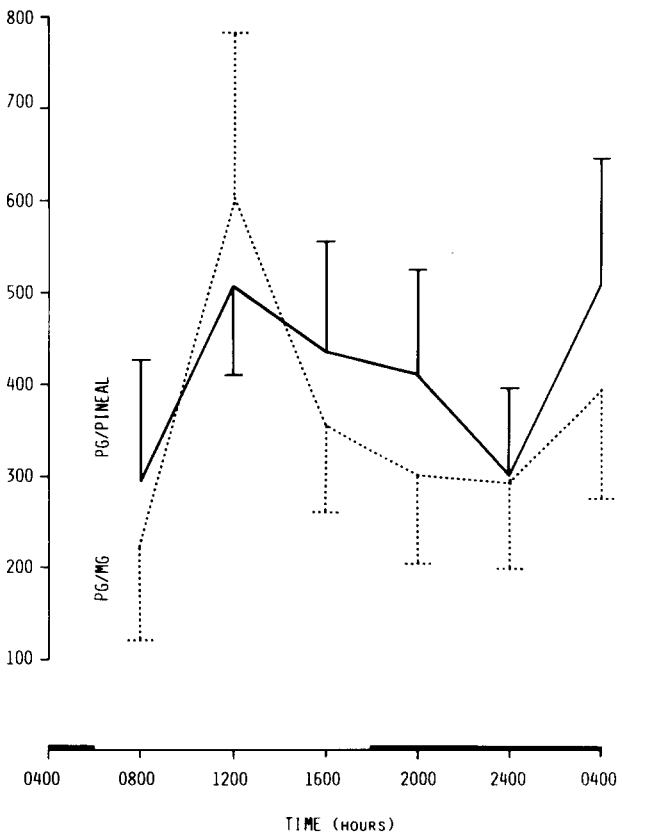

FIG. 6. Diurnal variation (±SEM) in pineals of MIF-1/Tyr-MIF-1-like immunoreactivity expressed per mg wet tissue or per whole pineal. The dark area along the abscissa represents the 12 hour period of darkness.

probably because of an affinity between the Tyr aromatic side chain and the dextran gel. An MIF-1 tetrapeptide with no aromatic residue should have eluted with MIF-1 or even earlier. It is likely that this tetrapeptide would be formed from a larger molecule and, indeed, some high molecular weight immunoreactive material did emerge near the void volume of the G-10 column. This precursor presumably would differ from oxytocin which contains MIF-1 as its C-terminal tripeptide but cystine rather than tyrosine as the adjacent amino acid. Oxytocin eluted in a completely different area from Tyr-MIF-1.

Measurement by RIA of MIF-1/Tyr-MIF-1-like immunoreactivity in rat pineal tissue showed that the levels were not reliably changed by several manipulations. However, it is possible that the large individual variation among rats obscured the tendency toward higher levels in pineals of rats which were hypophysectomized, kept in constant darkness for two weeks, or killed at noon in a 12:12 hour light: dark cycle.

The presence of MIF-1/Tyr-MIF-1-like material in pineal tissue does not necessarily indicate its synthesis there. It is likely that MIF-1 accumulates in the pineal, as suggested by its appearance there after IV administration [2,8]. The tendency in the present study toward more immunoreactivity in the pineals from hypophysectomized rats might be considered as further evidence in support of pineal trapping of peptides, since the absence of the pituitary seemed to prolong the disappearance of MIF-1 from blood [5,7]. Perhaps the accumulation of peptides in the pineal gland is part of a mechanism by which peripherally circulating peptides act on the brain. The peptides could act directly through the pineal or indirectly through a pineal product that would in turn affect the brain.

Since MIF-1 is the C-terminal tripeptide of oxytocin, it was not surprising that oxytocin cross-reacted about 5% with the antibody generated by injection of MIF-1. This made it theoretically possible that the RIA was measuring oxytocin or some other material not checked in the test system for specificity. If the pineal contained a few thousand pg of oxytocin, for example, then the few hundred pg of immunoreactivity measured by our assay could be due to oxytocin. Two recent papers have shown, however, that the rat pineal gland contains less than 7 pg/gland [3] and less than 2 pg/mg wet tissue [1]. Thus, the presence of oxytocin cannot account for our results. The immunoreactive material in pineal tissue most likely represents the presence of MIF-1-like and Tyr-MIF-1-like compounds.

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