EXISTENCE OF BIG AND LITTLE FORMS
OF LUTEINIZING HORMONE IN HUMAN SERUM

By

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ABSTRACT

Serum fractions from normal subjects obtained by gel chromatography have been investigated using three different assay systems: radioimmunoassay (RIA), radioligand receptor assay (RRA), and testosterone production assay (TPA). The bulk of immunoassayable and "bioassayable" LH-activity was found in two fractions differing widely in their molecular size. The slower moving component, designated as "little" LH, migrated identical to the radioiodinated pituitary hormone (LER 960) with a molecular weight of about 30,000, while "big" LH appeared in an elution volume consistent with a molecular weight range between 140,000 and 180,000. Concordance was seen between the LH-activities measured in all three assay systems. The RRA/RIA ratio varied between 1.6 and 8.9, the RRA/TPA ratio was close to unity. Treatment with 6 M urea and 0.1% mercaptoethanol and also, exposure to different pH values and salt concentrations did not change the elution position of the two LH components. Also, "big" and "little" LH appeared unaltered after re-filtration and no conversion each other could be found. In another experiment injection of gonadotrophin releasing hormone (Gn-RH) into a male induced a profound shift of LH towards the low molecular weight species. Kinetic uptake studies with "big" and "little" LH using RRA showed identical affinities to the receptor preparation. Ion exchange chromatography of serum, however, did not give two LH components, indicating no major differences in charge properties. This finding could be confirmed by preparative gel isoelectric focusing. The RRA potencies following gel filtration were in good agreement with that applied to the column, however, the immunological activities exceeded that of loaded by a factor 3-4. A new aspect of serum LH heterogeneity is the finding of a low molecular substance (mol. weight approximately 1000) in the outer dialysate of serum, which has LH like activity in all three assay systems.
Heterogeneity of proteohormones is a term used in different contexts: Thus, this term has been applied as “microheterogeneity” to the so-called iso­hormones of gonadotrophins, such as human chorionic gonadotrophin. This type of heterogenei­ty can be detected by techniques of very high resolving power, such as gel isoelectric focusing (Brossmer et al. 1971; Graesslin et al. 1972a) and is obviously based on charge differences of the molecular forms due to their different neu­ramic acid content (Graesslin et al. 1973).

Heterogeneity, however, also applies to qualitatively identical proteohormones differ­ing in their molecular size, in their relative immunoreactivity and their sur­vival time in the organism. Such type of heterogeneity has been shown for insulin (Steiner & Oyer 1967), parathyroid hormone (Berson & Yalow 1968), as well as for other proteohormones (Yalow & Berson 1970, 1973a; Goodman et al. 1974; Rabinowitz et al. 1974).

Although the presence of such different forms of peptide- or proteohormones has been clearly demonstrated by various techniques, only scant information is available about their biological significance.

While some forms of heterogeneity are apparently due to the presence of prohormones (Yalow & Berson 1973b) or degradation products, recent evidence indicates, that a change in the hormonal environment might induce an endocrine organ to produce different hormone forms. This has recently been shown by Bogdanove et al. (1975) who presented a line of evidence that the target organ (the gonad of the rat) not only dictates the amount of the trophic hormone to be produced but also the kind of the trophic hormone as well. Heterogeneity has also been shown for pituitary and urinary luteinizing hormone (LH) by Rabinowitz et al. (1974) who used radioimmunoassay and radioligand receptor assay techniques. Our group, on the other hand, extending earlier preliminary experiments of Wildt et al. (1973), has recently presented data to show the existence of two forms of serum-LH (Graesslin et al., in press).

It is the purpose of this investigation to confirm and to extend our previous observation on the size heterogeneity of LH in human serum, using various methods. Besides gel chromatography, this includes ion exchange chromatography, preparative isoelectric focusing and various treatments of serum samples prior to chromatography, such as urea- or mercaptoethanol treatment, and dialysis as well as exposure of serum to different pH values and salt concentrations. In addition, the kinetics of binding of “big” and “little” LH fractions to a receptor preparation was studied. For determination of LH activi­ties three assay systems have been used based on different endpoints: a radioimmunoassay (RIA), a radioligand receptor assay (RRA) and an in vitro bi­assay based on testosterone production (TPA).

