BIOSYNTHESIS OF A DISCRETE GLYCOPROTEIN: BOVINE LUTEINIZING HORMONE*

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Previous reports from this laboratory have dealt with the biosynthesis of growth hormone and prolactin in tissue slices,\(^1\) and of ACTH in both slices\(^2\) and a cell-free ribosomal system,\(^3\) derived from bovine anterior pituitary tissue. The present paper represents an extension of our research to luteinizing hormone (LH), a protein containing diverse carbohydrate components.\(^4\) The only studies to date on the in vitro biosynthesis of LH appear to be those of Wakabayashi and Tamaoki,\(^5\) and Samli and Geschwind.\(^6\) In both these investigations LH antiserum was used to precipitate the labeled hormone, following incubation of pituitary glands of rats (or other species) in medium containing isotopic leucine or glucosamine.

Apart from its intrinsic physiological importance, LH was chosen by us as a good model system for the study of glycoprotein synthesis, in view of increasing interest in the mechanisms of biogenesis of these complex proteins. Among the aspects currently receiving attention in several laboratories are the determination of the intracellular site of glycoprotein formation, the time sequence of carbohydrate incorporation as compared to polypeptide chain formation, and the elucidation of the enzymic pathways involved. Thus labeled glucosamine and UDP-galactose were found to incorporate into endogenous glycoprotein of liver microsomes\(^7,8\) and ascites carcinoma cell membranes\(^9\) in the presence of cell-free enzyme systems. Evidence that glycosylation of proteins can occur in the absence of protein synthesis was provided by studies such as those of Grebner et al.\(^10\) with mouse mastocytoma preparations, and Eylar and Cook\(^9\) with the above-mentioned ascites system. These last investigators proposed an intracellular mechanism by which carbohydrates, assembled by several enzymes, migrate to membrane regions bearing ribosomes, where they unite with completed polypeptide chains.

While the present communication is not designed to answer such questions in depth, it does provide a foundation for further mechanistic studies on a unique glycoprotein species. The small-scale adaptation of available preparative methods for ovine and bovine hormones has made it possible to isolate radioactive bovine LH in a high state of purity, following incubation of tissue slices in the presence of labeled amino acids and sugars, and to determine the influence of certain factors on the utilization of these two classes of LH constituents.

Materials and Methods.—(1) Hormone standards: samples of bovine LH were provided by the Hormone Research Laboratory, University of California, and by the Endocrine Study Section, National Institutes of Health. (2) Radioactive compounds: 4,5-\(\text{H}^2\)-L-leucine and 3,4-\(\text{H}^2\)-L-proline (both 5 c/mmole), and 2,3-\(\text{H}^3\)-DL-tryptophan and 6-\(\text{H}^3\)-D-glucosamine (both 0.2 c/mmole) were purchased from New England Nuclear Corp. 1-C\(^14\)-D-galactose (0.035 c/mmole) was obtained from Chicago-Nuclear Corp.

Preparation of antiserum: Rabbit antiserum to bovine LH (NIH-LH-B5) was prepared by a procedure which included removal of nonspecific antibodies by adsorption with normal bovine serum.\(^5\)
Incorporation experiments with anterior pituitary slices: The conditions were similar to those previously described.1 Eight tissue slices from about 1 gm of bovine anterior gland were usually incubated for 8 hr in 10 ml of Krebs-Ringer bicarbonate buffer containing 5 µc of a labeled amino acid or sugar.

Isolation of partially purified labeled LH: The procedure of Papkoff et al. for ovine hormone11 was scaled down as much as 1000-fold, and a number of minor modifications were introduced: following incubation of the slices, the medium was decanted from each flask, and the tissue was washed twice with 4 ml of Krebs buffer. Subsequent operations were performed at 0–4°. The slices were minced and homogenized (Teflon-glass homogenizer) with 10 ml of 0.15 M (NH₄)₂SO₄. The homogenate was adjusted to pH 4.0 with 1N HCl, and stirred for 1 hr. It was then centrifuged and the supernatant was decanted. The pellet was re-extracted with 0.15 M (NH₄)₂SO₄, pH 4.0, for 2 hr, followed by re-sedimentation. The two extracts were combined and adjusted to pH 3.0 by addition of 2 M metaphosphoric acid. The resulting pigmented precipitate was sedimented and discarded. The supernatant solution was brought to pH 6.7 by adding 2 N NaOH. Solid (NH₄)₂SO₄ was next added to half saturation (310 mg/ml). The precipitated crude LH was collected by centrifugation, and then resuspended in 1 ml of 0.2 M K₂HPO₄. The suspension was heated for 2½ min in a water bath at 58–60°, chilled in ice water, transferred to a cellophane dialysis bag, and dialyzed overnight against 0.01 M Na₂HPO₄.

