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Isolation and Properties of Subunits of Rat Pituitary Luteinizing Hormone*

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ABSTRACT: The isolation of subunits from rat pituitary luteinizing hormone (LH) is described. The subunits separate in the countercurrent distribution system: 40% (w/v) ammonium sulfate–0.2% dichloroacetic acid–1-propanol–ethanol (60:60:27:33). Two dissimilar subunits are obtained, that with a partition coefficient 0.1 is designated C-1, that with a partition coefficient of 10 is designated C-2. Similar subunits have previously been described for bovine and ovine luteinizing hormone. The C-1 subunit is characterized by a molar ratio 3:1 lysine:arginine, while C-2 has 9:1 arginine:lysine ratio. Although these ratios are not identical with those previously reported for LH subunits from other species they are similar. A higher content of tyrosine in C-1 than in C-2 also appears common, although the starting LH contained relatively little tyrosine compared to other proteins. The C-2 subunit was characterized by a very high proline content (the most abundant amino acid in this subunit and the starting LH) and a relatively high leucine content. Both subunits are glycopeptides containing glucosamine, galactosamine, mannose, galactose, and fucose; and each have a relatively high cystine content. Gel filtration studies and data on the Stokes radii are consistent with a molecular weight of approximately 15,500 for each subunit and 31,000 for the starting rat LH. The relative potency of the isolated rat LH was 1.55–1.72 units/mg measured by either the ovarian ascorbic acid depletion (OAAD) bioassay or radioimmunoassay (RIA), comparable to the potency of preparations from other species. The isolated subunits had 0.08 unit/mg by OAAD, but the RIA showed 0.07 unit/mg for C-1 and 1.1 units/mg for C-2. Thus a significant RIA reaction was retained for the C-2 subunit. Recombination of the two subunits in pH 7.0 buffer produced a 7-fold augmentation of the OAAD activity, which was essentially equipotent to the RIA response, both being about 35% of the starting LH potency.

Isolation of rat pituitary luteinizing hormone has been reported by three laboratories (Reichert and Midgley, 1968a; Ward *et al.* 1968; Fontaine and Burzawa-Gerard, 1968). Monroe *et al.* (1968) utilized purified rat LH[1] in the development of a radioimmunoassay for that hormone, but did not provide details of the preparative procedure. Ward *et al.* (1968) noted rather poor recoveries of rat LH and suggested this may be due to an unusually facile subunit dissociation of the hormone during its purification, although at that time subunits had not been demonstrated.

In this report, we wish to describe isolation of subunits of

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[1] Abbreviations used are: LH, luteinizing hormone; TSH, thyroid-stimulating hormone; RIA, radioimmunoassay; OAAD, ovarian ascorbic acid depletion. Luteinizing hormone subunit nomenclature has varied according to the laboratory involved. We originally designated an ovine S subunit and an A subunit (Ward *et al.*, 1966), shortly thereafter Papkoff and Samy (1967) designated C-I and C-II. In our studies on bovine LH (Reichert *et al.*, 1969) we designated C-1 and C-2. There is growing evidence that the subunits designated S-, C-I, or C-1 all have common properties which appear to relate to other glycopeptide hormones (*e.g.*, Liao and Pierce, 1970); the A subunit, C-II, or C-2 appears to possess a more specific character. In view of this J. G. Pierce and H. Papkoff (personal communication) have proposed a more uniform nomenclature. If uniformly adopted by those working in the field the C-1 subunit of the present study would become murine LH-α, and the C-2, murine LH-β.

rat LH and to present information on their molecular, biological, and immunological properties and on their chemical composition.

Materials and Methods

The rat luteinizing hormone was prepared as described by Ward *et al.* (1968), but with the additional precaution suggested in the discussion of that reference, namely, that highly substituted microgranular carboxymethylcellulose preparations be avoided. The more common carboxymethylcellulose (here Serva CM, 0.68 mequiv/g, Gallard-Schlesinger Co.) was used for the ion-exchange chromatography in the present studies. Subunits were isolated by the countercurrent distribution procedure of Papkoff and Samy (1967), but with the additional modifications employed by Reichert *et al.* (1969). Recombination studies of the isolated subunits were as described previously for bovine luteinizing hormone subunits (Reichert *et al.*, 1969). The ovine luteinizing hormone was prepared as described earlier (Ward *et al.*, 1967a).

