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OPTIMIZATION OF A MELANOTROPIN-RECEPTOR BINDING ASSAY BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We describe here a procedure, which should be generally applicable to a wide variety of polypeptide hormones, for analyzing with reversed-phase high-performance liquid chromatography the stability of radioiodinated ligands. Specifically, the integrity of mono [^{125}I]β-melanotropin during storage and under various assay conditions has been examined. The hormone was found to be quite stable during storage and when binding assays were conducted at 0–1°C. There was, however, a decrease in its stability when assays were conducted at higher temperatures (15, 25 and 37°C), the instability increasing with temperature and being greater when cells were present. Employing heat-inactivated instead of untreated bovine serum albumin in the buffer used in the assay provided only a modest improvement in the stability of [^{125}I]β-melanotropin.

INTRODUCTION

A useful probe for examining the interaction of a peptide hormone with its cell-surface receptor is a radiolabelled version of the hormone. Ideally, this ligand should have a high specific radioactivity (as can be attained by the incorporation of ^{125}I), full biological activity, minimal structural alteration and a high level of purity. We[1] have recently described a procedure for producing a labelled hormone, mono [^{125}I]β-melanotropin (β-MSH), which meets these criteria. Key to developing this technique was the use of reversed-phase high-performance liquid chromatography (RP-HPLC). Other laboratories have also found RP-HPLC to be valuable in purifying radioiodinated peptide hormones[2–4].

When conducting studies of the binding of the iodinated ligand to its receptor, it is useful to know the stability of the peptide under the assay conditions employed. If it is being broken down or converted to a less active form, this could seriously affect the data obtained and their subsequent interpretation. Of particular importance to assays with [^{125}I]β-MSH is the ease with which this hormone is oxidized to a less active form[1,5]. We describe here a simple procedure for analyzing the stability of [^{125}I]β-MSH using RP-HPLC, a technique which should be readily adaptable to

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other labelled hormones. The effects of temperature and of heat-inactivated bovine serum albumin (BSA) are examined and a temperature-dependent processing of [^{125}I]β-MSH is revealed.

EXPERIMENTAL

Iodination and purification of [^{125}I]β-MSH

The iodination of β-MSH was adapted from our earlier procedure[1] with the following modifications: IodoGen (Pierce), 2.2 μg (5 nmole) in 20 μl dichloromethane, was plated onto the bottom inside of 75 × 12 mm borosilicate glass tubes, evaporation of the solvent being carried out with a rotary evaporator at 37°C. The tubes were wrapped in aluminum foil and stored over Drierite in a dessicator until use. Each iodination was carried out in one of these tubes on ice with gentle stirring, the reaction mixture consisting of 5 mCi sodium [^{125}I]iodide (2.5 nmole; New England Nuclear) in approximately 10 μl 0.1 *M* sodium hydroxide, plus 100 μl borate buffer (9.5 g boric acid and 9.5 g sodium borate in 1 l distilled water, pH 8.2), and 11 μl (5 nmole) of HPLC-purified β-MSH in distilled water (1 mg/ml stock). Partially purified β-MSH was a gift from Dr. Saul Lande of our department, and was purified further by the method of Lambert and Lerner[6] before use in these experiments. The iodination was terminated after 20 min by removing the reaction mixture from the IodoGen-coated tube and adding the mixture to 0.9 ml borate buffered saline[1] containing dithiothreitol (0.75 *M*; Sigma) and gentamicin (100 μg/ml; Gibco). This solution was then filtered through a sterile 0.22-μm Gelman Acrodisc into a sterile 15-ml centrifuge tube and incubated at 37°C for approximately 24 h. At the end of this time, the solution was passed through a Sephadex G-10 column (22 cm × 0.45 cm^2), which was eluted with distilled water. The labelled and unlabelled MSH was eluted in the void volume. The peak fractions were pooled and applied to a HPLC system consisting of two Waters Assoc. M6000A pumps, a 660 programmer, a U6K injector, a μBondapak C_{18} column (30 × 0.39 cm), a Perkin-Elmer LC75 UV–VIS detector and a Houston Instruments dual pen chart recorder. The components of the injected solution were eluted from the column using a linear gradient of increasing acetonitrile (Baker, HPLC-grade), 19–27% over 25 min, with 1% triethylammonium phosphate (TEAP) pH 3.00 (Pierce) as the primary eluent. The flow-rate was 2 ml/min. Aliquots (0.6 ml) were collected with a Gilson FC-100K fraction collector and tested for radioactivity (^{125}I) with an LKB MiniGamma counter. The fractions containing monoiodo-β-MSH (peak A in Fig. 1), characterized in our previous work[1], were then chromatographed on a column of Sephadex G-10 (18 cm × 1.77 cm^2), eluted with 1 m*M* dithiothreitol in Ca^{2+}/Mg^{2+}-free phosphate buffered saline[7] to separate the [^{125}I]β-MSH from the acetonitrile and TEAP. Fractions (1 ml) were collected in siliconized (Sigmacote, Sigma) glass tubes. The [^{125}I]β-MSH was eluted in the void volume, and the peak fractions were pooled into one or two siliconized tubes. These were stored under an argon atmosphere on ice.

