MSH-Release Inhibiting Factors: Recent Studies

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The evidence which has finally resulted in the general acceptance of the concept that there are substances present in hypothalamic tissue which inhibit the release of melanocyte-stimulating hormone (MSH) from the pituitary glands of mammals as well as amphibians has been summarized in several recent reviews (1–3). Although the chemical structures of several compounds with such activity are known, it is still uncertain which one of these, if not a different one, is the actual hormone.

Both a tripeptide, Pro-Leu-Gly-NH$_2$, and a pentapeptide, Pro-His-Phe-Arg-Gly-NH$_2$, have been isolated from bovine hypothalamic tissue and structurally identified (4, 5). Since purification of the bovine extract involved heating to boiling in dilute acetic acid followed by rapid cooling (6), the recent suggestion by Hadley, Bower, and Hruby (7) that hypothalamic extracts heated to boiling under undefined conditions lose their ability to inhibit MSH release probably can be dismissed as a technical problem. The assay used for following the isolation of the two peptides is based on the lightening of the skin of a frog which has been previously darkened by destruction of the hypothalamus (8, 9). Pro-Leu-Gly-NH$_2$ can be formed by incubating oxytocin with an enzyme found in hypothalamic tissue and has been reported previously to inhibit MSH release in the rat also (10). However, Bower et al. (11) did not find any MSH-release inhibiting factor (MIF) activity of Pro-Leu-Gly-NH$_2$ (MIF-I) in the frog or rat. Why they failed to find activity in the frog, Rana pipiens, is easy to understand: they injected MIF-I into the dorsal lymph sac, a procedure already shown to be ineffective, and they also incubated MIF-I with pituitaries for 4 hr, a time which results in essentially complete inactivation of the material (9). It is also possible that an inactive (12) batch of MIF-I was tested. The reason for the inability of Bower et al. to find the activity of MIF-I which had been reported by Celis et al. (10) for rats is not known. Alternatively, Bower et al. proposed that tocinoic acid, Cys-Tyr-Ile-Gln-Asn-Cys-OH, the cyclic pentapeptide ring of oxytocin, or its amide, tocinamide, is more likely to be MIF (11). Interestingly, Celis et al. (13) have suggested that if the

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tocinoic acid ring is opened, the resulting hexapeptide, H-Cys-Tyr-Ile-Gln-Asn-Cys-OH, is the MSH-releasing hormone (MRH) but they found no activity of tocinoic acid in the rat (14).

Thus, four compounds have been found by different investigators to possess MIF activity. Only Pro-Leu-Gly-NH₂ (MIF-I) and Pro-His-Phe-Arg-Gly-NH₂ (MIF-II) have been isolated from hypothalamic tissue, but it is reasonable to expect that the other two compounds may also be present in hypothalamic tissue.

SKIN LIGHTENING EFFECTS IN THREE SPECIES OF AMPHIBIANS

When Hruby et al. reported (15) that tocinoic acid and tocinamide inhibit MSH release from the bullfrog (Rana catesbeiana) and the toad (Bufo marinus) but not the frog (Rana pipiens), the question of species specificity was raised. Their findings were based upon an in vitro assay, presumably involving incubation for 4 hr (15). We tested tocinoic acid and MIF-I in the same three species of amphibians used by Hruby et al., namely R. pipiens, B. marinus, and R. catesbeiana. Using doses of tocinoic acid as high as 100 µg, we were unable to find significant (melanocyte index less than 4.0) lightening (8) of these animals darkened (melanocyte index of 5.0) by destruction of the hypothalamus. Doses of 10 ng, 50 ng, 100 ng, 1 µg, 10 µg, 50 µg, and 100 µg were tested. In the frog and bullfrog, there was a tendency for slightly more lightening with increasing doses of tocinoic acid. In the toad, however, the maximum lightening of the skin seemed to occur with a dose of 1 µg, not with doses 10 and 100 times greater or smaller. Nevertheless, at no dose was the reduction in melanocyte index as great as that seen with only 50 ng of MIF-I; this lightening became greater with increasing doses of MIF-I but was less evident with smaller doses.

The biological activity of MIF-I determined in the frog was not seen when a similar assay procedure was used in the other two species. That is, MIF-I, when applied to the pituitary glands of either the toad or bullfrog in doses from 10 ng to 100 µg, did not reduce the melanocyte index in their skin to 4.0. Application to the pituitary of 100 µg MIF-II resulted in significant reduction in the melanocyte index of the frog, but no lightening of the toad or bullfrog. With N. Yanaihara, we considered the unlikely possibility that during the structural determination on a microscale of MIF-II, which has certain amino acids in common with the active core of MSH, a tryptophyl residue might have been overlooked. Accordingly, Yanaihara synthesized Pro-His-Phe-Arg-Trp-Gly-NH₂ as well as MIF-II. At a dose of 100 µg, MIF-II significantly lightened the lesioned R. piliens, as expected. The hexapeptide containing Trp (Pro-His-Phe-Arg-Trp-Gly-NH₂) was not active in the frog but did have a slight tendency to lighten the lesioned toad. Pressinoic acid (40 µg) and pressinamide (110 µg) were inactive in all three species of amphibians, as had been found previously by Hruby et al. (15). We also failed to find significant lightening in any of the three species with 100 µg tocinamide as well as two other analogs of MIF-I and MIF-II synthesized by Yanaihara: pyro-Glu-His-Phe-Arg-NH₂ and Ac-Pro-Leu-Gly-NH₂. The slight lightening caused by 100 µg doses of the acetylated MIF-I (Ac-Pro-Leu-Gly-NH₂) occurred in all three species of amphibians tested. Perhaps in our assay system there are variations in the rate of penetration of certain test substances into the pituitary after being applied to its surface, or maybe materials applied to the surfaces of the pituitary glands from different species are subject to varying rates of inactivation. These
findings support the concept that species specificity may exist for the compounds proposed to have MIF activity, but the specificity appears to differ among different laboratories using different techniques of assay.