Partial purification (at room temperature) was achieved by adsorption of the crude LH on a 1 × 6-cm column of SE-Sephadex C-50. Upon passage of about 15 ml of 0.01 M Na₂HPO₄ through the column, a large proportion of inactive protein was removed. The LH component was then eluted by changing to 0.1 M Na₂HPO₄. The individual effluent fractions of the LH peak were pooled, dialyzed overnight against water, and the dialysate was lyophilized. The radioactivity of the residue was measured in a liquid scintillation spectrometer, as previously described.12

Final purification of labeled LH by Sephadex gel filtration: The method of Reichert and Jiang13 was applied to the partially purified LH from the above SE-Sephadex C-50 chromatography stage. However, operations were conducted on the pooled hormone derived from five tissue slice experiments. A 1.2 × 50-cm column was employed, and two components were obtained: a peak very low in LH activity; closely followed by a second component derived from very high LH potency. Aliquots of the individual fractions collected were taken for radioactivity and optical density measurements.

Biologic assay of LH: The biological activity of the two fractions obtained by Sephadex G-100 gel filtration was determined by the rat ovarian ascorbic acid-depletion method. Animals were primed with 75 IU of PMS and 50 IU of HCG, and used 6 days later. The standard was NIH-LH-B3 (bovine), which had a potency of 1.02 × NIH-LH-S1. The first component was tested at 10 and 50 µg against two levels of the NIH standard, while the second fraction was tested at 0.1- and 0.5-µg doses. These assays were performed by Dr. Donald C. Johnson of the University of Kansas Medical Center.

Agar gel diffusion analysis: The Ouchterlony double-diffusion method was used for immunological characterization of the purified LH isolated from pituitary slices, and also to detect trace contamination by serum proteins. NIH-LH-B5 and highly purified Li-Papkoff LH were used as standards.

Results.—The crude labeled LH preparations derived from tissue slice incubation experiments were first fractionated on SE-Sephadex C-50 into: (a) a major unabsorbed component, which was found to be virtually devoid of LH activity; and (b) a much smaller adsorbed and eluted peak, which corresponded to authentic LH. Figure 1 shows the distribution of radioactivity between these two fractions in experiments with isotopic leucine and galactose. Very similar results were obtained when radioactive proline and glucosamine were employed (data not given). The time course of incorporation of two radioactive amino acids and
a sugar into partially purified LH is shown in Figure 2. It may be seen that the three curves had rather similar shapes.

The type of fractionation achieved in the further purification of LH by Sephadex G-100 gel filtration is illustrated in Figure 3. It was important to compare the biological activity of the two components. The four-point assay by the rat ovarian ascorbic acid-depletion method gave values of 0.0317 U/mg and 2.76 U/mg for the first and second Sephadex fractions, respectively. In both cases, the dose-response lines were perfectly parallel. The biological activity of the second peak compares favorably with values reported by other investigators for the highly purified hormone: bovine LH, 2.41 U/mg; ovine LH, approximately 2 U/mg. 11

Immunoassay of purified LH by the Ouchterlony agar gel method is shown in
Figure 4. It may be seen that the H³-leucine-labeled hormone gave a single sharp precipitin line, confluent with those formed by the Li-Papkoff and NIH standards, against antiserum. A faint cross-reaction against antibovine serum was observed with both the radioactive LH and the NIH standard, but not with Li-Papkoff LH. Similar results were obtained with LH preparations derived from other tissue slice incubations with labeled proline, galactose, and glucosamine.

The distribution of radioactivity observed between the two Sephadex G-100 components in several experiments is summarized in Table 1. With H³-leucine, about 40 per cent of the isotope was recovered in the second peak. In view of the very low tryptophan content reported for ovine LH,¹¹ it is significant that the H³-amino acid gave rise to labeling almost exclusively in the first peak. The small radioactivity in the LH region is considered negligible. With both labeled galactose and glucosamine, the greater part of the radioactivity was found in the LH region.

Figure 5 shows the refiltration on Sephadex G-100 gel of a part of the LH from the C¹⁴-galactose experiment of Table 1. A relatively large proportion of highly purified LH carrier had been mixed initially with the isotopic preparation.

### Table 1. Resolution of labeled LH from inactive protein component by Sephadex G-100 gel filtration.

<table>
<thead>
<tr>
<th>Labeled compound employed</th>
<th>Radioactivity Recovered (cpm)</th>
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<tbody>
<tr>
<td></td>
<td>In first component</td>
</tr>
<tr>
<td>H³-leucine</td>
<td>5,100</td>
</tr>
<tr>
<td>H³-tryptophan</td>
<td>950</td>
</tr>
<tr>
<td>H³-galactose</td>
<td>1,130</td>
</tr>
<tr>
<td>H³-glucosamine</td>
<td>290</td>
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The fractions corresponding to the central portion of each peak were retained as in Fig. 4. The yield of LH (chiefly endogenous hormone) was approximately 0.4 mg/gm of original anterior tissue.
optical density and radioactivity readings were both confined almost exclusively within a single peak. It could be calculated that most of the ultraviolet absorbance was contributed by the LH standard.