Binding assay

The binding buffer consisted of 15 m*M* N-(2-hydroxyethyl-1-piperazine-N'-ethane sulfonic acid (HEPES) (Research Organics) in Ham's F10[8] (MA Bioproducts, Walkersville, MD, U.S.A.) plus 5 mg/ml bovine serum albumin (BSA, Frac-

tion V; Sigma). Depending on the experiment (see Results) the BSA either was not modified or was heated in distilled water at 60°C for 1 h to remove potential protease activity[9], then lyophilized. Following addition of BSA to the binding buffer, the pH was adjusted to 6.8 and the solution was filtered through a Nalgene sterilization filter unit (Type S). Gentamicin was then added to a final concentration of 50 μg/ml. Experiments were carried out both with and without cells (see Results), the cells being Cloudman S91 melanoma, CCL 53.1, obtained from the American Type Culture Collection and maintained as described previously[1]. In all experiments, volumes of 750 μl were incubated in siliconized glass vials (5.5 cm × 4.9 cm^2) in a water-bath with shaker (90 rpm) at various temperatures, depending on the experiments (see Results). Each solution contained binding buffer, with or without cells, and [^{125}I]β-MSH, diluted from the stock preparation to a final concentration of 1 n*M*. In those experiments in which cells were included, at a concentration of $4.2 \cdot 10^6$–$7.2 \cdot 10^6$ cells per ml, the solution was removed from the vial at the appropriate time and centrifuged in a Beckman Microfuge B for 1 min to pellet the cells. Aliquots of the cell-free supernatants were then subjected to HPLC analysis.

HPLC analysis of [^{125}I]β-MSH stability

The HPLC apparatus and conditions used for analyzing the stability of [^{125}I]β-MSH were the same as those used in its purification (see above), except for the following modifications. Instead of the steel μBondapak C_{18} column, a Waters radial compression module (RCM-100), equipped with a Waters μBondapak C_{18} Radial-Pak cartridge plus C_{18} guard insert, was used. When examining the integrity of [^{125}I]β-MSH following different periods of storage, approximately 20 μl of the stock were injected. For analyzing the effects of different incubation conditions, 0.6-ml aliquots of the [^{125}I]β-MSH-containing binding buffer solutions were subjected to HPLC analysis at the appropriate time.

RESULTS

Our iodination procedure yields a mixture of peptides which are efficiently resolved by RP-HPLC (Fig. 1a). As has been characterized more fully previously[1], unlabeled β-MSH is eluted ahead of iodinated β-MSH, between fractions 35 and 40 under the present conditions (not shown). Oxidized monoiodo[^{125}I]β-MSH is eluted slightly before the unlabelled β-MSH. Monoiodo (peak A) and diiodo (peak B) β-MSHs are eluted around fractions 60 and 75, respectively. Of these, monoiodo-β-MSH is the form with full biological activity[1], and hence is the one used in these experiments.

Under the conditions used to store the monoiodo-β-MSH (see Experimental), the peptide is quite stable (Fig. 1b and c). An aliquot removed from the stock solution of [^{125}I]β-MSH after 5 days of storage yields, in HPLC, a single major peak, preceded by some minor contaminants (Fig. 1b). A similar result is obtained after 15 days of storage (Fig. 1c). Between these two analyses, the system was used extensively in examining the stability of the label under different binding assay conditions. During this time, as contaminants built up on the guard insert, the back pressure increased, the tailing of the [^{125}I]β-MSH peak decreased and the retention times of the [^{125}I]β-MSH and contaminants on the column gradually decreased.

