A NEW PROCEDURE FOR LABELLING LUTEINIZING HORMONE WITH TRITIUM

P. DE LA LLOSA†, P. MARCHE††, J. L. MORGAT†† and M. P. DE LA LLOSA-HERMIER†

† *Laboratoire des Hormones Polypeptidiques du CNRS, 91190 Gif-sur-Yvette, France*

†† *Service de Biochimie, Département de Biologie, CEN Saclay, BP 2, 91 Gif-sur-Yvette, France*

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1. Introduction

Studies of the specific binding of glycoprotein hormones to their receptors require labelled compounds of high specific radioactivity. The most frequent procedure for labelling glycoprotein hormones is iodination, but incorporation of tritium into the glycoprotein molecule by modification of the sialic acid residues has also been employed for this purpose [1,2]. We reported recently [3,4] that reductive methylation of ovine luteinizing hormone, using the procedure of Means and Feeney [5], could be achieved without loss of biological activity. We now report a procedure for the tritiation of LH* by reductive methylation of its lysine residues with tritiated $NaBH_4$. The tritiated LH derivative fully retains its biological potency when measured by the ovarian ascorbic acid depletion bioassay of Parlow [6]. Our experiments show this derivative to be of advantage for the study of the binding of this hormone to its receptors [7,8].

2. Experimental

2.1. *Materials*

Ovine luteinizing hormone, prepared as described elsewhere [9], was a generous gift of Dr Jutisz; its potency (Batch P73bd) was approximately 2.70 X NIH-LH-S11 (confidence limits for P of 0.05 = 1.81–4.14) as assayed by the Parlow test. Tritiated $NaBH_4$ (5–20 Ci/mmole) was purchased from NEN Chemicals and Biogel P-100, used for purification, was supplied by Bio Rad. All other chemicals and solvents were of analytical grade from Prolabo or Merck. Amino-acid analyses were performed in a 22-hr Technicon Autoanalyzer; a special gradient, containing propanol, was used to separate lysine, dimethylated lysine and monomethylated lysine [5]. Tritium determinations were made with a Packard Tri-Carb liquid scintillation counter. Absorbance was measured with a Beckman Spectrophotometer Acta C II. ^3H compounds were stored in liquid nitrogen. Biological potency was assayed by the ovarian ascorbic acid depletion bioassay [6]. The solutions assayed were prepared in polyallomer tubes (Beckman centrifuger tubes) because adsorption of methylated LH on this material is negligible [4].

2.2. *Preparation of tritiated methylated luteinizing hormone*

The methylation reaction was accomplished at 4°C. 0.6 mg of tritiated $NaBH_4$ (dissolved in 0.5 ml of a borate buffer pH 9, 0.2 M) was added to 4 mg of ovine LH dissolved in 0.5 ml of the same buffer. Ten µl of 10 M formaldehyde were then slowly introduced by repetitive additions, as recommended by Means and Feeney [5].

After maintaining the reaction mixture at 4°C for 1 hr, 1 ml of pyridinium acetate (pH 5, 2.5 M) was added to decompose the unreacted borohydride. Labile hydrogens were then removed by successive flash evaporation with distilled water. The volume was finally reduced to 1 ml.

* LH will be used as an abbreviation of luteinizing hormone.