From such a study we hoped to gain more detailed information about the nature and stability of the forms of serum LH. Such information will hopefully lead to clarify the biological significance, and to explain the significant dif­
ferences in serum LH measurement which have been observed by several investigators (Karg et al. 1969; Qazi et al. 1974; Reichert & Leidenberger 1975, in press; Leidenberger et al. 1976) when different assay systems have been used.

MATERIALS AND METHODS

1. Blood sampling. – Venous blood samples were collected from normal male subjects between 23 and 38 years of age. The blood was allowed to stand for 1 h at room temperature followed by 2 h at 4°C. The blood samples were then centrifuged at 2000 × g and immediately subjected to chromatography.

2. Gonadotrophin releasing hormone test. – In order to follow changes of serum “big” or “little” LH concentrations after application of gonadotrophin releasing hormone (Gn-RH, purchased from Hoechst, Frankfurt, Germany), 100 μg of Gn-RH was injected intravenously into a male volunteer. Blood samples were drawn immediately before and 40 min after this injection.

3. Gel chromatography. – The serum (5 ml) samples were applied to a 2.5 × 92 cm column of Sephadex G 200 at 4°C using 0.1 M Tris-HCl-buffer, pH 8.0, containing 0.2 M NaCl. For comparison, two other buffer systems were used: 0.1 M phosphate-buffer, pH 5.0, containing 0.2 M NaCl, and 0.3 M Tris-buffer, pH 8.0, containing 0.5 M NaCl.

To protect endogenous LH from damage by proteolytic enzymes 500 units of Trasylol/ml (Bayer, Leverkusen, Germany) was added to the serum sample and to buffer in a few studies.

The columns were calibrated using blue Dextran and three different proteins of known molecular weight: human gamma globulin (mol. wt. 160 000), albumin (mol. wt. 70 000) and chymotrypsinogen A (mol. wt. 25 000), all purchased from Serva, Heidelberg, Germany. Highly purified human pituitary LH (LER 960) was labelled with the chloramin T method of Greenwood et al. (1963) as modified by Leidenberger & Reichert (1972). This marker has been incubated with serum at 37°C for 30 min prior to gel filtration.

The fractions were monitored at 278 nm and were collected in volumes of 10 ml. Tubes were pooled into 9 main fractions as indicated in Fig. 1, then dialysed for 48 h at 4°C against distilled water under gentle agitation and lyophilized.

Prior to gel filtration, few sera were treated with 6 M urea (final concentration) for 60 min at 37°C or with 0.1% mercaptoethanol, which also has been added to the buffer. Most native individual sera, however, were chromatographed without any prior treatment.

4. Ion exchange chromatography. – Ion exchange chromatography of serum was performed on QAE-Sephadex A 50 (column size: 2.5 × 32 cm) using 0.04 M imidazole-HCl-buffer, pH 6.4, and a stepwise increase of concentration with NaCl to 0.08 M, 0.15 M and 0.3 M at the same pH. Ten ml fractions were pooled, dialyzed against distilled water and lyophilized.

5. Preparative isoelectric focusing on thin layers of granulated gels. – Preparative isoelectric focusing runs in 1.2 mm layers of Sephadex G 75 superfine (pH range 3.5–10) were performed according to Radola (1971). 20 × 20 cm glass plates and the DE-double
Elution pattern of 5 ml native male serum after gel filtration on Sephadex G 200 (2.5 x 92 cm column). Immunological and biological LH-activities were predominantly found in the two zones indicated as shaded areas. For calibration proteins of known mol. weight have been used: gamma globulin (160 000), albumin (70 000), chymotrypsinogen A (25 000), and labelled hLH (30 000), which eluted at the positions as marked by arrows.

chamber of Desaga, Heidelberg, Germany, were used. The carrier ampholytes (Ampholine) were purchased from LKB-Produkter, Stockholm. Between 22 and 25 mg of the “little” or “big” LH fractions obtained after gel chromatography were applied to two separate gel slabs near the anode. Focusing started with 10 V/cm, the voltage being increased to 30 V/cm during the course of the experiment. Total focusing time was 5 h. After the run of each LH form, the slabs were cut into 4 zones corresponding to different pH areas (pH 4-5; 5-6; 6-7; 7-8), transferred to small columns and eluted with distilled water. The protein recovery was 60%. Prior to LH-assaying, the carrier ampholytes were removed by dialysis. In order to determine the isoelectric points of the protein fractions, small portions of the granulated gel along the pH gradient were suspended in distilled water and the pH was measured with a microelectrode (Ingold, Frankfurt, Germany).