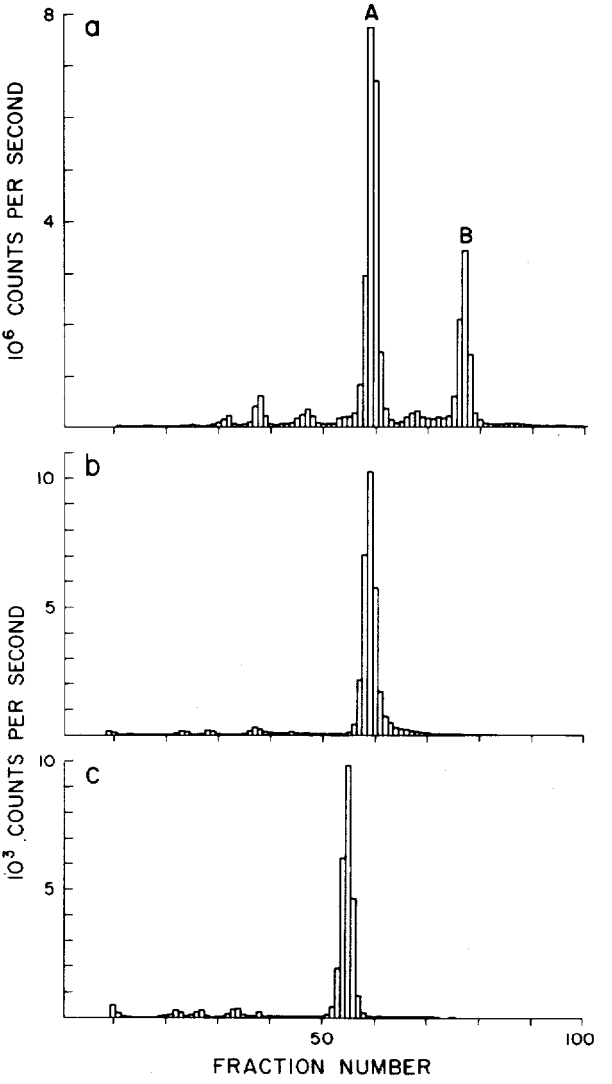

Fig. 1. HPLC elution profiles of β-MSH following iodination (a), monoiodo-[^{125}I]β-MSH after 5 days of storage (b) and monoiodo-[^{125}I]β-MSH after 15 days of storage (c). For iodination, storage and HPLC procedures, see Experimental. Ordinate: a, 10^6 counts per second (cps) per fraction; b and c, 10^3 cps. Abscissa: fraction number (0.6-ml fractions).

After [^{125}I]β-MSH was incubated with shaking in binding buffer containing untreated BSA plus cells in an ice–water bath, most of the radioactivity is eluted as unmodified [^{125}I]β-MSH (peak D, Fig. 2a and b). Besides the minor contaminants present in the stock solution (Fig. 1b and c), two additional peaks (C and E) are seen in Fig. 2a and b. These are relatively insignificant, but their levels increase with incubation time (a, 1.5 h; b, 3 h). It should be noted that whereas peak C is consistently present when these experiments are repeated, peak E is more variable. When cells were excluded from the binding buffer, only peak C is detected as an additional

