ON THE MECHANISM OF REVERSIBLE INACTIVATION OF LUTEINIZING HORMONE BY UREA

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Treatment of luteinizing hormone (LH) by urea is known to produce its total or partial inactivation (Ellis, 1961 ; Adams-Mayne and Ward, 1964 ; Papkoff, 1965 ;Visutakul and al., 1966). According to Jirgensons (1960) this treatment does not alter the optical rotatory dispersion characteristics of LH, suggesting a low α-helical content in this protein. It seemed therefore difficult to explain the inactivation of LH by urea considering only alteration of its secondary structure. Beside its action on the secondary structure, urea is known to act on the dissociation of a polymer protein into subunits (Reithel, 1963). In this respect one should mention that Li and Starman (1964) reported, on the basis of sedimentation studies at pH 1,3, that the LH molecule is a dimer which can be dissociated at this pH.

In this note are presented some results showing that treatment of LH by urea causes a dissociation of the active dimer into inactive subunits, and that this reaction is reversible.

EXPERIMENTAL

Purification of LH. Ovine LH was purified according to a procedure which will be published elsewhere (Jutisz and Courte). The specific activity of this preparation was 2 - 2,5 x NIH-LH-S3 * as listed by Parlow's method (1961) ; it appeared to be homogeneous according to several criteria.

* Reference preparation supplied by the Endocrinology Study Section, NIH.

Treatment by urea. LH was dissolved in a freshly prepared 8 M solution of urea (3 µg/ml) and kept for 24 h at 25° C ; it was then filtered on a Sephadex G-100 column.

Gel filtration. The columns used to separate native LH, denatured LH and urea, were made from Sephadex G-100. Usually an analytical column, 0,9 x 124 cm, was used ; it was calibrated with blue-dextran, with different proteins (cytochrome c, ribonuclease, ovalbumin, bovin serum-albumin) and with NaCl. A solution of pyridine acetate 0.05 M, pH 5 was employed as eluant. A linear correlation between $K_D^{1/3}$ and the equivalent Stokes radii of these proteins was obtained (Porath, 1963 ; Siegel and Monty, 1966).

When native LH was filtered on a Sephadex G-100 column, as indicated above, it gave a K_D of 0.30 (corresponding to an apparent Stokes radius of 30 ± 1 Å). After treatment by urea , a new constituent (LH-U) appeared, separate from the first one (Fig. 2), with a K_D = 0.45 (apparent Stokes radius of 24 ± 1 Å). The ratio between the molecular size of the two forms of LH, $(30/24)^3$, can thus be estimated as approximately equal to 2.

Transformation of native LH into LH-U is a function of several factors : concentrations of LH and urea, time and temperature. More details on this reaction will be given elsewhere. In the conditions mentioned above, this transformation of native LH to LH-U was complete in 24 h ; it was of about 50 % after 90 min of treatment. On the other hand, it was verified that the treatment of LH by urea was not accompanied by a modification of the UV-spectrum between 250 and 350 mµ.

Sedimentation studies. Ultracentrifugation experiments were performed in a Spinco Model E analytical ultracentrifuge *. The fraction corresponding to the denatured LH, resulting from the filtration on the Sephadex column, was concentrated by ultrafiltration in a collodion bag and equilibrated against sodium acetate buffer, pH 5 (0.1 N with respect to Na^+). The final concentration of protein was

* All sedimentation studies were done by the Station centrale d'ultracentrifugation du C.N.R.S. (Paris). We acknowledge this help.

10 mg/ml in one case and 15 mg/ml in the other. These solutions were kept at 4° C for 24 h before ultracentrifugation at 10° C. With the first (10 mg/ml) solution, a 12 mm type, synthetic-boundary cell was used, with a rotor speed of 59,780 rpm. With the second (15 mg/ml) solution, a single-sector 12 mm cell was employed at a speed of 67,770 rpm. Another experiment was done in the same conditions, after diluting the last solution 3 times.

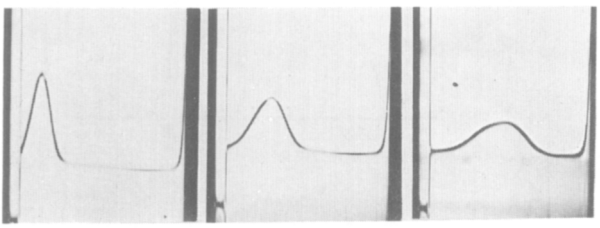

Fig. 1. Sedimentation pattern of LH dissociated by urea and partially reassociated at 56, 120 and 200 min.