Luteinizing hormone activity was determined by the ovarian ascorbic acid depletion assay (Reichert and Parlow, 1963) with results expressed in terms of NIH-LH-S1.

Immunoassay of rat LH and its subunits was done using the radioimmunoassay (RIA) procedure described by Niswender *et al.* (1968). Briefly, this RIA utilizes an antiserum to ovine LH with radioiodinated ovine LH as the label and rat LH as the reference standard. The purified ovine LH used in this system was prepared as described by Reichert and Parlow (1963) and had a specific activity of 1.73 units/mg. The rat LH reference standard (LER-1240) was prepared as reported by Reichert and Midgley (1968a) and had a specific activity of 0.59 NIH-LH-S1 unit/mg. The antiserum and varying amounts of rat LH are incubated for 24 hr, followed by addition of radioiodinated LH. After a further 24-hr incubation, an antiserum to rabbit γ-globulin was added to precipitate the antibody-bound radioiodinated hormone. After 72 hr, the samples were centrifuged, the supernatants decanted, and the radioactivity of the precipitate measured in a Packard Autogamma scintillation spectrometer.

Each preparation was tested in duplicate at each of four to six dose levels in the RIA. A negative sigmoidal relationship was observed between log of the dose of unlabeled hormone or subunits and the percentage of [^{131}I]LH bound to antibody. Transformation to a linear relationship was accomplished by converting, into logits, the response in the region of 20–80% of the counts per minute bound to antibody (Rodbard *et al.*, 1968).

Statistics for linearity, homoscedasticity of variance, precision and relative potency estimates of the bioassay and RIA were calculated by the parallel line assay method of Finney (1964) using a RCA 70–55 computer and a program developed at the Emory University Computer Center.

Acrylamide gel electrophoresis was done at pH 9.5 or 8.25, in Tris-glycine buffer (0.04 M). Samples were run at 4 mA/tube for 3 hr, on 7.5% gels. Staining was with Buffalo Black or Amido Schwarz dye; destained by electrophoresis, 2 mA/gel, run perpendicular to the long axis. Other details of the gel electrophoresis were essentially as described by Liao *et al.* (1969) for bovine thyrotropin.

Sufficient material was not available to permit estimation of sedimentation coefficients, so that molecular weight determinations based on this approach were not possible. The difficulties in assigning molecular weights to glycoproteins in general and luteinizing hormone in particular when using the technique of gel filtration through columns calibrated with proteins of known molecular weight (Whitaker, 1963; Andrews, 1964) have been discussed elsewhere (Ward and Arnott, 1965; Reichert and Jiang, 1965). Although the gel filtration data was obtained during the purification studies the molecular weight estimates on this basis were expectedly high. Rather the Stokes radii and diffusion coefficients of rat LH and rat LH subunits were determined by gel filtration of ^{131}I-labeled preparations using procedures described in detail in earlier studies with bovine LH and bovine LH subunits (Reichert *et al.*, 1969) and with human LH (Ryan, 1969). The labeling procedure employed here was essentially that described by Niswender *et al.* (1968). Approximately 2.5 μg of LH or subunit in 5–10 μl of water was mixed with 20 μl of 0.5 M sodium phosphate buffer (pH 7.5) and reacted for 2 min with 1 mCi of carrier-free [^{131}I]NaI (Cambridge Nuclear Corp.) and 30 μg of chloramine-T in 20 μl of 0.05 M sodium phosphate buffer (pH 7.5). The reaction was stopped by adding 100 μg of sodium metabisulfite in 50 μl of 0.05 M phosphate buffer; 170 μl of a solution containing 1 g of potassium iodide, and 16 g of sucrose per 100 ml of water was then added to the reaction vial. This was then diluted with 0.05 M phosphate buffer (pH 7.5) to give a final solution having 1×10^6 cpm/ml. The labeled hormone was then filtered through a 2.5×100 cm column of Sephadex G-100 (Pharmacia) in the cold, and the tube in the elution profile having the highest count was used for calculation of average distribution coefficient. From this the Stokes radius and diffusion coefficient (*vide infra*) were calculated.