**STABILITY**

Part of the problem may lie in the relative instability of MIF-I as determined in the lesioned frog. When MIF-I is shaken in a Dubnoff metabolic incubator at 35–37°C, it rapidly loses activity. This occurs in Kreb's ringer bicarbonate, (with or without calcium), frog ringer's solution, saline, and serum from the rat, frog, or human being. It does not seem to make any great difference whether MIF-I is shaken in an atmosphere of nitrogen or 95% oxygen–5% carbon dioxide. Coating the incubation beakers with silicon or using cerebral spinal fluid instead of serum also fails to maintain activity. Although the loss of activity is retarded when higher concentrations are used (e.g., 1 mg/ml instead of 100 μg/ml), there still is significant loss of activity in less than 2 hr.

Even if the material is refrigerated at 4°C, it appears to retain its biological activity in the lesioned frog for less than 4 days. Under these conditions, an acidic solution seems to result in less loss of activity than saline. More MIF activity is retained by storage of the solution at −20°C (Table 1), although the duration of this benefit is limited to less than 10 days.

It is not known whether the instability of MIF-I explains the difficulty we have been having in demonstrating its activity in the rat in an in vivo situation. We have tried several different systems without much success. After plasma MSH levels have been elevated by such compounds as phenothiazines or haloperidol, injection of MIF-I or tocinoic acid into the lateral ventricle, third ventricle, hypothalamus, or pituitary gland did not consistently result in decreased plasma levels of MSH. Incidentally, ketamine also was found not to cause any change in plasma MSH levels. Initially, in preliminary studies performed with J. Sandow, direct infusion of MIF-I into the pituitary portal system seemed to inhibit MSH release into the plasma, but the variation among samples was so great that the effect was not statistically significant.

![Table](attachment:table.png)

**TABLE 1**

**Activity in Lesioned Frogs of MIF-I after Storage for 4 Days**

| Diluent                | Concentration (mg/ml) | 4°C (refrigerator) (melanocyte index) | −20°C (freezer) (melanocyte index) |
|------------------------|-----------------------|--------------------------------------|-----------------------------------|
| 0.1 M acetic acid      | 10                    | 4.25                                 | 4.05                              |
|                        | 1                     | 4.30                                 | 4.15                              |
|                        | 0.1                   | 4.35                                 | 4.30                              |
| 0.1 N hydrochloric acid| 10                    | 4.28                                 | 4.05                              |
|                        | 1                     | 4.30                                 | 4.07                              |
|                        | 0.1                   | 4.35                                 | 4.25                              |
| Saline                 | 10                    | 4.45                                 | 4.22                              |
|                        | 1                     | 4.42                                 | 4.30                              |
|                        | 0.1                   | 4.45                                 | 4.32                              |

*a The melanocyte index is inversely related to melanocyte-lightening (melanin granule aggregation) activity; 50 ng of MIF-I consistently lowers the melanocyte index from 5.0 to less than 4.0.
EFFECTS OF MIF ON THE CENTRAL NERVOUS SYSTEM (CNS)

We have reported that MIF-I is active in several animal models of Parkinson's disease and mental depression and a preliminary report indicates that it may also be beneficial in patients suffering with parkinsonism (16-19). These effects do not require the presence of the pituitary gland and thus show for the first time that hypothalamic hormones may have extraendocrine effects.

One of these animal models involves potentiation by MIF-I of the behavioral effects of DOPA (16). Table 2 indicates that some of the other substances discussed here (in the present paper) also potentiate the effects of DOPA. Thus, MIF-II, tocinioic acid, and MSH all have some activity in this test system, but none of them are as active as MIF-I. That this finding cannot be explained by a simple effect of MIF-I or MSH upon motor activity has been shown in a separate study measuring activity where body weight, food intake, and water ingestion were also unchanged by MIF-I or MSH (20).

Although a pilot study seemed to indicate that MIF-I shared with MSH the ability to inhibit step-down latency in rats in a passive avoidance situation, no statistically significant difference was found (21). Habituation to a loud buzzer did not seem to be affected by administration of MIF. It also appeared that MIF had a slight tendency to delay extinction of an operant response for food in a Skinner box, but again the wide variation among the rats made it impossible to draw any conclusions.

The mechanism by which MIF-I exerts actions upon the CNS is not known. Frogs pretreated with a monoamine oxidase inhibitor and reserpine show a slight loss of the righting reflex (22). We found this effect to be greatly enhanced by the tricyclic antidepressants but unchanged by MIF-I. This system is considered (22) to involve 5-hydroxytryptamine in the CNS. In another study, a dose of 100 μg 6-hydroxydopamine did not lighten the lesioned frog. Furthermore, after 5 days of treatment of mice with MIF-I (5-20 mg/kg), the total brain content of dopamine, norepinephrine, or serotonin did not show any significant change (23). The mechanism of action of MIF-I on the CNS is not known, but, at the moment (January 1973), it is still important to demonstrate that CNS actions of MIF do occur.

SUMMARY

The existence of a hypothalamic inhibitor of MSH release is now well established. Four compounds, two of which have been isolated from hypothalamic tissue, have been reported to have MIF activity. Species specificity appears to be involved although problems in stability of the compound may also contribute to
the problems encountered. Apart from the actions of MIF mediated by MSH upon the skin color of amphibians, these compounds also have an effect upon the nervous system. Thus, MIF affects tissue derived from the neural crest both directly (CNS) and indirectly (melanocytes).

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