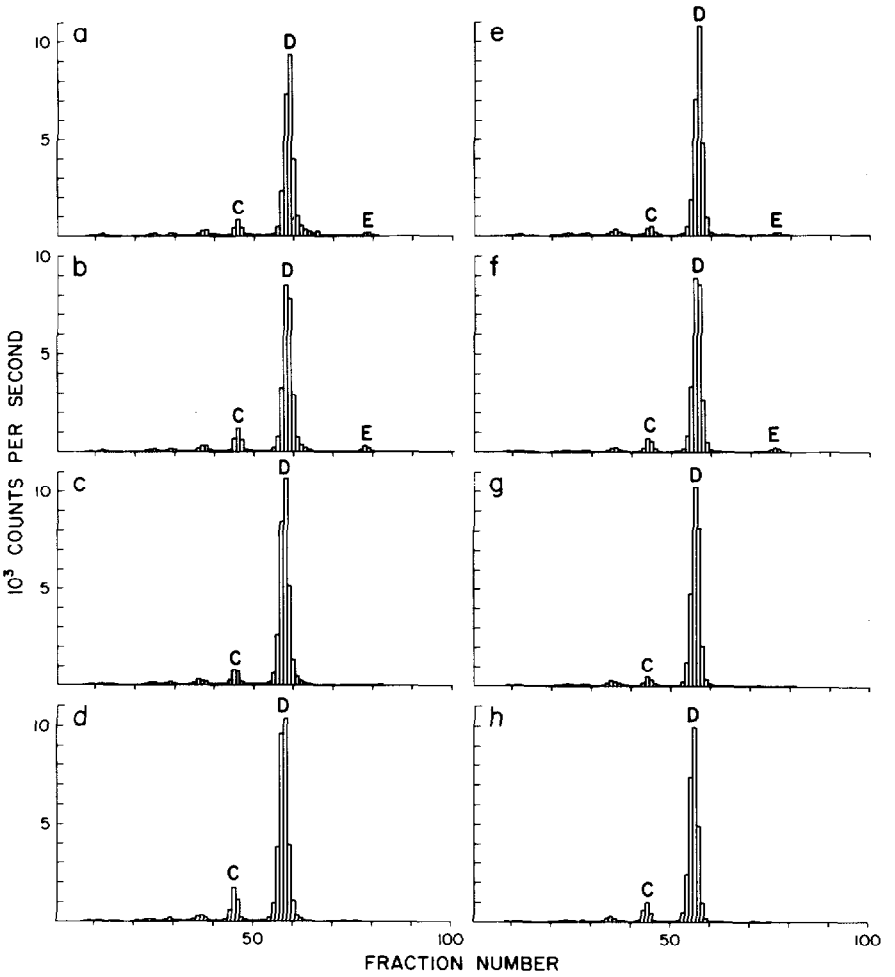

Fig. 2. HPLC elution profiles of [^{125}I]β-MSH incubated in binding buffer at 0–1°C. For incubation conditions and HPLC analysis see Experimental, except binding buffer contained [^{125}I]β-MHS and (a) cells and untreated BSA for 1.5 h, (b) cells and untreated BSA for 3 h, (c) untreated BSA without cells for 1.5 h, (d) untreated BSA without cells for 3 h, (e) cells with heat-inactivated BSA for 1.5 h, (f) cells with heat-inactivated BSA for 3 h, (g) heat-inactivated BSA without cells for 1.5 h and (h) heat-inactivated BSA without cells for 3 h. Ordinate: 10^3 cps per fraction. Abscissa: fraction number (0.6-ml fractions).

contaminant (Fig. 2c and d). This again increases with time (c, 1.5 h; d, 3 h). After [^{125}I]β-MSH was incubated with shaking with cells in binding buffer containing heat-inactivated BSA in a water-bath, the elution profile is very similar to that when untreated BSA is used (Fig. 2e and f *vs.* Fig. 2a and b, respectively). Peaks C and E are slightly smaller when heat-inactivated BSA was used, but they still increase with time (e, 1.5 h; f, 3 h). As was the case for binding buffer containing untreated BSA, when cells were omitted from binding buffer containing heat-inactivated BSA, only peak C is observed as an additional contaminant (Fig. 2g and h), which again increases with time (g, 1.5 h; h, 3 h).