Amino acid analyses were done with a Beckman Spinco Model 120C amino acid analyzer equipped with an expanded-range card and high-sensitivity cuvet. All analytical samples were weighed on a Cahn electrobalance and dried *in vacuo* to constant weight during weighing. To check the accuracy of all subsequent aliquot handling, internal standards of norleucine were introduced at the time of hydrolysis. Samples were hydrolyzed *in vacuo* with 6 N HCl for 24 or 72 hr at 110°. Extrapolations for zero-time values of amino acids which decompose during hydrolysis were as described by Hirs *et al.* (1954). Valine, isoleucine, and leucine "extrapolated" values are the maximum yield from the 72-hr hydrolysis, since dipeptides incorporating these amino acids are known to be resistant to hydrolysis. Half-cystine values were also confirmed with a cysteic acid analysis of a performic acid oxidized sample.

Hexosamines were determined according to Walborg *et al.* (1963) and neutral sugars by the method of Lamkin *et al.* (1966). Tryptophan was assayed by the method of Bencze and Schmid (1957). Amino-terminal groups were estimated by the dansyl procedure (Gray, 1967; Woods and Wang, 1967). Carboxyl-terminal amino acids were estimated by catalytic hydrazinolysis and carboxypeptidase digestion as described for other luteinizing hormone preparations (Ward *et al.*, 1967b).

Analytical values have been calculated on the basis of a 31,000 molecular weight for intact rat luteinizing hormone and 15,500 for each of the subunits. The former value is assumed by analogy to LH preparations from other species, all of which have closely similar molecular weights of about 31,000 (Ward *et al.*, 1968; Reichert and Jiang, 1965), and the latter, by analogy to studies with subunits of bovine (Reichert *et al.*, 1969), ovine (Papkoff and Samy, 1967), and human (Reichert and Midgley, 1968b) LH, all of which seem to have molecular weights approximately one-half that of the intact molecule.

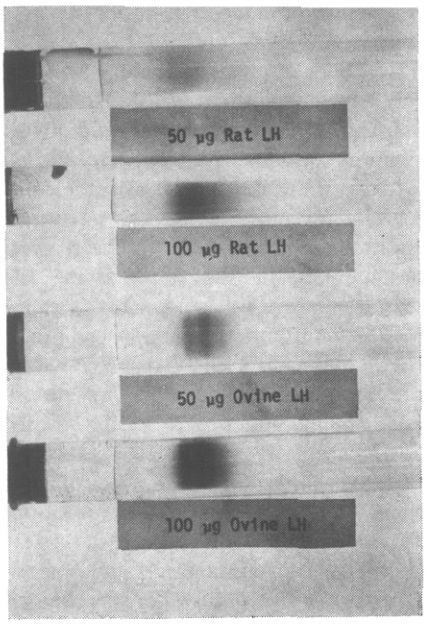

FIGURE 1: Disc electrophoresis of rat LH compared to ovine LH. Running pH 9.5; 7.5% gels; 4 mA/tube per 3 hr. The origin was to the left, at the end of the label; migration was toward the anode on the right.

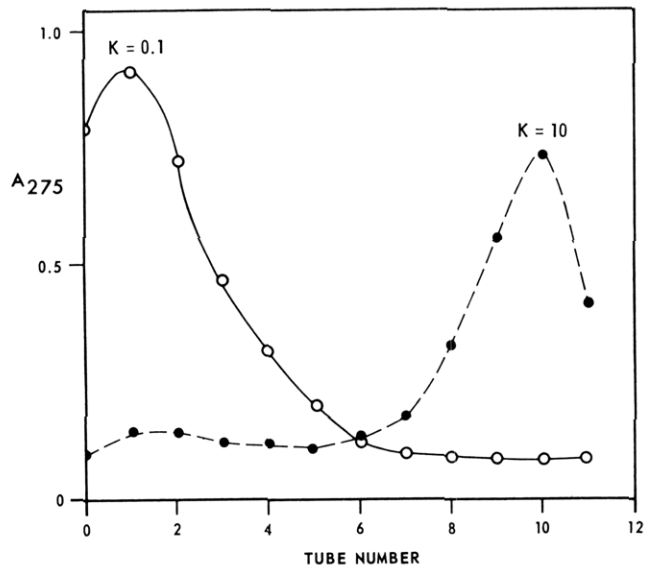

FIGURE 2: Countercurrent distribution pattern obtained with 15 mg of rat LH in the solvent system described by Papkoff and Samy (1967), 1 ml/phase, 11 transfers, room temperature. For the ultraviolet absorption meaurements 0.7-ml aliquots of each phase were taken and 0.1 ml of water added. Open circles = lower phase; solid circles, dotted line = upper phase. Recovery of material and bioassay data are presented in Tables I and II. The material on the left side (tubes 0–4) was designated C-1, that on the right C-2 (tubes 7–11).

