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SITE OF INACTIVATION OF MELANOCYTE-STIMULATING HORMONE-RELEASE-INHIBITING HORMONE BY HUMAN PLASMA*

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Abstract—Since the main melanocyte-stimulating hormone (MSH)-release-inhibiting factor or hormone (MIF or MRIH) has been isolated from bovine hypothalami and its structure determined to be H-Pro-Leu-Gly-NH_2, the corresponding synthetic hormones H-Pro-[^{14}C]Leu-Gly-NH_2 and [^{3}H]Pro-Leu-Gly-NH_2 were prepared to facilitate studies on the inactivation of MIF. Incubation of [^{14}C]MIF for 1 hr at 37° with human plasma, followed by recovery and bioassay, showed complete inactivation. At 0°, inactivation was negligible. The radioactivity recovered from the inactivated products of MIF incubated at 37°, and from the active, intact hormone incubated at 0°, was similar. Thin-layer chromatographic and electrophoretic separation of the inactivation products of [^{14}C]MIF yielded four peptide materials which were identified by amino acid analyses, Edman–dansyl degradation, and measurement of radioactivity as: proline, [^{14}C]Leu-Gly-NH_2, and traces of Pro-[^{14}C]Leu-Gly-OH and [^{14}C]Leu-Gly-OH. The peptides, Pro-Leu-Gly-OH, Leu-Gly-NH_2 and Leu-Gly-OH, were then synthesized and shown to be inactive in the MIF assay. Electrophoretic and chromatographic comparison of the mobilities of these synthetic peptides and proline with that of the purified inactivation products obtained from labeled or unlabeled MIF confirmed Leu-Gly-NH_2 and proline as the main fragments as well as traces of Pro-Leu-Gly-OH. These studies show that incubation of MIF with human plasma caused cleavage of its Pro-Leu bond and destruction of biological activity.

AN INTRICATE system exists for control of the release of melanocyte-stimulating hormone (MSH) from the pituitary gland.[1–3] This control is mediated by the hypothalamus through neurohumoral substance(s) designated MSH-release-inhibiting factor or hormone (MIF or MRIH).[4] Two MIF-active peptides were isolated from bovine hypothalami.[5, 6] One was active at doses of 10–50 ng[7] and was found to be a tripeptide, H-Pro-Leu-Gly-NH_2.[5] The second MIF-active peptide, which possessed only 20 per cent of the biological activity of H-Pro-Leu-Gly-NH_2, was found to be a pentapeptide having the structure H-Pro-His-Phe-Arg-Gly-NH_2.[6] These two peptides were synthesized; the respective biological activities and chromatographic and electrophoretic mobilities of the synthetic and natural materials were compared and found to be similar. The peptide possessing the highest MIF activity, H-Pro-Leu-Gly-NH_2, also forms the C-terminal tripeptide tail of oxytocin. An enzyme contained in rat

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hypothalami can liberate the tripeptide side chain, which may then inhibit the release of pituitary MSH.[8] This tripeptide, H-Pro-Leu-Gly-NH_2, presumably may be the main MIF.

After the report[9] that synthetic tocinoic acid, the ring structure of oxytocin (Cys-Tyr-Ileu-Gln-Asn-Cys-OH), inhibits MSH release from rat pituitaries, we compared the biological activities of the natural bovine MIF, synthetic H-Pro-Leu-Gly-NH_2, and also of tocinoic acid and tocinamide.* The cyclic hexapeptide and its amide were inactive at doses larger than 50 μg,[6] whereas the tripeptide, Pro-Leu-Gly-NH_2, was active at nanogram levels. We also observed the rapid inactivation of this tripeptide while it was in contact with plasma under various conditions. To perform a systematic study on the fate of MIF during its inactivation with human plasma, we synthesized H-Pro-[^{14}C]Leu-Gly-NH_2 ([^{14}C]MIF) and [^{3}H]-Pro-Leu-Gly-NH_2 ([^{3}H]-MIF). This paper reports the results of our investigations on the inactivation of MIF and its ^{14}C and ^{3}H analogues by human plasma.