The effect of a classical inhibitor of protein synthesis on the incorporation of labeled amino acids and sugars into LH is shown in Table 2. It may be seen that puromycin inhibited the utilization of leucine and proline by approximately 80 per cent. The incorporation of radioactive glucosamine and galactose into LH was inhibited to a lesser extent (about 30%) by puromycin.

Table 2. Effect of puromycin on the incorporation of labeled amino acids and sugars into purified LH.

<table>
<thead>
<tr>
<th>Labeled compound</th>
<th>Inhibition of incorporation (%)</th>
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<tr>
<td>H3-leucine</td>
<td>80</td>
</tr>
<tr>
<td>H3-proline</td>
<td>78</td>
</tr>
<tr>
<td>H3-glucosamine</td>
<td>30</td>
</tr>
<tr>
<td>C14-galactose</td>
<td>27</td>
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The concentration of antibiotic was 0.15 mg/ml of medium. The results are expressed relative to control experiments performed in the absence of puromycin.

Discussion.—The results obtained with different isotopic compounds in the present experiments cannot be compared quantitatively because of several variables, including different specific radioactivities, possible loss of hormones from slices during incubation,\(^\text{13}\) different degrees of abundance of the amino acids and carbohydrates in the glycoprotein hormone, and variations in the activity of slices prepared at different times. Also, corrections have not been made for the different counting efficiencies of H\(^3\) and C\(^14\) in the liquid scintillation spectrometer. One factor which was explored was the ease of penetration of the labeled amino acids and sugars into the tissue slices. In each case studied, it was found that 40–50 per cent of the radioactivity was removed from the medium in about ten hours (data not shown). A similar result was obtained by Gan et al.\(^\text{14}\) with thyroid slices and C\(^14\)-D-glucosamine.

Despite the several uncertainties and variables mentioned above, the radioactivity recovered in purified LH was found to represent approximately 0.05–0.1 per cent of the total isotope employed in the various experiments. While these values are small, they seem reasonable in relation to the low concentration of LH in pituitary tissue and the probable complexity of the biosynthetic process involved. We have observed somewhat higher efficiencies of labeled amino acid utilization (about 0.2–1%) in comparable studies on ACTH, growth hormone, and prolactin, which are simpler in structure and relatively more abundant in anterior tissue.

In studies based on isotopic incorporation experiments, it is important to demonstrate that the labeling process provides a valid measure of the synthesis of the protein in question. Several criteria have been employed for this purpose in the present paper: (1) the radioactive product of the biosynthetic process was isolated by a well-established chemical procedure; (2) during purification, this preparation exhibited the same chromatographic and gel filtration properties as the authentic hormone; (3) the final isolation step was shown to yield a protein
with very high biological activity; (4) in Ouchterlony double-diffusion analysis, radioactive LH preparations from the slice incubations showed precipitin lines confluent with LH standards, indicating immunological identity; (5) an amino acid (tryptophan) which does not occur to a significant extent in the hormone molecule, did not give rise to appreciable labeling in purified LH when employed in radioactive form. While the molecular weight and amino acid composition of the purified radioactive LH preparations have not yet been determined, their biological potency relative to the best available hormone standards makes it appear unlikely that our biosynthetic products were seriously contaminated with impurities.

The limitations which the tissue slice technique impose on studies of reaction mechanisms are well recognized. However, it has been possible to draw some helpful inferences from the present experiments. Although the labeled carbohydrate substrates were not employed in nucleotide form, it seems likely from other studies on glycoprotein synthesis that they were converted into uridine diphosphate derivatives (UDP derivatives) within the pituitary cells, prior to utilization. The absence of an initial lag phase in the rate curve for incorporation of labeled carbohydrate into LH suggests that an endogenous protein acceptor was available. This view is in agreement with the opinions of several investigators \(^8\) that such incorporation occurs at a late stage in glycoprotein synthesis, after completion of the polypeptide chain.

The experiments with puromycin provide only limited support for the above mechanism, inasmuch as the antibiotic drug had some inhibitory effect on the incorporation of two labeled carbohydrates into LH, even though it exerted a stronger action in the case of isotopic amino acids. It should be pointed out in this connection that different laboratories have reported a range of results with puromycin in glycoprotein synthesis. This situation is not surprising in view of the variety of biological systems and experimental methods employed. To cite examples, puromycin exerted no appreciable effect on glucosamine incorporation into (mixed) glycoproteins with a preparation from Ehrlich ascites cells, while amino acid utilization was strongly depressed.\(^8\) On the other hand, the same drug caused varying degrees of inhibition of carbohydrate incorporation into thyroglobulin with either slice or particulate preparations of thyroid, under conditions which completely blocked leucine utilization.\(^16\)

More definitive experiments require cell-free biosynthetic systems. Recently in our laboratory a pituitary polysome preparation was shown to be active in the biosynthesis of ACTH, growth hormone, and prolactin.\(^17\) The availability of this system, coupled with newer information on hormone chemistry and structure, should facilitate basic studies on the mechanisms of formation of LH and the other unique glycoprotein hormones of the adenohypophysis.

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