Results

The fractionation data for the fresh-frozen, lyophilized rat pituitaries is summarized in Table I. This tabulation utilizes the same designation as in our previous report (Ward *et al.*, 1968) which may be consulted for further details on the fractionation.

Disc electrophoresis of the rat luteinizing hormone and subunits, in several experiments, produced very faint bands after electrophoresis and staining with Amido Schwarz or Buffalo Black. Bands were detectable at the following positions measured in mm from the sample origin: rat luteinizing hormone, 20 mm (2 mm width); subunit C-1, 20 mm (1 mm width); subunit C-2, 23 mm (0.5 mm width). Distances represent migration toward the anode. In several runs, staining was too poor to photograph. The reversed-current tubes showed no detectable bands. In view of the very poor staining of this material (similar difficulty had been observed with several earlier preparations of rat LH), it was decided to compare rat and ovine LH under identical conditions. This comparison is illustrated in Figure 1. It is seen that the 50-µg sample of ovine LH stained better than 100 µg of rat LH, while the 50-µg pattern for rat LH was faintly discernable. The extent of migration was comparable for both rat and ovine LH, but the pattern was more diffuse for rat LH. Both preparations gave two heavier staining bands superimposed on a broad, diffuse, very lightly stained band. This type of pattern has been seen repeatedly, although the double-band character may not be apparent for the heavier staining portion in all runs. Perhaps the gel electrophoresis pattern for luteinizing hormone represents a complex combination of LH and its subunits. Although the thyrotropin content of this rat LH preparation may be as high as 2.5% (*vide infra*) this apparently contributes little to the patterns observed, since the contamination of the ovine LH by thyrotropin is an order of magnitude less, but the character of the patterns is the same.

The pattern obtained when the rat luteinizing hormone was submitted to countercurrent distribution is shown in Figure 2. The material with a partition coefficient of 0.1 was designated subunit C-1, while that with a partition coefficient of 10 was designated subunit C-2. This nomenclature is consistent with that employed with bovine luteinizing hormone (Reichert *et al.*, 1969), but drops the earlier notation of A and S subunits (chains) used with ovine luteinizing hormone (Ward *et al.*, 1966). The reasons for this will become apparent in the discussion of the COOH-terminal groups. Subunits were recovered from the solvent by salt removal through a UM-05 Diaflo membrane (Amicon Co.).

Biologic and immunologic analysis of the rat luteinizing hormone and its subunits are presented in Table II and Figure 3. Both subunits possessed a potency approximately $^1/_{20}$ that

TABLE I: Summary of Fractionation for Rat LH and Rat LH Subunits.

Fraction[a]	Yield (mg)
Starting material (lyophilized pituitaries)	8100
Gonadotropin fraction ($G_1 + G_2$)	232
Carboxymethylcellulose (Serva-CM) chromatography, LH fraction (D)	57.9
Purified rat LH from Sephadex G-100 chromatography (RLH)	32.6[b]
15 mg of RLH subjected to countercurrent distribution (Figure 2)	
Subunit C-1	6.4[b]
Subunit C-2	6.3[b]

[a] Letter designations correspond to fractions previously described in detailed fractionation scheme (Ward *et al.*, 1968). [b] Bioassay data and immunoassay data in Table II.

TABLE II: Biologic and Immunologic Assay of Rat Luteinizing Hormone and Its Subunits.