Sedimentation patterns obtained with the 15 mg/ml solution are shown in Fig. 1. The boundary peak, apparently symmetrical after 56 min and 120 min, becomes quite asymmetrical after 200 min of centrifugation. The $S_{20,w}$ values established at different protein concentrations were, respectively, 15 mg/ml : 1.98 S, 5 mg/ml : 1.92 S, 10 mg/ml (synthetic boundary cell) : 1.87 S.

An aliquot of the 15 mg/ml protein solution was recovered after centrifugation and submitted to filtration on the Sephadex column. The pattern of this filtration obtained by O.D. measurements at 275 mμ is given in Fig. 2. Thus the heterogeneity of this sample, observed by ultracentrifugation, is confirmed. Two components were separated, the first one, A, which behaves on Sephadex as native LH represents 45 % of the total amount, the second one, B, behaving as LH-U, represents 55 %.

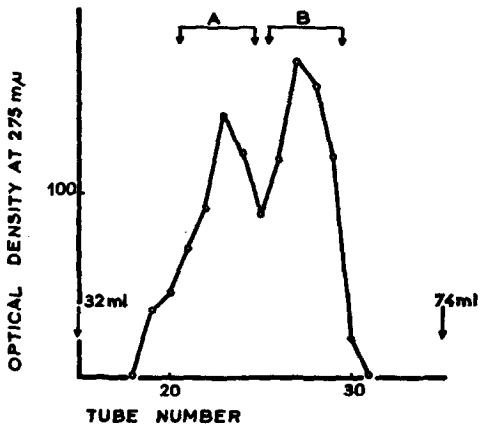

Fig. 2. Filtration pattern on a Sephadex G-100 column of LH dissociated by urea and partially reassociated. Fractions of 2.1 ml.

We have found for native LH in the above mentioned conditions $S_{20,w}$ = 2.36 S. Assuming for the sample examined by ultracentrifugation a mean value for $S_{20,w}$ = 1,9 S and taking into account the proportion (α) of components A and B in the mixture, the sedimentation constant of LH-U could be estimated as approximately equal to 1.5 S if the following formula is used : $s = (1-\alpha)s_{LH} + \alpha s_{LH-U}$ (Kawahara and al., 1965).

Assuming as a rough approximation the relation :

$$\left[\frac{S_{LH}}{S_{LH-U}}\right]^{3/2} \simeq \frac{M_{LH}}{M_{LH-U}} ,$$

the molecular weight (M) of LH-U should be half of that of LH.

Biological activity. Table I gives the biological activity of native LH, of LH treated by urea and freshly isolated by gel filtration, and of the two components A and B (Comp.A, Comp.B) recovered by gel filtration from the sample submitted to ultracentrifugation. Assays were done by Parlow's (1961) method and the results expressed in terms of NIH-LH-S3.

T A B L E I

BIOLOGICAL ACTIVITY OF DIFFERENT FORMS OF LH
(IN TERMS OF NIH-LH-S3)

Sample	R.P.* & 95 % Confidence limits
LH(native)	1.83 (1.02 - 3.26)
LH-U	0.17 (0.10 - 0.29)
Comp. A	1.50 (0.94 - 2.38)
Comp. B	0.16 (0.09 - 0.28)

* relative potency

DISCUSSION

Treatment of native LH by urea produces a modification of its molecular weight and size, suggesting an alteration of the quaternary structure of this protein. The reversibility of the dissociation of this polymer makes the precise determination of the physico-chemical constants of its subunits difficult. The results obtained suggest however that in the molecules produced by treatment by urea the molecularweight and size correspond to half those in the original molecule. The behaviour of the subunits obtained during gel filtration seems to indicate that they have similar or identical molecular size. Nevertheless, for the time being, no data are available on the primary structure of the two moieties of LH polymer. It was shown that after elimination of the denaturing agent it was possible in certain conditions to reconstitute the polymer which then behaved during gel filtration as did the original molecule.

As can be seen from Table I there is a close correlation between the quaternary structure and the biological activity of the LH molecule. Dissociation of the dimer is accompanied by a drastic loss of biological activity which can be almost entirely restored when the dimer is reconstituted.

Reconstitution of the LH polymer is dependant on the monomer concentration : for low concentrations, 0.1 mg/ml and less, the rate of reassociation is very low (at 4° C it is negligeable after 2 months). For concentrations of 10-20 mg/ml reassociation reaches 50 % in 24 h. After that, it proceeds very slowly without becoming complete (In 3 months at 4°C in

a 10 mg/ml solution the reassociation reaches about 65 % as judged by gel filtration).

Preliminary investigations showed that other denaturing agents, such as guanidine, as well as treatments at a pH values below 4 and above 12 caused dissociation of the LH polymer, apparently in the same way as urea.

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