EXPERIMENTAL PROCEDURE

Purification of MIF. MIF was extracted from bovine hypothalami and purified 11,000 times by filtration through Sephadex columns. Further purification of this material was effected by preparative thin-layer chromatography (TLC).[5] The pure material corresponding in structure to H-Pro-Leu-Gly-NH_2 was homogeneous by TLC and thin-layer electrophoresis (TLE) and active in doses of 10–50 ng/frog. This was used as a standard. Synthetic MIF and its ^{14}C and ^{3}H analogues, H-Pro-[^{14}C]Leu-Gly-NH_2 and [^{3}H]Pro-Leu-Gly-NH_2, were prepared in our laboratory by Merrifield's solid phase method.[10] *N*-tertiary butoxy carbonyl amino acids, including *t*-BOC-[^{14}C]leucine (161·2 mCi/m-mole), *t*-BOC-[^{3}H]proline (800 mCi/m-mole) and *t*-BOC-glycine resin ester were purchased from Schwarzman (Orangeburg, N.Y.). The details of the syntheses were described elsehwere.† The synthetic peptides were recrystallized from 60 per cent aqueous methanol and were found to be homogeneous by TLC and TLE and biologically active. The [^{14}C]MIF (0·6 mCi/m-mole) and [^{3}H]MIF (2·1 mCi/m-mole) showed a single radioactive spot in the thin-layer chromatogram (Fig. 1). H-Pro-Leu-Gly-OH, H-Pro-Leu-OH, H-Leu-Gly-NH_2 and H-Leu-Gly-OH were also synthesized by the solid phase method.

Recovery of MIF and its inactivation products. After incubation with freshly drawn human plasma, the proteins were precipitated with a very slight excess of 5 per cent aqueous trichloroacetic acid[11] and centrifuged. The clear supernatant was removed, lyophilized and washed six times with absolute ether to remove traces of tricholoroacetic acid. The resultant residue was extracted with 0·2 N acetic acid and relyophilized. The solid material was extracted with 70 per cent aqueous methanol and evaporated *in vacuo*. The product thus obtained was repurified by preparative TLC and the major spots were cut out and eluted.

Bioassay for MIF. A modified assay *in vivo*,[7] based on the measurement of aggregation of melanin granules in dermal melanocytes of the skin (skin lightening) of frogs which have been darkened by destruction of hypothalamus, was adapted for the estimation of the biological activities of the purified incubation products. In this assay, small

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doses (10–50 ng) of MIF, when applied directly to the surface of the pituitary on the lesioned frog, markedly lightened its skin.

Homogeneity and composition tests. Thin-layer chromatography and electrophoreses were carried out in plates coated with 250 μm layer of cellulose MN 300 HR (Nacherey, Nagel & Company, Duren, West Germany) or Avicel microcrystalline cellulose (Brinkmann Instruments, Inc., Westbury, N.Y.). Desaga–Brinkmann equipment was used for separations. The spots were visualized by spraying with chlorine-*o*-tolidine reagent[12] or with ninhydrin reagent.[13] Amino acid analyses of the purified inactivation products were performed in an automatic Beckman-Spinco model 120-C-analyzer, provided with microcuvettes and 1-mV range card. The samples were hydrolyzed in 6 N HCl for 22 hr at 110° in evacuated sealed tubes. Edman–dansyl sequential degradation of the peptides was performed as described previously.[5, 14]

Incubation experiments. MIF (labeled or unlabeled) was incubated in undiluted fresh human plasma at 37° for 1 hr. One milliliter of plasma was used for every 100 μg MIF. Aliquots of this were used for bioassay and for measurement of radioactivity. A saline control with the same concentration of MIF (100 μg/ml) in saline, without plasma and a plasma control without MIF were incubated simultaneously at 37°. Analogous experiments were also conducted at 0°. The radioactivity was measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Company, Downers Grove, Ill.).

Labeling with [^{14}C]diazomethane. Ethereal [^{14}C]diazomethane was prepared from [^{14}C]diazald (*N*-methyl[^{14}C]nitroso-*p*-toluene sulfonamide, 5 mCi/m-mole), purchased from New England Nuclear Corp. and standardized as described previously.[11] This was utilized in estimating the free —COOH groups in the inactivated peptides recovered from the incubation experiments.