Preparation	Bioassay[a]			Radioimmunoassay[b]			Ratio of Bioassay: Immunoassay
	Potency (units/mg[c])	95% Limits	λ	Potency (units/mg[d])	95% Limits	λ	
Rat LH	1.55[e]	0.96–1.21	0.099	1.72	1.65–1.78	0.018	0.90
C-1 subunit	0.080	0.10–0.055	0.138	0.067	0.065–0.069	0.010	1.19
C-2 subunit	0.078	0.11–0.056	0.131	1.11	1.08–1.14	0.010	0.070
C1 + C2[f]	0.55	0.74–0.38	0.113	0.47	0.45–0.48	0.012	1.17

[a] Bioassay by ovarian ascorbic acid depletion method (Reichert and Parlow, 1963). Potency is expressed relative to NIH-LH-S1. All assays were of the 2 + 2 design, with $n = 20$. [b] For details of radioimmunoassay, see text. [c] Units expressed in terms of NIH-LH-S1. One unit is equivalent to 1 mg of the reference preparation. [d] The reference preparation for radioimmunoassay was the rat LH fraction LER-1240, having a biologic activity of 0.60 NIH-LH-S1 unit/mg. [e] TSH contamination of the rat LH was tested by the thyroidal ^{32}P-uptake method as was described earlier for ovine LH (Reichert and Parlow, 1963). The TSH potency was 1.85 units/mg, with 95% confidence limits of 1.43–2.38 units/mg. The index of precision for the assay was 0.126. [f] Conditions for the recombination experiment were as described for bovine LH subunits (Reichert *et al.*, 1969). Expected biologic potency (average of subunits) if no augmentation were obtained would be 0.079 unit/mg. Expected immunologic potency (average of subunits) if no augmentation were obtained would be 0.58.

of the intact molecule. After recombination, this low potency was augmented to a highly significant degree (7-fold) over the predicted average if no augmentation were obtained. The radioimmunoassay analysis of the intact LH, subunit C-1 and the recombined subunits agreed very closely with the biological analysis (Table II). A major difference, however, was in the immunologic potency of subunit C-2. This subunit contained 64% of the immunologic activity present in the intact LH, while subunit C-1 contained approximately 3.8% that of the intact precursor. These results will be considered further in the Discussion.

The Stokes radius of each subunit was 2.20 mμ, and the diffusion coefficient was 7.76×10^{-7} cm² sec. These values are similar to, but not identical with, those obtained for bovine LH and bovine LH subunits (Reichert *et al.*, 1969). The differences are rather small, however, and are not considered significant. They also tend to support the assumptions made earlier that the molecular weight of the rat LH and subunits are closely similar to those of the ovine and bovine hormone and subunits (*vide supra*).

The amino acid and carbohydrate composition of rat luteinizing hormone and its subunits is presented in Table III. Although the ratio of lysine to arginine is approximately 1 in the intact rat luteinizing hormone this ratio differs considerably in the subunits (*e.g.*, 10:3 for C-1 and 1:9 for C-2). The C-2 subunit has an exceptionally high proline, valine, and leucine content. Both subunits are rich in cystine. The C-1 subunit has two more residues of tyrosine than the C-2 subunit which is reflected in the relative absorbance of the subunit in patterns in countercurrent distribution (Figure 2). The intact rat luteinizing hormone contained no detectable tryptophan as measured by the method of Bencze and Schmid (1957). Accordingly, this measurement was not done on the isolated subunits.

After dansylation of rat luteinizing hormone or its subunits only *O*-dansyltyrosine or ε-dansyllysine was detected in the hydrolysates, suggesting a blocked NH_2 terminus on both subunits or NH_2-terminal heterogeneity.

The carbohydrate analyses indicate the C-1 subunit contains more carbohydrate than its C-2 counterpart, although the analyses for the two subunits do not account for all the carbohydrate in the original luteinizing hormone. The 2-hr hydrolysates gave slightly higher values for all sugars except glucosamine and mannose; the tabulated values thus represent the 2-hr hydrolysis values except in the two cases cited. Although the values for the sugars are maximum observed, the values are considered minimum values for the carbohydate content due to the analytical problems involving hydrolysis of neutral and amino sugars (see Discussion). Every effort was made to minimize these errors by using optimal conditions

