CARBOXYMETHYLATED (His)^2 LUTEINIZING HORMONE–RELEASING HORMONE: A NEW CONJUGABLE DERIVATIVE

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1. Introduction

Three main groups of antigens are used to induce antibodies to synthetic LH–RH* (table 1): (a) unmodified LH–RH [1–3]; (b) LH–RH conjugated to a carrier molecule [4–8]; (c) modified LH–RH conjugated to a carrier molecule [4,8–12].

An enhancement of the antigenicity of LH–RH may be expected by its coupling to an appropriate carrier molecule. The coupling may be carried out on unmodified LH-RH or on modified LH–RH containing the appropriate NH_2 or COOH functional groups.

The LH–RH may be conjugated by the bis-diazotized benzidine (BDB) reaction [4,5,7] which will modify the tyrosine and histidine residues.

The appropriate NH_2 or COOH functional groups could be provided by peptides derived from LH–RH [4,8–10], or by introducing chains containing NH_2 or COOH groups [11,12].

Obviously, all methods of conjugation, except the last two, have very little probability of succeeding and their results are, as yet, not very convincing [6,8,13].

All the antigens described up to now have given rise to cross-reactions with various peptides and metabolites of LH–RH. As the extent of the cross-reaction seems to depend on the kind of derivative of LH–RH which is conjugated to the carrier molecule [4,6,8,10,11], a new derivative of LH–RH is proposed in which the terminal groups of the decapeptide are respected.

This work describes a method for introducing one carboxyl group into the LH–RH molecule by carboxymethylation of its histidine residue.

The new compound can be conjugated to the amino groups of the carrier protein by the classical carbodiimide method [14].

2. Materials and methods

2.1. *Reaction of LH–RH with iodoacetate*

Synthetic LH–RH (40 μmol/ml) and iodoacetic acid (Merck, 800 μmol/ml) were each dissolved in 2 ml of water and adjusted to pH 9 at 40°C by the addition of 2 M NaOH.

To the iodoacetate solution, labelled [^{14}C]iodoacetic acid was added (5 μCi in 0.1 ml of water) and the pH was reajusted as necessary. Immediately afterwards, the two reagents were brought together and the reaction was carried out in the dark for 1.5 hr at 40°C under a stream of nitrogen. The pH was kept at 9 [15] by automatic addition of 0.04 M NaOH (pH stat).

After the elapsed time, the solution was freeze-dried and the dry residue was dissolved in 0.5 ml of 0.1 molar acetic acid. An aliquot of 10 μl was taken for measuring the radioactivity and the remainder

* *Abbreviations:* CM, carboxymethyl; BDB, bis-diazotized benzidine; ECDI, ethylcarbodiimide; DCDI, dicyclohexylcarbodiimide; BSA, bovine serum albumin; HSA, human serum albumin; b-Tg, bovine tyroglobulin; LH–RH, luteinizing hormone–releasing hormone.

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Table 1
Antigens used to induce antibodies to LH–RH

Form of LH–RH	Carrier molecule	Coupling agent	No. of LH–RH molecules per carrier molecule	Reference
Unmodified LH–RH	–	–	–	1, 2, 3
Unmodified LH–RH	BSA	BDB	No data	4, 5
Unmodified LH–RH	b-Tg	BDB	No data	7
Unmodified LH–RH	BSA	ECDI	No data	6, 8
3-10 LH–RH octapeptide	BSA	ECDI	No data	8
LH–RH des NH_2	BSA	Chloroformate	± 20	4
LH–RH des NH_2	HSA	–	± 10	9
LH–RH des NH_2	BSA	ECDI	No data	10
1-glutaryl-1-despyroglutamyl-LH–RH	Polylysine	DCDI	No data	11
2,5 (diazoparaphenyl acetic acid) LH–RH	BSA	ECDI	± 27	12

was put on a column (1.5 × 20 cm) filled with carboxymethyl cellulose in the H^+ form (Whatman CM32). The column was eluted first with 0.1 M acetic acid and then with an ammonium acetate gradient up to 0.4 M (see results and figure).

The elution of the column was monitored by measuring the absorption at 280 nm (LKB Uvicord) and by the radioactivity of a 20 μl aliquot (Nuclear Chicago Liquid Scintillation Counter).

The LH–RH and LH–RH derivatives were detected by ultraviolet spectrophotometry of each fraction (300–250 nm, Coleman Perkin Elmer Spectrophotometer 124 D).