RESULTS AND DISCUSSION

After incubation with human plasma at 37° for 1 hr, MIF showed a complete loss of biological activity (Table 1), while a similar incubation at 0° showed only 8 per cent inactivation. The slight loss of biological activity observed in this study after incubation of 100 μg MIF in saline at 37° for 1 hr was more pronounced when lower concentrations of MIF were used. After extraction and uprification of [^{14}C]MIF incubated at 37°, 86 per cent of the radioactivity was recovered from the pure peptides, an amount which compared favorably with the 88 per cent recovery for the material incubated at 0°. Of the total radioactivity, 12–14 per cent remained bound to the plasma proteins in both cases (37° and 0° incubation experiments), as demonstrated by the radioactivity measured in the plasma residue. TLC and TLE of the purified inactivation products of MIF, the amino acid analyses, and Edman–dansyl degradation identified proline and Leu-Gly-NH_2 as the main products (75 per cent). Pro-Leu-Gly-OH and Leu-Gly-OH were also found in very small quantities (11 per cent, Table 2). Comparison by TLC and TLE (Figs. 2, 3) of the purified inactivation products—proline, Leu-Gly-NH_2, Pro-Leu-Gly-OH and Leu-Gly-OH—with their synthetic equivalents indicated the respective mobilities to be similar. This confirms the identification of the inactivation products. Additional evidence for the identity of the purified products of incubation of the labeled MIF was obtained by measurement of radioactivity after separation. Thus, after incubation and separation of the inactivation products of [^{14}C]MIF, 75 per cent of the radioactivity was recovered

TABLE 1. RESULTS OF THE BIOASSAY OF MIF BEFORE AND AFTER INCUBATION WITH HUMAN PLASMA*

Group no.	Description of sample	Dose (ng/frog)	Melanocyte index (mean ± S.E.M.)	Biological activity (%)
1	[^{14}C]MIF after incubation with human plasma at 37°, 1 hr, and recovery	150	4·75 ± 0·06	00·00
2	[^{14}C]MIF + saline at 37°, 1 hr	150	4·125 ± 0·04	86·00
3	[^{14}C]MIF with human plasma at 0°, 1 hr, and recovery	150	3·925 ± 0·05	92·00
4	[^{14}C]MIF + saline at 0°	150	3·75 ± 0·02	98·00
5	Serum control	500	4·675 ± 0·07	00·00
6	Saline control	500	4·985 ± 0·06	00·00
7	MIF, bovine (Pro-Leu-Gly-NH_2)	150	3·625 ± 0·02	100·00
8	Synthetic Pro-Leu-Gly-NH_2	150	3·615 ± 0·06	100·00
9	[^{14}C]MIF	150	3·675 ± 0·03	100·00
10	[^{3}H]MIF	150	3·605 ± 0·02	100·00

* The test material dissolved in saline was applied to the surface of the pituitary gland (with a 1-μl pipette) of a frog[7] which has been darkened by destruction of the hypothalamus.[13] A 1-hr response brought about the maximum decrease in the melanocyte index (MI) and was used in the assay. The MI is inversely related to melanocyte lightening (melanin granule aggregation) activity.

TABLE 2. AMINO ACID COMPOSITION AND PERCENTAGE RECOVERY OF RADIOACTIVITY AND BIOLOGICAL ACTIVITY OF PURIFIED PRODUCTS OF MIF INCUBATED AT 37° AND 0° FOR 1 hr WITH PLASMA

Group no.	Description of sample	Amino acid composition	Sequence by Edman-dansyl procedure	Recovery of radioactivity (%)	Biological activity (%)
1	[^{14}C]MIF after incubation with human plasma at 37°, 1 hr, and recovery	(a) Proline 1	(a) Proline	0	0
		(b) Glycine 1 and leucine 1	(b) Leu-Gly-NH_2	75	0
		(c) Proline 1, glycine 1, and leucine 1	(c) Pro-Leu-Gly-OH	11	0
2	[^{14}C]MIF + saline at 37°, 1 hr	Proline 1, glycine and leucine 1	Pro-Leu-Gly-NH_2	98	86
3	[^{14}C]MIF + human plasma at 0°, 1 hr, and recovery	Proline 1, glycine 1, and leucine 1	Pro-Leu-Gly-NH_2	88	92
4	[^{14}C]MIF + saline at 0°, 1 hr			98	98
5	Serum control	Traces of all amino acids		0	0
6	Saline control	Blank		0	0
7	MIF, bovine (standard)			0	100
8	Synthetic Pro-Leu-Gly-NH_2	Not analyzed		0	100
9	[^{14}C]MIF	Proline 1, glycine 1, and leucine 1		100	100