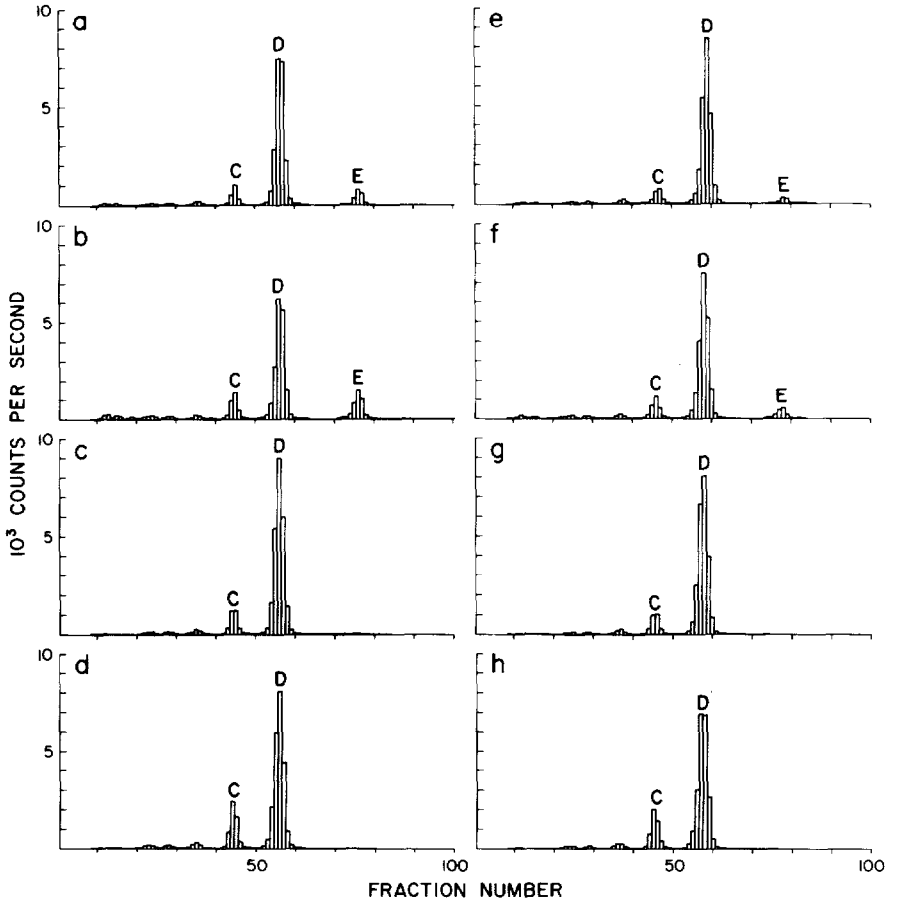

Fig. 3. HPLC elution profiles of [^{125}I]β-MSH incubated in binding buffer at 15°C. With the exception of the temperature, all methods and conditions for a–h were the same as those described in Fig. 2.

The elution profiles of [^{125}I]β-MSH incubated at 15°C are similar to those after incubation at 0–1°C (Fig. 3 *vs.* Fig. 2, respectively). When cells were present, both peaks C and E appear and increase with time (Fig. 3a, b, e and f). As at 0–1°C, the contribution of peak E varies with experiment. The peaks are slightly lower when heat-inactivated BSA was used in place of untreated BSA (Fig. 3e and f *vs.* a and b). When cells were excluded, only peak C is present as an additional contaminant (Fig. 3c, d, g and h). This is again slightly lower when heat-inactivated BSA was used in place of untreated BSA and increases with incubation time. Peaks C and E are higher, and peak D ([^{125}I]β-MSH) lower, in experiments at 15°C compared to parallel experiments at 0–1°C (Figs. 3 *vs.* Fig. 2).

The sizes of peaks C–E differ even more from those obtained at 0–1°C when incubations were carried out at 25°C (Fig. 4 *vs.* Fig. 2), although the trends noted at 0–1°C and 15°C are similar at 25°C. Again, both peaks C and E appear when cells were present and increase with time (Fig. 4a, b, e and f), with a concomitant decrease in the size of peak D ([^{125}I]β-MSH). And again, peak E is more variable than C