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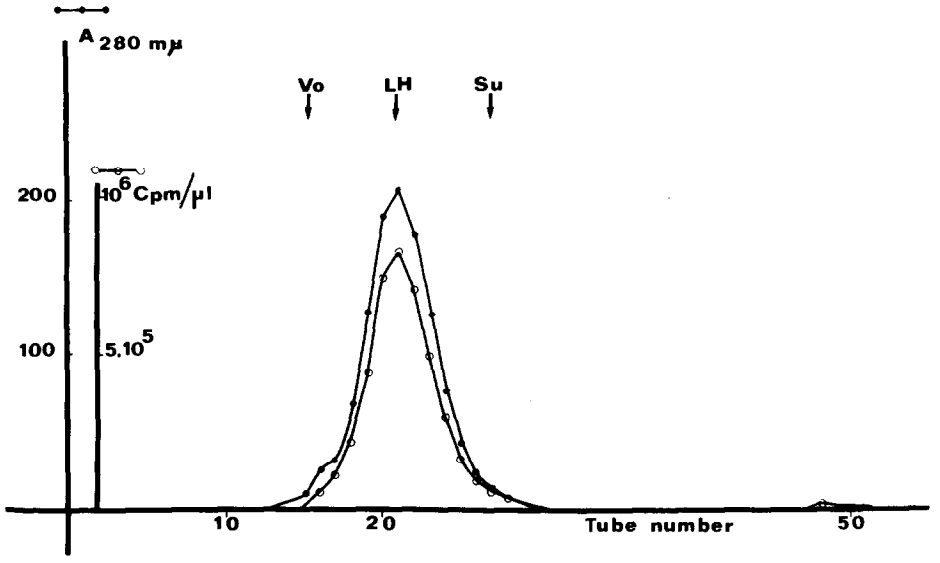

Fig. 1. Gel filtration of tritiated methylated luteinizing hormone on a Biogel P-100 column. Arrows indicate the elution volumes corresponding to the void volume (Vo), native luteinizing hormone (LH) and LH subunits (Su).

2.3. *Purification*

The tritiated material was then filtered on a Biogel P 100 column (0.9 X 125 cm) in order to obtain its purification. The column was eluted by pyridinium acetate 0.05 M pH 5 at 4°C. Fig. 1 shows a typical elution pattern. (Elution patterns obtained using absorbance measurements or tritium counting are superposable.) The same pattern was observed with all the active preparations: only one symmetrical peak is obtained at the elution volume corresponding to that of undissociated LH. Dissociated subunits or denatured material (eluted in the void volume) appear only when the specified conditions of methylation were not respected (for instance, the temperature). They are clearly separated from the native dimer by this method, as has been established in the preparation of other LH derivatives [10]. The elution peak corresponding to the pure tritiated hormone was then lyophilized and its weight determined. The yield was 78%. Labelled methylated luteinizing hormone was redissolved in a phosphate buffer 0.1 M, pH 7.4 (0.2 mg/ml) solution. This solution was stored in liquid nitrogen.

2.4. *Degree of methylation*

As reported [4], small differences in the degree of alkylation are observed after reductive methylation of luteinizing hormone, depending on the conditions of the reaction.

Table 1 shows the degree of alkylation of two preparations of tritiated methylated LH. Under the conditions described above, the methylation of 75%

Table 1
Degree of alkylation and specific radioactivity of tritiated methylated luteinizing hormone

Methylated derivative	Dimethyl Lys	Monomethyl Lys	Free Lys	Ci/mmole
Preparation 72	60	10	30	98
Preparation 73†	84	6	10	35

† The specific radioactivity of the tritiated $NaBH_4$ used in this experiment was 6.51 Ci/mmole.

Table 2
Biological activity of tritiated methylated luteinizing hormone (LH)

Period of storage at −196°C	Relative potencies in terms of native LH	Relative potencies in terms of unlabelled methylated LH†
First month	1.69 (0.83–3.4) 2.07 (0.88–4.8)	1.12 (0.54–2.3)
8th month	1.00 (0.55–1.82)	0.62 (0.34–1.10)

Relative potencies and 95% confidence limits are expressed in terms of either native luteinizing hormone or methylated luteinizing hormone.

† The relative potency of this preparation with respect to native LH was measured in three separate bioassays: March 1972: 1.43 (1.01–2.02); May 1973: 1.76 (0.65–4.8): March 1974: 1.52 (0.68–3.46).

of the lysine residues is easily achieved, but 90% of alkylation is unusual.