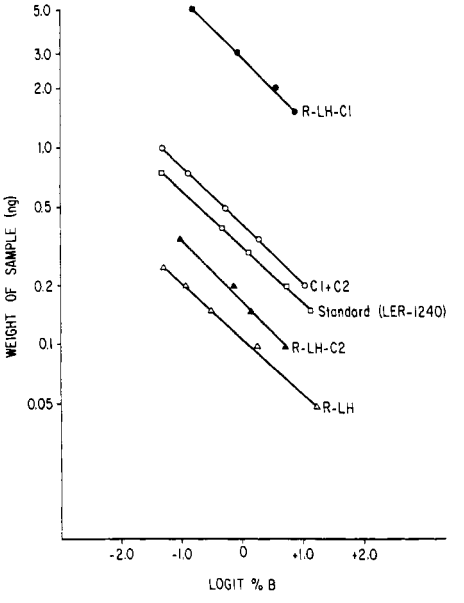

FIGURE 3: Radioimmunoassay data for rat LH and its subunits. RLH = rat LH; R-LH-Cl or C2 designates subunits obtained in Figure 2; Cl + C2 represents the recombination experiment (see Table II); the reference standard is described in the text.

TABLE III: Composition of Rat Luteinizing Hormone and Its Isolated Subunits.

Amino Acid or Sugar	Subunit C-1		Subunit C-2		Rat Luteinizing Hormone	
	μmole/mg[a]	Residues/Mole[b]	μmole/mg[a]	Residues/Mole[b]	μmole/mg[a]	Residues/Mole[b]
Lysine	0.672	10	0.090	1	0.351	11
Histidine	0.232	4	0.083	1	0.163	5
Arginine	0.207	3	0.597	9	0.380	12
Aspartic acid	0.440	7	0.380	6	0.423	13
Threonine	0.518	8	0.494	8	0.486	15
Serine	0.461	7	0.556	9	0.455	14
Glutamic acid	0.435	7	0.365	6	0.411	13
Proline	0.431	7	1.308	20	0.877	27
Glycine	0.304	5	0.478	7	0.375	12
Alanine	0.512	8	0.576	9	0.496	15
Half-cystine	0.602	9	0.648	10	0.569	18
Valine	0.282	4	0.617	10	0.454	14
Methionine	0.190	3	0.119	2	0.119	4
Isoleucine	0.259	4	0.222	3	0.237	7
Leucine	0.271	4	0.737	11	0.518	16
Tyrosine	0.298	5	0.169	3	0.228	7
Phenylalanine	0.204	3	0.263	4	0.214	7
Glucosamine	0.498	8	0.261	4	0.409	13
Galactosamine	0.090	1	0.064	1	0.095	3
Galactose	0.093	1	0.047	1	0.048	2
Mannose	0.192	3	0.151	2	0.196	6
Fucose	0.028	1	0.060	1	0.051	2

[a] Average or extrapolated values from 24- and 72-hr hydrolyses (amino acids); neutral and amino sugars are maximal values, 2- or 8-hr hydrolysates. [b] Nearest integer, assuming subunit mol wt 15,500; intact LH = 31,000.

of hydrolysis and sample preparation for the neutral and amino sugar analyses (see Methods).

The COOH-terminal amino acid analyses of rat luteinizing hormone and its subunits as detected by hydrazinolysis and carboxypeptidase A digestion is summarized in Table IV. Serine and leucine are the principal COOH-terminal amino acids detected by either method. From the subunit analyses, the serine is derived from C-1 and the leucine from the C-2 subunit. The presence of the other amino acids detected in the analyses will be discussed in comparison to observations on luteinizing hormone preparations from other species (see Discussion).

Discussion

The analytical values presented were obtained under conditions which permitted the maximum information to be obtained with the minimal consumption of very scarce material. For example, the analyses of amino acids are based on two analyses of each sample, but without sufficient replication to estimate a standard deviation. The sugar analyses are considered minimal values since a study of different hydrolysis times to obtain maximal yield was not possible. In spite of the very limited number of analyses possible, the analyses are internally consistent within the limitations of accuracy of the methods employed. For example, the total analysis of the subunits accounts for 240 residues, while the intact luteinizing hormone accounts for 236 residues; a difference of less than 2%.

It has been noted that ovine and bovine thyrotropin preparations have both amino acid and sugar composition that closely resembles luteinizing hormone (Pierce and Wynston, 1960; Ward *et al.*, 1961; Walborg and Ward, 1963; Liao *et al.*, 1969). This relationship is emphasized further by the observation of Fontaine and Burzawa-Gerard (1966) that mammalian luteinizing hormone has a thyrotropic effect in fish. Recently, Liao and Pierce (1970) showed bovine thyrotropin and bovine luteinizing hormone possessed a common (apparently identical) type of subunit.