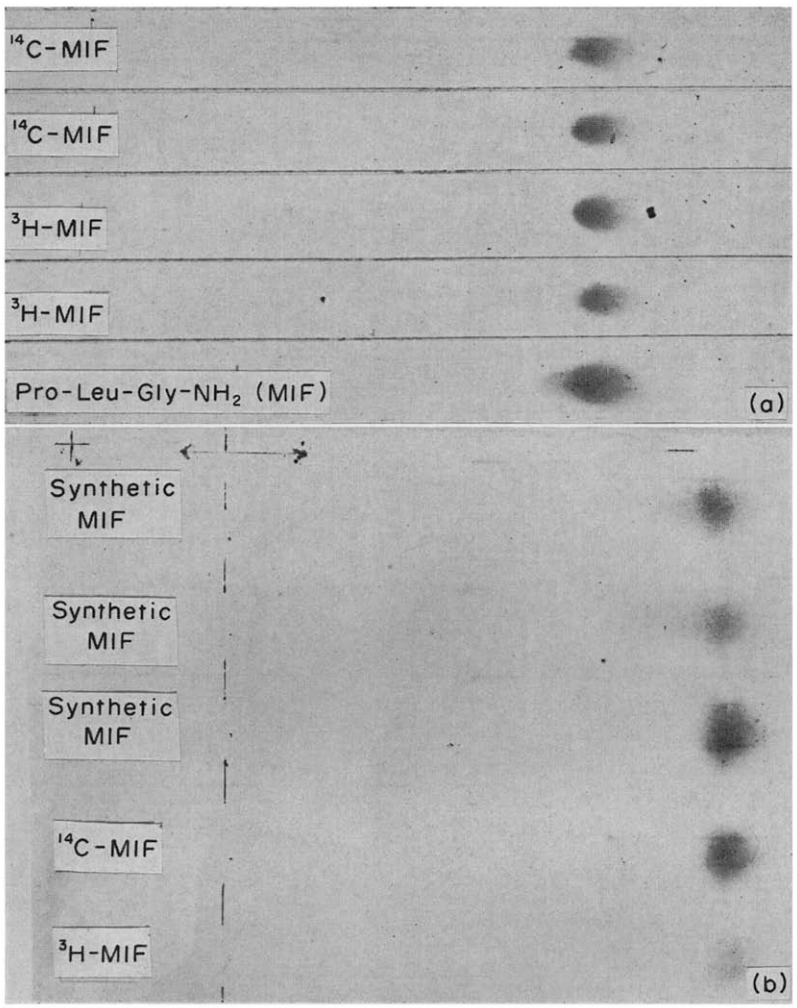

FIG. 1. Thin-layer chromatography and eletcrophoresis of synthetic MIF, [^{14}C]MIF and [^{3}H]MIF. (a) TLC on cellulose. Solvent system: 1-BuOH–AcOH–H_2O (4:1:5); (b) TLE on cellulose. Pyridine acetate buffer, pH 4·5; 310 V, 12·5 mA, 9°, 4 hr.

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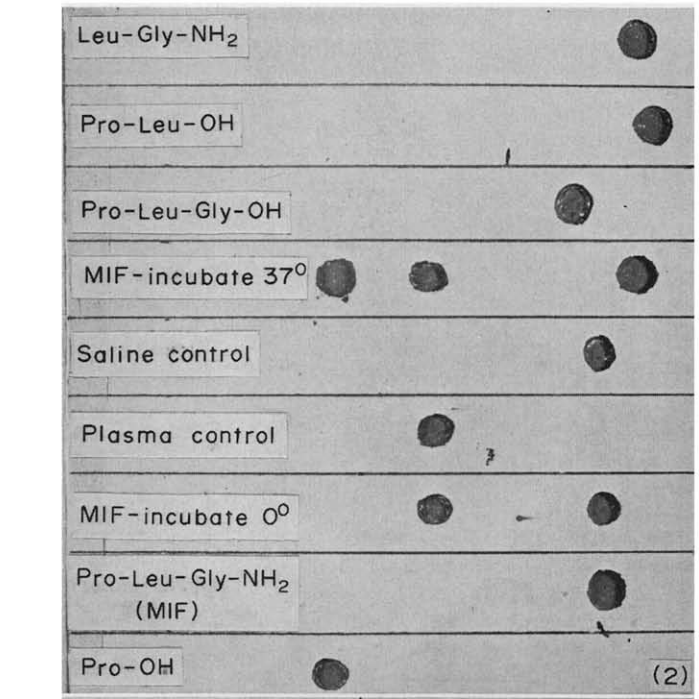