6. Dialysis of serum samples. – Individual and pooled serum samples were dialyzed against a 20 fold volume of either distilled water or 0.1 M Tris-HCl-buffer, pH 7.2, for 72 h at 4°C under gentle agitation. The outer dialysate was lyophilized and used for direct measurement of LH-like activity. Further treatment of this dialysate consisted of gel chromatography on Sephadex G 50 medium (column dimensions: 1 x 85 cm, fractions of 4.3 ml, 4°C). The columns were calibrated with albumin (mol. wt. 70 000), chymotrypsinogen A (25 000), cytochrome C (13 000) and tryptophane (204), purchased from Serva, Heidelberg, Germany. Fractionation of the outer dialysate was monitored at 280 nm.
7. Assay systems used for determination of LH activity. – a. The radioimmunoassay (RIA) used is based on the double antibody technique of Midgley (1966). LER 960 was used as antigen, LER 907 as reference preparation. Serum LH determinations were performed with untreated serum.

b. The radioligand receptor assay (RRA) was performed as described by Leidenberger & Reichert (1972). For determination of LH activity serum had to be pre-treated with 8–10% cold ethanol (final concentration) or cold ether prior to the assay, in order to remove method interfering substances. The ethanol treatment has been described in detail by Leidenberger et al. (1976).

c. The testosterone production assay (TPA) was performed as reported by Lichtenberg & Pahnke, in press). This assay procedure is a modification of the TPA originally described by Van Damme et al. (1974). To use this assay for serum determination, serum has to be pre-treated with cold ether in order to remove method interfering substances (Lichtenberg, unpublished results). For determination of LH activity, the salt free, chromatographed and lyophilized serum fractions were dissolved in 0.1 M Tris-HCl buffer, pH 7.4 to make 1% solutions, and diluted further as required for the assay. The detection limit is 20–25 µIU LH (LER 907).

8. Kinetics of binding of “big” and “little” LH to a receptor preparation. – Since binding characteristics of “big” and “little” LH compared to that of the reference preparation LER 907 could not be evaluated by directly labelling these crude fractions, an indirect way to compare the uptake kinetics relative to each other was selected: since it has been shown previously (Reichert et al. 1973b) that the initial rate of uptake of LH in the receptor assay, also used in this study is directly proportional to the hormone concentration, “big” and “little” and pituitary LH (LER 907) were diluted to the same concentration (125 mIU/ml). Using the receptor assay conditions, dose-response curves were obtained. However, in order to determine the LH uptake after various times, pre-incubation times were varied, as shown in Fig. 5 (5, 10, 20, 40, 80 min) and the LH not bound after the pre-incubation times indicated, was removed by centrifugation and the supernatant (1800 × g, 15 min, 0–4°C). The pellet was then washed one time. The receptor sites not blocked by cold LH after the different pre-incubation times, were then occupied by a further incubation of the re-suspended pellet for 2 h (37°C) with 2 ng of [125I]HCG. Thereafter, the amount of the tracer hormone bound was determined as indicated in the description of the RRA. For each preparation five dose response curves were obtained, for which the 50% – intercept could be read or extrapolated (Fig. 5).

9. Reference preparation. – For all assays the pituitary FSH/LH reference LER 907 was used. Activities were expressed as mIU, assuming 48 IU/mg for LER 907.

RESULTS

1. Gel chromatography

A typical elution pattern of protein measurement after fractionation of 5.0 ml of serum on Sephadex G 200, showing three well separated peaks, is documented in Fig. 1. In total, 42 sera have been investigated following gel chromatography. Immunoassayable and bioassayable LH-activities were predominantly found in two zones. These two zones differ widely in their molecular size. The apparent
Comparison of "biological" and immunological LH activities in the serum fractions obtained from the chromatographic run of Fig. 1 as measured in the three assay systems.

Molecular weight of the faster migrating fraction was found to be between 140,000 and 180,000. This hormonal form of LH has been designated as "big" LH. The more retarded peak, designated as "little" LH showed a molecular size between 20,000 and 30,000, on the basis of the protein markers used for calibration (Fig. 1). [125I]hLH appeared in the two last fractions overlapping with the low molecular weight zone of serum-LH; no radioactivity was found in the area characteristic for "big" LH.