Reported potencies for mammalian thyroid stimulating hormone have generally ranged from 20 to 100 units per mg. Condliffe *et al.* (1969) reported mouse thyrotropin with a potency of 73 units/mg. Assuming rat TSH to have a similar potency the TSH contamination would amount to 2.5% (Table II) for the preparation described in this report. In view of the close similarities for luteinizing hormone and thyrotropin preparations that have been described it is unlikely this contaminant has significantly altered the composition values reported herein. In any event, the very serious losses of luteinizing hormone activity during our previous attempts to remove TSH (Ward *et al.*, 1968) made it inadvisable to make a similar attempt in the present study. The foregoing assumption relative to rat TSH composition may soon be clarified, for Martin and Reichlin (1970) have referred to a description of rat TSH which is in preparation.

The COOH-terminal data in Table IV, when both hydrazinolysis and carboxypeptidase data are considered, clearly indicate a COOH-terminal leucine on the C-2 subunit and a COOH-terminal serine on the C-1 subunit. The nature of the penultimate residues cannot be stated. Release of tyrosine

from the C-1 (S subunit) of ovine LH was interpreted as evidence for a tyrosylserine sequence by our laboratory (Ward *et al.*, 1969) and by Samy *et al.* (1969). Nevertheless, detailed sequence studies of the tryptic peptides of this subunit (Ward *et al.*, unpublished observations) clearly demonstrate this tyrosine release resulted from a chymotryptic-type cleavage further down the peptide chain followed by carboxypeptidase A attack. Since the Worthington carboxypeptidase A preparations utilized in all these studies are DFP-treated and stated to possess very low trypsin or chymotrypsin contamination, it appears the ovine luteinizing hormone C-1 subunit is extremely susceptible to attack by chymotrypsin in this position. By analogy, the rat LH-C-1 subunit also may possess this susceptibility. This is further indicated by the considerably slower release of tyrosine compared to serine from the C-1 subunit, and the virtual absence of tyrosine released from the intact LH. For these reasons, we do not believe the penultimate amino acid residue of either C-1 or C-2 subunit can be deduced from the present data.

The relative reactivities of rat LH, its subunits and the recombined subunits in the OAAD assay and by RIA is given in Table II, and the results of the RIA analysis are also shown in Figure 3. The slope of all preparations are similar. A comparison of the potency estimates of these preparations by bioassay and by RIA shows excellent agreement in all instances, save for the C-2 subunit. The C-2 subunit retained about 65% of the immunologic activity of the intact LH, but only 5% of its biologic activity. These results provide striking evidence for the nonidentity of sites for immunologic and biologic activity in the rat LH molecule. Similar results have been obtained with ovine (Reichert and Treadwell, 1967) and human LH (Reichert, 1969; Reichert and Midgley, 1968b); also see pertinent abstracts by Parlow (1970) and Papkoff *et al.* (1970).

The electrophoresis studies of the subunits of rat LH failed to demonstrate conditions for suitable staining. Lack of material prevented extension of these studies. Since some staining was observed it is probable, given adequate supply of the subunits, suitable conditions could be established for this staining. There was sufficient rat LH remaining to undertake the studies shown in Figure 1. On a unit weight basis a lesser affinity for the dye as compared to ovine LH was demonstrated. This difference is real since the two preparations had approximately the same protein content and biological potency on a unit weight basis. The character of the pattern at pH 8.2 or 9.5 deserves comment. The LH preparations from beef and sheep pituitaries all show this tendency to form two heavy staining bands on a more diffuse background as shown for both rat and sheep LH in Figure 1. As noted above, the resolution of the two heavy-staining bands may not always be apparent. This property of LH does not seem to depend upon the method of preparation, but seems to be a general characteristic of LH preparations submitted to acrylamide gel electrophoresis at this pH. For example, bovine LH by two different isolation procedures shows discernable double-banding appearance (Reichert *et al.*, 1969; Liao *et al.*, 1969). The study of Liao *et al.* (1969) is particularly noteworthy since the pattern for the same bovine LH preparation shows a broad single band character in their Figure 2b but a double band in Figure 2c. In contrast, similar preparations submitted to acrylamide gel electrophoresis at pH 4.5 show a single band which is more diffuse than sharp (*e.g.*, Ward *et al.*, 1967a; Papkoff and Gan, 1970). At pH 4.0 or lower one might expect acid dissociation of the subunits, but the double-banded type of pattern obtained at pH 9.5 probably is more complex, since subunit dissociation is not appreciable in the absence of high concentrations of urea or guanidine hydrochloride.