A first determination of the number of carboxy-

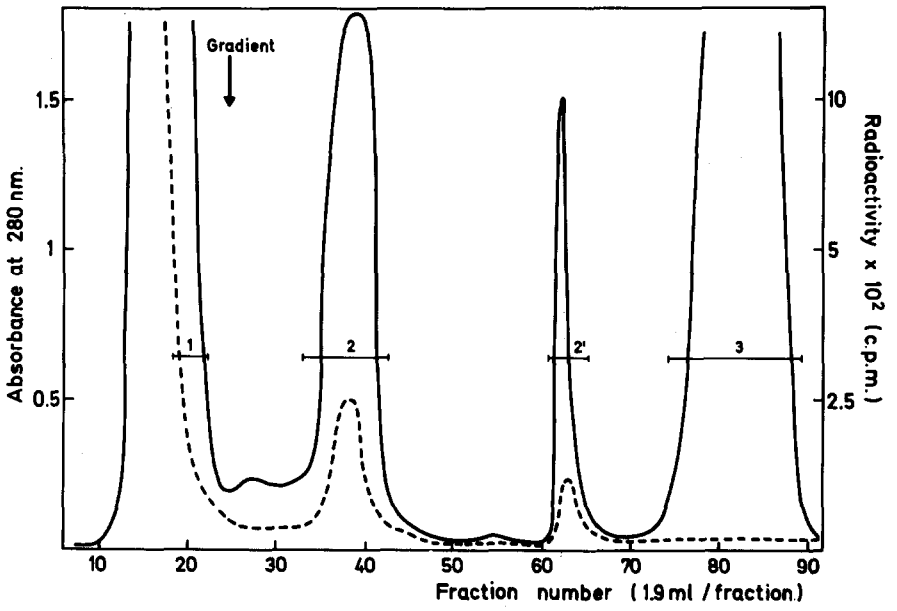

Fig. 1. Chromatography of the products of reaction of 80 μmol LH–RH with 1600 μmol iodoacetic acid in 4.1 ml of H_2O at 40°C, pH 9 (pH stat), 1.5 hr. Column was filled (1.5 × 20 cm) with CM32 (H^+ form). Elution with 0.1 M acetic acid until tube 25 where an ammonium acetate gradient is applied (see Materials and methods). Elution followed by A_{280} (——) and by the radioactivity (– – –). Peak 1 (Fr. 17–Fr. 22): mixture of 1.3 di-CM-LH–RH and iodoacetic acid. Peak 2 (Fr. 33–Fr. 43): mixture of 1 and 3 mono-CM-LH–RH. Peak 2′ (Fr. 61–Fr. 65): mixture of 1 and 3 mono-CM-LH–RH. Peak 3 (Fr. 74–Fr. 86): unmodified LH–RH.

methyl groups introduced into LH–RH, in each peak was obtained by the measurement of the radioactivity compared to the absorption at 280 nm (molar extinction of LH–RH was taken as 6737).

These results were confirmed and extended by amino acid analysis after the acid hydrolysis of the sample (6 M HCl at 110°C, in evacuated sealed tubes, 24 hr).

Carboxymethylation was also carried out on *N*-acetyl-histidine [14] in the same conditions but for 1.5, 24 and 72 hr.

The position of 1 and 3 monocarboxymethyl-his and of 1,3-dicarboxymethyl-his were reported relative to a standard mixture of amino acids [16].

3. Results and discussion

The profile of elution of chromatography on CM32 is shown in the figure. The different products of peaks 1, 2, 2′ and 3 were eluted following their decreasing acidity as expected: di-, mono-, and non-carboxymethylated-LH–RH.

Amino acid composition of the peaks is given in table 2. No other residues outside histidine are modified by the reaction of LH–RH with iodoacetate.

The number of carboxymethyl groups per molecule of LH–RH is 1 for peak 2 and 0.9 for peak 2′.

Peak 1 consisted of a mixture of 1.3-di-CM-LH–RH and iodoacetic acid. It was resolved on a longer column of CM32 (1.5 × 70 cm) eluted with 0.1 M acetic acid. The purified peptide had 2.1 carboxymethyl groups.

The total yield of carboxymethylation is around 50%. The same yield is observed when *N*-acetyl-his is carboxymethylated in the same conditions, and an unimportant increase in this yield is observed when the time of reaction is prolonged up to 3 days.

These data could indicate that the histidine in LH–RH has a normal accessibility to the reagent at pH 9 which is unaffected by either the neighbouring residues or by peptide conformation.

The identical amino-acid composition of peaks 2 and 2′ could be attributed to a 'salt effect' already observed in gel filtration. When a salt solution of pure LH–RH is eluted with 0.1 M acetic acid out of a column filled with Biogel P_2, Sephadex G-15 or G-25, two fractions are collected which have the same LH–RH amino acid composition. The degree of separation of these two peaks seems to be dependent on the gel pore size.

Until now, no satisfactory explanation of this phenomenon has been given.

Table 2
Amino acid composition of hydrolysis of fractions eluted from the chromatography on CM32 products of the reaction of LH–RH with iodoacetate (40°C, pH 9, 1.5 hr) (see fig.)

Amino acids	No. of amino-acids residues per molecule				
	Unmodified synthetic LH–RH	Peak 1 1.3 di-CM-LH–RH	Peak 2 1 and 3 mono-CM-LH–RH	Peak 2′ 1 and 3 mono-CM-LH–RH	Peak 3 LH–RH
1.3 di-CM-his	0	0.6	0	0	0
Ser	1	0.8	0.9	0.9	0.8
Glu	1	1	1	1	1
Pro	1	1	1	1	1
1-CM-his	0	0	0.3	0.3	0
Gly	2	2.1	2.3	2.1	2.1
3-CM-his	0	0	0.7	0.6	0
Leu	1	1	1	1	1
Tyr	1	1	1	1	1
His	1	0	0.05	0.1	1
Arg	1	1.4	1	1	1
Trp	1	–	–	–	–

4. Conclusions

The monocarboxymethylated LH–RH, as a new derivative for conjugation, presents two advantages.

First, it still contains the normal glycine amide and pyroglutamic terminal groups.

Second, the new compound provides a well defined antigen which can react through its unique carboxyl with the amino groups of the carrier molecule, forming a stable amide bond.

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