Fig. 2 shows a comparison of LH activities found in nine fractions obtained after gel filtration of serum samples, as tested in the three assay systems against the same reference preparation. Fig. 2 is a typical example of the fractionation of an individual serum and shows the distribution of LH-activities.
Table 1.
LH-activities of "big" and "little" LH fractions from 15 individual sera following gel chromatography as measured by RIA and RRA. LH potencies have been expressed in mIU/mg protein.

<table>
<thead>
<tr>
<th>Serum sample No.</th>
<th>&quot;big&quot; LH</th>
<th></th>
<th></th>
<th>&quot;little&quot; LH</th>
<th></th>
<th></th>
<th>Ratio of &quot;big&quot;/ &quot;little&quot; LH (RRA)</th>
<th>Ratio of &quot;big&quot;/ &quot;little&quot; LH (RIA)</th>
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<td></td>
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<td>RRA mIU LH/mg</td>
<td>RRA/RIA</td>
<td>RIA mIU LH/mg</td>
<td>RRA mIU LH/mg</td>
<td>RRA/RIA</td>
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72–92% of the LH-activity, as found in all three assays, was recorded in the two main LH containing zones. In few cases, activity in the RRA and in the TPA reached maximum values of up to 2000 mIU/mg protein and even more, in both the “big” and “little” LH areas.

There was a good correlation between LH-activities measured in the two “bioassays”, both in respect to the elution pattern and in respect to its relative amounts (Fig. 2).

Also, the peaks of radioimmunoassayable LH-activity were eluted in the same area as the “bioassayable” LH-containing fractions (Fig. 2) and there was concordance in nearly all fractions. However, RIA-LH was always lower than RRA- or TPA-LH, when expressed in mIU/mg protein. The RRA/RIA ratio varied between 1.6 and 8.9 (see Table 1), while the RRA/TPA ratio was close to unity (0.8–1.1).

We consider the elution- and LH-pattern of Fig. 1 as typical; however, there was a great variation in the ratio between the amount of “big” and “little” LH found (see Table 1). In a number of cases, even complete absence of one of the two LH containing fractions was recorded and in few cases, some

Fig. 3.
Elution profiles of LH activities from six different individual sera. The fractions F1–F9 of this figure correspond to fractions F1–F9 of Fig. 1. Note the variability of the “big” and “little” LH area in the six sera shown.

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activity was found in an area corresponding to a molecular weight of approximately 60,000. This must be inherent to the serum samples themselves because, in all cases tested, LH-like activities were fairly concordant in all three assay systems. This variability is illustrated in Fig. 3 and in Table 1. Fig. 3 shows the LH-activities of fractions obtained from 6 individual runs.

2. Recovery

The LH-activities eluted from the column as tested by RRA indicated a 65–80 \% recovery. However, the immunological activity exceeded that loaded by a factor of 3 to 4 (see also Discussion). It should be mentioned here, that determination of immunoreactive LH in the serum sample to be loaded has been done in native serum, while for RRA part of the sample to be chromatographed had to be pre-treated (see Method section).

3. Stability of “big” LH

Several other experiments were made in order to examine the possibility that “big” LH is converted to “little” LH during gel filtration. After separate re-filtration of both LH forms under identical conditions, all the LH-activity in the effluent appeared in a single peak, which migrated in a manner typical for “little” and “big” LH, respectively (Fig. 4). Both forms appeared also to be stable against different physico-chemical treatments. Thus, treatment of the sera with 6 m urea or 0.1 \% mercaptoethanol prior to gel filtration did not significantly effect the LH profile of the serum pattern. In addition, pre-treatment with trasyol or variation in buffer and pH (0.1 m phosphate-buffer, pH 5.0, or 0.3 m Tris-HCl-buffer, pH 8.0, containing 0.5 m NaCl) did not change the elution position and the ratio of the LH fractions, compared to untreated controls.

4. Ion exchange chromatography

Besides gel filtration, serum was also subjected to ion exchange chromatography, a method based on a different separation principle.