2.5. *Specific radioactivity*

The specific radioactivities obtained with two distinct preparations are indicated in table 1. These were determined by counting tritium radioactivity of samples whose mass was established by weight measurements as well as by absorbance measurement. The differences between the values shown in table 1 are due to the different specific radioactivities of the tritiated borohydride used.

2.6. *Biological activity*

The biological potency of the labelled hormone was measured by the ovarian ascorbic acid depletion bioassay during the first month after its preparation and 8 months later. Table 2 shows the relative potencies observed, using as standard, either unlabelled native LH or methylated non-tritiated LH containing 75% of modified lysine residues. As can be seen, no significant differences exist between the biological potency of freshly prepared [^{3}H]methylated LH and unlabelled methylated LH, but the relative potency of labelled methylated LH is about twice that of the native LH. Differences in the degree of methylation seem to have little influence, if any, on the biological activity. The labelled methylated LH reported in table 2 contains 90% of its lysine residues modified. Similar results were obtained previously with the unlabelled methylated LH [4]. However, after 8 months of storage in liquid nitrogen, the relative potency of [^{3}H]methylated LH would appear to decrease to about half of the original activity. This would suggest some radiolysis.

3. Discussion

As would be expected, methylation of luteinizing hormone with tritiated borohydride can be achieved with similar results to those observed when unlabelled $NaBH_4$ was used [4].

It is interesting to compare the specific radioactivity obtained with that expected from theoretical considerations. Reductive methylation is achieved probably by the addition of two atoms of hydrogen to the double bond of a Schiff base, one of these hydrogen atoms being exchangeable. As the α-amino group of the α-subunit of LH was probably methylated to the same extent as the ε-NH_2, it appears (table 1, preparation 73) that our results agree with the theoretical value. It may be concluded that, as claimed by Means and Feeney [5], reductive methylation is a specific reaction which affects only free amino groups. On the other hand, the amino groups of glucosamine and galactosamine do not seem to react. This would suggest that, as firstly proposed by Ward and Coffey [11], they are blocked (acetylated).

The potency of methylated derivatives is greater than that of native LH in the ovarian ascorbic acid depletion test. However, preliminary work in our

laboratory (C. Hermier, M. Evrard, P. Lebouleux, P. De La Llosa, unpublished results) indicates that the differences in potency between methylated and native LH, observed in the Parlow test, could be due to differences in clearance rates. When ovaries from pseudopregnant rats are incubated in vitro for 3 hours in the presence of LH or its derivatives [12], no significant difference is found in the stimulatory potencies of native LH, unlabelled methylated LH and labelled methylated LH.

Methylated LH derivatives offer several advantages over the iodinated LH, (i) the degree of methylation and the corresponding variation in biological activity can be controlled more easily than in the case of iodination [13], (ii) the specific radioactivity and the biological potency are stable for several months, (iii) reductive alkylation is a very gentle reaction which allows incorporation of a considerable number of tritium atoms in the LH molecule without danger of secondary reactions. Other methods proposed for the incorporation of tritium into the gonadotropin molecules involve more drastic reactions, such as oxidation of sugar residues [1,2]. Moreover, this last reaction precludes the introduction of a large number of tritium atoms in the molecule. Reductive methylation using [^{11}C]formaldehyde can also be employed to incorporate atoms of ^{11}C into the LH molecule, as will be published elsewhere. The only limitation to the generalization of this procedure for labelling hormones arises from the fact that the alteration introduced in the $-NH_2$, even though small, may have, in some cases, a considerable effect on biological activity.

Tritiated methylated LH has been used in our laboratory for the investigation of the binding of LH to bovine corpora lutea. The K_D found, using either the homogenate pellet or purified plasma membranes incubated with this derivative, are in good agreement with the results found by other authors [7,14,15]. Furthermore, by using the [^{3}H]methylated LH, evidence was obtained for the binding of ovine LH to a soluble macromolecular constituent within the bovine corpus luteum cytosol fraction [8].

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