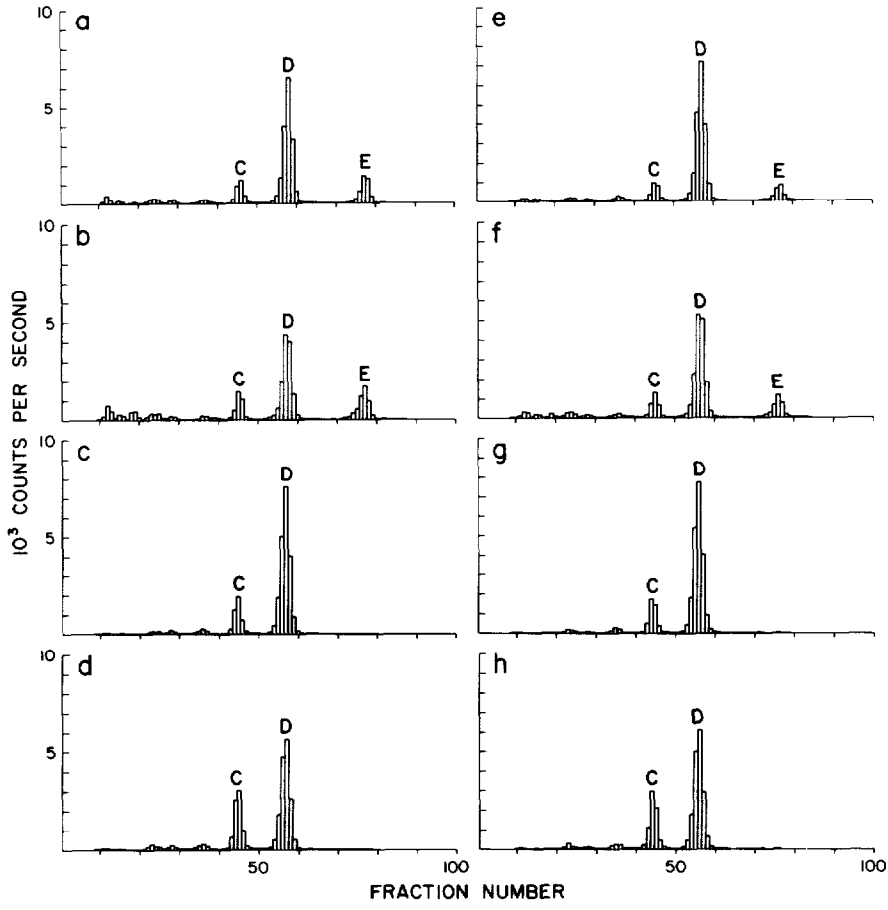

Fig. 4. HPLC elution profiles of [^{125}I]β-MSH incubated in binding buffer at 25°C. Other than the temperature, all methods and conditions for a–h were the same as those described in Fig. 2.

when experiments are repeated. Peaks C and E are slightly lower and D is higher when the [^{125}I]β-MSH was incubated in the presence of heat-inactivated, as opposed to untreated, BSA (Fig. 4e and f *vs.* a and b). When cells were omitted from the binding buffer, only peak C occurs as an additional contaminant, increasing in size with time (Fig. 4c, d, g and h).

When [^{125}I]β-MSH was incubated with cells at 37°C, the resulting HPLC elution profiles differ markedly from those obtained from 0–1°C incubations (Fig. 5 *vs.* Fig. 2). In addition to peaks C and E, which develop at lower temperatures (Figs. 2–4), two additional components become significant, peaks A and B, which are eluted early. They occur whether or not heat-inactivated BSA (Fig. 5e and f) was used in place of untreated BSA (Fig. 5a and b). Peaks A, B, C and E form at the expense of peak D ([^{125}I]β-MSH), which is substantially decreased even after 1.5 h in the presence of cells (Fig. 5a and e). After 3 h of incubation with cells, even less of peak D is present, and peaks C and E also decrease (Fig. 5b and f); these decreases are accompanied by increases in peaks A and B. When cells were absent during incubations at 37°C (Fig. 5c, d, g and h), peaks A and B do not occur, C being the only contaminant in addition to those already present in the stock solution of [^{125}I]β-MSH. The proportion of peak C in the incubation mixture increases markedly with