In this system most of the “bioassayable” and immunoassayable LH activity was eluted from the column in the unadsorbed protein fraction which was developed with the imidazole-HCl starting buffer. Increasing of the buffer concentration to 0.08 m resulted in the elution of only minute amounts of LH-activity, while no further LH activity could be released with additional increases of the buffer concentrations. Obviously, we are not able to differentiate the LH fractions found by gel filtration with this method which is based on charge differences.
Elution pattern and LH activities (RRA) after re-chromatography of the isolated “big” LH and “little” LH fractions from the run in the upper part. Re-chromatography was performed under conditions identical to those of the first chromatographic step.

5. Isoelectric focusing of “big” and “little” LH fractions

Twenty-five mg of “big” and 22 mg of “little” LH was subjected to preparative gel isoelectric focusing on slabs of Sephadex G 75 sf. In both runs LH-activity could only be detected in the pH range of 7–8, indicating that both forms cannot be separated on the basis of their isoelectric values within the range used. This finding essentially agrees with the results of ion exchange chromatography. The pI range of 7–8 for both serum LH forms appears to be identical to that found for pituitary LH.

6. Kinetics of binding of “big” and “little” LH to a receptor preparation

Binding characteristics of hormone binding to its specific receptor sites in a target organ can be used to detect differences in LH species. This has been shown for LH from different animal species (Leidenberger & Reichert 1973; Reichert et al. 1973a), using slope differences of dose-response curves in the same receptor assay as used here. No such slope differences between “big” and “little” LH could be detected in this study using standard conditions and LER 907 as reference preparation.
Kinetics of binding of "big", "little" and pituitary LH (LER 907). Each point in the figure represents the 50% intercept of dose-response curve obtained with the preparations indicated, using the pre-incubation time on the ordinate. This figure shows that an inverse relation exists between the pre-incubation time and the dose required to obtain 50% tracer uptake inhibition. The identical slopes suggest that no major difference exists between the three preparations in respect to their receptor binding affinities.

Elution pattern and profile of LH activity of sera after chromatography on Sephadex G 200. The upper part shows serum before, the lower part 40 min after intravenous injection of 100 μg of gonadotrophin releasing hormone into a male. LH activities have been measured by RRA.
The indirect approach of studying uptake kinetics of the two forms has been chosen, because it has not been possible to label them for direct kinetic studies. Thus, the binding affinity of cold pituitary LH has been compared relative to that of “big” and “little” serum-LH. Fig. 5 is a logarithmic plot of the doses needed after various pre-incubation times to inhibit tracer uptake by 50%. It illustrated, that no significant differences exist between pituitary LH and the two forms in serum under the conditions used.

7. Gonadotrophin releasing hormone test

One hundred µg of Gn-RH was injected into a male. Serum samples were drawn before and 40 min after injection. Both samples were simultaneously applied to two columns and the fractions obtained tested in the RRA (see Fig. 6). A very high LH value (2000 mIU/mg) was found in the zone of “little”, whereas “big” LH was absent (lower part of Fig. 6). Considerable LH activity, however, was also found in a zone corresponding to a molecular size of approximately 60,000. In contrast, “little” LH was absent in the control run, which showed high activity in the area of “big” LH. In this experiment injection of Gn-RH resulted in a profound shift of the LH species into the lower molecular weight area. The biological significance of this finding is not clear and should be carefully studied.

Fig. 7.

Elution pattern of the outer dialysate of normal male serum after chromatography on Sephadex G 50. The shaded area represents the zone of LH-activity corresponding to a molecular weight of approximately 1000. Note that LH-activity in fraction 4 has been found in all three assay systems.
8. "Mini" LH in the outer dialysate of human serum

Individual and pooled serum samples were dialyzed against 0.1 M Tris-HCl-buffer, pH 7.4, or against distilled water. The outer dialysate was concentrated by lyophilization and tested for LH potency by RIA, RRA and TPA: activities were found in all three assays.

The freeze-dried outer dialysis was further fractionated by gel filtration on Sephadex G 50. Fig. 7 illustrates such a chromatographic fractionation starting with 15 ml of a normal male serum. The fractions obtained were tested for LH potency. The first peak seen in Fig. 7 represents traces of albumin as identified by polyacrylamide gel electrophoresis. LH-activity was found in the shaded area (fraction 4) corresponding to a molecular size range between 1000 and 1500 daltons. In all three assays this "mini" LH fraction gave dose-response curves parallel to that of the pituitary reference preparation (LER 907). When calculated in terms of mIU LH/ml of original serum this fraction had 2.7 mIU LH/ml by RIA, 29 mIU LH/ml by RRA and 24 mIU LH/ml by TPA.