TABLE IV: COOH-Terminal Amino Acids of Rat Luteinizing Hormone and Its Subunits as Detected by Hydrazinolysis and Carboxypeptidase A Digestion.

Amino Acid	Catalytic Hydrazinolysis[a]		Carboxypeptidase A Digestion[a,b]		
Detected	24 hr	40 hr	15 min	45 min	4 hr
		C-1 Subunit			
Asp	0.132	0.100			
Thr	0.041	0.033	0.043	0.032	0.089
Ser	0.553	0.531	0.696	0.693	0.883
Leu	Trace	0.034	0.029	0.022	0.044
Tyr			0.146	0.226	0.807
		C-2 Subunit			
Asp	0.144	0.137			
Thr	Trace		0.045	0.050	0.059
Ser	0.174	0.171	0.088	0.082	0.111
Leu	0.225	0.323	0.580	0.618	0.708
		Rat Luteinizing Hormone			
Asp	0.060	0.075	0.093	0.070	0.093
Thr			0.142	0.156	0.170
Ser	0.585	0.589	0.996	1.000	1.020
Ile	0.030	0.060			0.044
Leu	0.595	0.645	0.610	0.627	0.606
Tyr			Trace	Trace	0.115

[a] Values are mole per mole of luteinizing hormone or subunit. The subunits contained no bound amino acid (Holcomb *et al.*, 1968); the luteinizing hormone values have been corrected for the following bound amino acids: Asp, 0.060; Thr, 0.011; Ser, 0.220; Gly, 0.140; and Ala, 0.105. [b] Enzyme–Substrate (1:40, w/w); digestions at 37°.

We believe all LH preparations yet described possess an intrinsic heterogeneity which relates both to the terminal portions of the peptide chains (Reichert, 1966; Ward *et al.*, 1967b) or the carbohydrate moieties (Fujino *et al.*, 1968). The heterogeneity referred to has been studied for our own LH preparations and confirmed in some cases with preparations furnished by our colleagues, but may be deduced from the nature of the gel electrophoresis patterns published from various laboratories (*vide supra*). Indeed Sherwood *et al.* (1970) have made an extensive study of the heterogeneity of ovine luteinizing hormone, which reminds one of an early study by Jutisz and Squire (1958). A heterogeneity determined by the carbohydrate portion of human chorionic gonadotropin has also been described (Bell *et al.*, 1969). The heterogeneity we refer to is one obtained with closely related molecules with biological activity and is commonly encountered with glycoproteins (see Discussion, Fujino *et al.*, 1968). The electrophoresis patterns as in Figure 1 serve more to characterize the material than to demonstrate purity in the usual sense.

Data are available on the subunit composition and properties from bovine (Reichert *et al.*, 1969; Liao *et al.*, 1969; Papkoff and Gan, 1970) and ovine (Papkoff and Samy, 1967; Ward *et al.*, 1969) luteinizing hormone. The complete amino acid sequence of the ovine luteinizing hormone A subunit (closely related to the C-2 subunit herein described) has also

been reported from our laboratory (Liu *et al.*, 1970). The subunits from the species so far studied in detail have the following similarities. (1) They separate with approximately the same partition coefficients in the countercurrent distribution system of Papkoff and Samy (1967) as used in the present study. (2) The subunit with the low partition coefficient, designated S subunit, C-I, or C-1 in various studies, is characterized by a high (approximately 3:1) lysine-to-arginine ratio, a greater tyrosine content compared to the other subunit, and a COOH-terminal serine. (3) The subunit with the high partition coefficient, designated A subunit, C-II, or C-2 in various studies, is characterized by a high arginine to lysine ratio, and a high proline and leucine content. (4) Both subunits have a high cystine content and both are glycoproteins containing glucosamine, galactosamine, mannose, galactose, and fucose. (5) Both of the subunits have approximately equal molecular weight of the order 15,500. Both subunits are relatively basic proteins in their electrophoretic behavior. (6) The C-2 subunit appears to have the greatest immunological reactivity with antibodies prepared against intact LH.

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