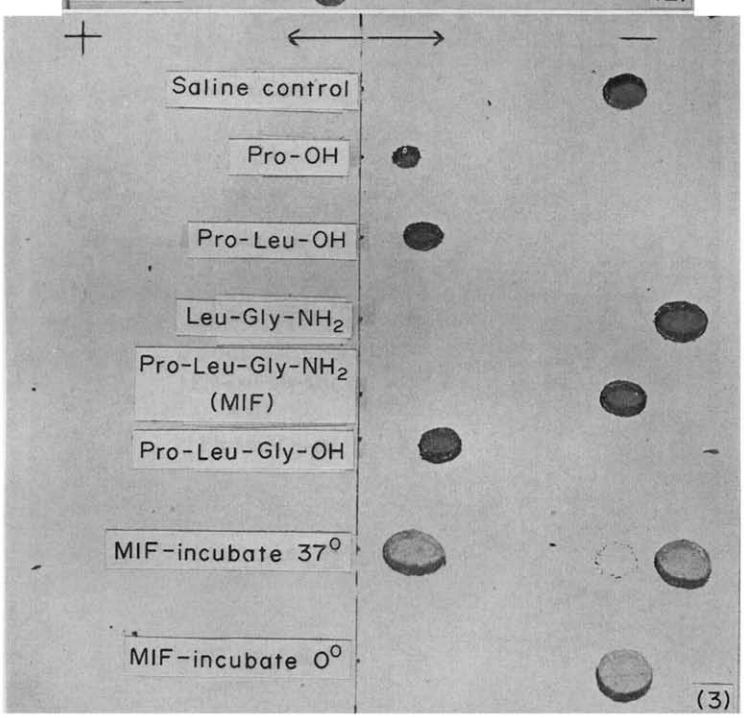

FIG. 2. Thin-layer chromatography of the purified products of MIF, incubated with human plasma at 37° and 0°, along with standards on cellulose (250 μm, layer). Solvent system: 1-BuOH–AcOH–H_2O (4:1:5).

FIG. 3. Thin-layer electrophoresis of the purified products of MIF after incubation with human plasma at 37° and 0°, along with standards, on cellulose (250 μm, layer). Pyridine acetate buffer, pH 4·5, 310 V, 7·5 mA, 10°; 2 hr, 40 min.

from the area corresponding to Leu-Gly-NH_2 and 11 per cent from that of Pro-Leu-Gly-OH. The rest of the radioactivity was found in the precipitated plasma proteins. Similarly, during incubation of [^{3}H]Pro-Leu-Gly-NH_2, 74 per cent of the radioactivity was recovered from the area corresponding to proline and 8 per cent from the Pro-Leu-Gly-OH area; 18 per cent was not separable from the plasma proteins.

These studies demonstrate that MIF is rapidly inactivated by human plasma. The inactivation is the result of cleavage of this tripeptide at the Pro-Leu- bond, with the production of proline and Leu-Gly-NH_2 as the primary products. This cleavage is probably brought about by the influence of plasma enzymes.[15,16] The presence of traces of Pro-Leu-Gly-OH after incubation of MIF suggests that the second metabolic pathway probably involves the cleavage of the —COOH-terminal amide. It is interesting to note that in the inactivation by human plasma of the thyropropin-releasing hormone (TRH), which is also a tripeptide amide (pyro-Glu-His-Pro-NH_2),[11,17] the major pathway is de-amidation with the production of TRH-free acid (pyro)-Glu-His-Pro-OH. The structural vulnerability of MIF to attack by the plasma enzymes may partially explain the absence of its biological activity in lightening skin during an assay involving injection of very large doses of MIF into the dorsal lymph sac or into the aortic trunk of the lesioned frog.[7]

Our inactivation studies *in vivo* with [^{14}C]MIF revealed its half-life to be about 10 min[18] as compared with a half-life of 4 min for [^{14}C]TRH. It was also demonstrated that [^{14}C]MIF accumulates in the pituitary and the pineal gland of rats. The accumulation of radioactivity in the pineal suggests a role for this gland in a complex hypothalamic–pineal–pituitary axis[19,20] involving MIF, melatonin and MSH.

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