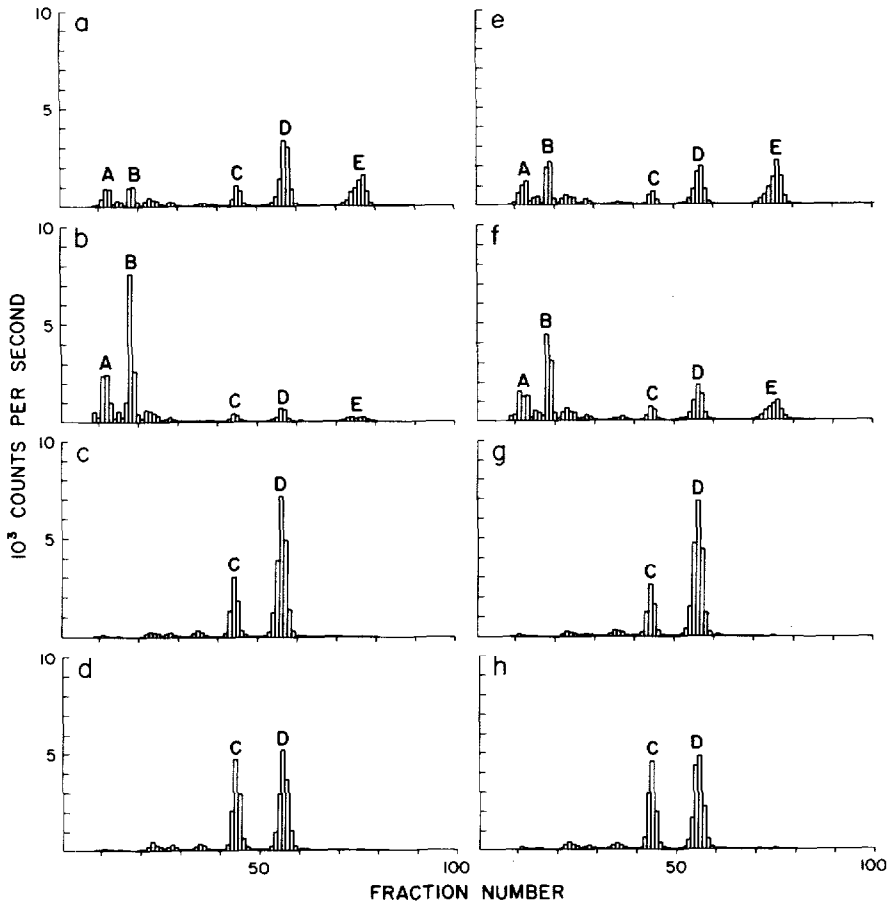

Fig. 5. HPLC elution profiles of [^{125}I]β-MSH incubated in binding buffer at 37°C. Other than the temperature, all methods and conditions for a–h were the same as those described in Fig. 2.

time to become a major component (almost as much as the [^{125}I]β-MSH) by 3 h (Fig. 5d and h).

DISCUSSION

The significance of the material presented in this contribution is three-fold: A rapid, simple procedure is described for monitoring the stability of radiolabelled peptides during storage and under various assay conditions; two parameters for optimizing β-MSH receptor-binding assays have been examined; and temperature-dependent processing of [^{125}I]β-MSH by melanoma cells has been demonstrated.

Traditionally, the integrity of radiolabelled peptide hormones, if examined at all, has been monitored by such procedures as trichloroacetic acid precipitation or gel filtration. Such techniques, while revealing gross structural changes in the ligand, would be less likely to reveal smaller changes. For some peptide hormones small modifications, such as loss or alteration of one or two amino acids, may have little effect on receptor-binding characteristics, and hence an ability to detect only major structural changes may be quite sufficient. In others, such as β-MSH, even a minor change like the oxidation of methionine can have a substantially deleterious effect

on biological activity[1,5,10], and possibly on the binding of the hormone to its receptor. We have demonstrated both previously[1] and now again (Fig. 1) that RP-HPLC is an effective tool for separating biologically active monoiodo-β-MSH from unlabelled β-MSH, diiodo-β-MSH, oxidized monoiodo-β-MSH and a variety of other components. By changing our earlier procedure[1] to the use of the Radial-Pak cartridge/guard insert system, we have developed a technique that retains resolving power while circumventing the problem of destroying expensive steel columns. When optimizing conditions for separating a labelled hormone from structurally related contaminants, the procedure will not necessarily elute other components injected with the hormone. For instance, the relatively large injection volumes we use in analyzing the stability of [^{125}I]β-MSH in binding buffer results in large amounts of BSA being applied to the column, and this protein does not appear to be eluted under our conditions. Similarly, should cells containing the labelled hormone be disrupted and the extract be applied to an HPLC system to examine the integrity of the hormone, it is likely that a large amount of material will accumulate at the top of the column. In both cases, the adsorbed material would eventually increase the back pressure and reduce the resolution until the column would have to be discarded or repacked. In the system described here, adsorption takes place on the inexpensive, disposable guard insert. Even if a large amount of material should be deposited on the cartridge/insert such that breakthrough occurs and the Radial-Pak cartridge itself becomes contaminated, the cartridge can still be replaced at a substantially lower cost than a steel column. By using this system we have demonstrated that [^{125}I]β-MSH is quite stable under the storage conditions we routinely use (Experimental). Also, under the conditions of the binding assay described here, there is no conversion of [^{125}I]β-MSH to its biologically less active oxidized form, although other components do appear. Although we have not yet identified these peaks, this should be possible by comparing their retention times with those of likely metabolites of monoiodo-β-MSH. Our system should be readily adaptable to stability tests for other radiolabelled peptide hormones under various incubation conditions. It may also be useful in examining the intracellular processing of [^{125}I]β-MSH, as well as other hormones.

Two parameters for optimizing a test of the binding of [^{125}I]β-MSH to melanoma cells have been examined in this report. One is the use of heat-inactivated as opposed to untreated BSA; the other is the use of different temperatures. In the former case, employing heat-inactivated instead of untreated BSA provides only a modest improvement in the stability of [^{125}I]β-MSH, especially when no cells are present in the binding buffer. When cells are present, the degree of protection offered by heat-inactivation of the BSA increases somewhat, but not systematically, with increasing temperature.

We have found temperature to be an important factor in controlling the stability of [^{125}I]β-MSH in the binding buffer, especially if cells are present. At 0–1°C, most of the radioactive material is present as intact [^{125}I]β-MSH, with only a few minor contaminants even after 3 h of incubation. At 15°C the [^{125}I]β-MSH is somewhat less stable, and two of the minor contaminants noted at 0–1°C increase at the expense of the ligand. This instability with increasing temperature is more apparent when incubations are carried out at 25°C, and becomes quite striking at 37°C.

At all test temperatures, it was noted that a peak (E) which is eluted after [^{125}I]βMSH occurs in the binding buffer only when cells are also present. At lower temperatures (0–1, 15 and 25°C), this cell-associated contaminant (metabolite?) increases in amount with time and increasing temperature, at the expense of [^{125}I]β-MSH. At 37°C this contaminant, [^{125}I]β-MSH, and the peak which is eluted just

prior to [^{125}I]β-MSH (C) are converted to earlier eluted peaks (A and B). The fact that peaks A and B are eluted from the column very early is consistent with the possibility that they are breakdown products of [^{125}I]β-MSH (*e.g.*, monoiodotyrosine), although this remains to be proved. The nature of the late-eluting peak (E) is more equivocal. It could be simply a degradation product of [^{125}I]β-MSH which happens to be less polar than the original ligand. Alternatively, it could be intact or processed [^{125}I]β-MSH bound to some cellular component, the complex being released by the cells. One candidate for such a component would be a fragment (subunit?) of the receptor for MSH. It is unlikely that the cell-associated peaks are simply artifacts resulting from the release of proteolytic enzymes from dead or dying cells. As determined by trypan blue exclusion, cell viability was about 95%. Also, the high level of BSA relative to [^{125}I]β-MSH in the binding buffer would be expected to limit non-specific degradation of the peptide.

One hypothesis on the action of MSH states that the hormone is internalized by the cell[11,12], the hormone–receptor complex being translocated to premelanosomes, subcellular organelles in which melanin synthesis, the principal cellular activity under the control of MSH, occurs. It remains to be determined whether or not the cellular processing of [^{125}I]β-MSH detected in the binding buffer reflects an intracellular metabolism of the peptide. How the processing of [^{125}I]β-MSH relates to the physiological behavior of the hormone is another unanswered question. It may be that the peptide, after activating the cell, is being specifically degraded (either intra- or extracellularly), thus terminating the signal. Alternatively the β-MSH, following binding and internalization, may be modified, the newly altered MSH(s) serving as intracellular messengers of MSH action. One approach which will help answer these and other questions will be the analysis by RP-HPLC of [^{125}I]β-MSH and its products extracted from cells exposed to the hormone.

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REFERENCES

1 D. T. Lambert, C. Stachelek, J. M. Varga and A. B. Lerner, *J. Biol. Chem.*, 257 (1982) 8211.
2 N. G. Seidah, M. Dennis, P. Corvol, J. Rochemont and M. Chretien, *Anal. Biochem.*, 109 (1980) 185.
3 C. B. Heward, K. L. Kreutzveld, M. E. Hadley, B. Larsen, T. K. Sawyer and V. J. Hruby, in M. Seiji (Editor), *Pigment Cell 1981. Phenotypic Expression in Pigment Cells*, University of Tokyo Press, Tokyo, 1981, p. 339.
4 D. I. Buckley, J. Hagman and J. Ramachandran, *Endocrinology*, 109 (1981) 10.
5 C. B. Heward, Y. C. S. Yang, T. K. Sawyer, M. D. Bregman, B. B. Fuller, V. J. Hruby and M. E. Hadley, *Biochem. Biophys. Res. Commun.*, 88 (1979) 266.
6 D. T. Lambert and A. B. Lerner, submitted for publication.
7 R. Dulbecco and M. Vogt, *J. Exp. Med.*, 99 (1954) 167.
8 R. G. Ham, *Exp. Cell Res.*, 29 (1963) 515.
9 P. Cuatrecasas and M. D. Hollenberg, *Advan. Protein Chem.*, 30 (1976) 251.
10 D. T. Lambert and J. M. Varga, in M. Seiji (Editor), *Pigment Cell 1981. Phenotypic Expression in Pigment Cells*, University of Tokyo Press, Tokyo, 1981, p. 347.
11 J. M. Varga, G. Moellmann, P. Fritsch, E. Godawska and A. B. Lerner, *Proc. Nat. Acad. Sci. U.S.*, 73 (1976) 559.
12 G. Moellmann, J. M. Varga, E. Godawska, D. T. Lambert and A. B. Lerner, *J. Cell Biol.*, 72 (1978) 196a.