This experiment has been repeated for several times with similar results. This low molecular material derived from serum showed LH activity which is in the same order of magnitude as serum-LH, proven in three different assay systems.

DISCUSSION

Heterogeneity of human pituitary LH is an experimentally well documented fact, both, in respect to microheterogeneity (Saxena & Rathnam 1971; Graesslin et al. 1972b), and in respect to differences in immunoreactivity (Rabinowitz et al. 1974). The data summarized in this publication offer new aspects by providing evidence for heterogeneity of serum LH, in addition to what is already known about heterogeneity of pituitary LH.

To our knowledge, this is the first clear demonstration of the existence of different forms of serum LH, distinguishable from each other by markedly different molecular weights: 140 000–180 000 for "big" LH as compared to 20 000–30 000 for "little" LH, and about 1000 for "mini" LH.

Since it could not be excluded, that storage of serum can cause conversion of one LH form into the other – as it has been shown for "big" and "little" growth hormone (Goodman et al. 1974) – only fresh sera have been applied onto the columns. In our experiments serum LH activity following gel chromatography was determined in three in vitro assay systems. Quantitation of LH activity in assays measuring different parameters (antigen-antibody reaction, receptor binding and steroid production) seemed to be a necessary precaution, because it is well documented, that serum constituents can interfere non-specifically with such assays. It is only recently, that it has been shown, how relatively
Insensitive assays such as LH receptor assays can be affected by such serum factors (Leidenberger et al. 1976). For determination of LH activity in chromatographed serum fractions, this seems, however, to be a minor problem, since the various serum constituents are fractionated during chromatography and a good correlation between RRA- and TPA-LH values can be shown (see Fig. 2).

In contrast, immunoreactivity of the same serum fractions of Fig. 2 is consistently lower, as it is reflected by RRA/RIA ratios between 1.6 and 8.9 (Table 1). Though lower in their absolute immunoreactive LH activities, all fractions showed concordance of their LH activities in all three assay systems.

An important finding is the stability of the “big” LH fraction under various conditions: neither chromatography in 0.1 m phosphate-buffer, pH 5.0, nor in Tris-HCl-buffer, containing 0.5 m NaCl made “big” LH disappear. Also, pre-treatment of serum with 6 m urea or 0.1 % mercaptoethanol prior to gel chromatography did not affect the “big” LH fraction. The relative stability of “big” LH is also illustrated in Fig. 4, which shows re-chromatography of the isolated “big” and “little” LH fractions.

Except for the size heterogeneity, no qualitative differences could be detected between “big” and “little” forms of serum LH and pituitary LH: All three forms of LH studies here showed kinetics of binding to a receptor preparation not distinguishable from each other (Fig. 5). Also, determination of the isoelectric points of the “big” and “little” LH fraction by isoelectrofocusing as described above, gave an isoelectric range for both serum LH forms between 7 and 8. This finding is in good agreement with the isoelectric range found for pituitary LH (Saxena & Rathnam 1971; Graesslin et al. 1972b), and can be confirmed using ion exchange chromatography.

We conclude from these studies, that “big” LH is a rather stable form and is most likely not due to loose attachment of pituitary to a serum protein or due to aggregation. This view is supported by the stability of “big” LH under the various conditions, by the result of re-chromatography (Fig. 4) and by the finding that highly purified 125I-labelled human pituitary LH (LER 960) did not appear in the “big” LH area, when pre-incubated with serum prior to gel chromatography. Analogous observations have been made by Goodman et al. (1974) who found a relatively stable form of “big” growth hormone in human plasma.

An interesting feature whose biological significance awaits clarifications is the rather big variability of the relative amounts of “big” and “little” LH present in individual sera (Fig. 3, Table 1).

While the physicochemical characteristics of “big” LH are nearly identical with the characteristics of a “big” pituitary TSH, which has been characterized using similar techniques (Ehrhardt 1976, personal communication), our finding of two molecular size species disagrees with that of Qazi et al. (1974) who found only one area of biological activity after gel chromatography of human plasma.
This discrepancy might be due to the small number of samples examined, or more likely, due to different experimental conditions (dialysis of serum prior to chromatography, use of post-menopausal sera and Sephadex G 100 for gel chromatography).

A provocative new aspect of the already complex features of heterogeneity of pituitary and serum LH is the consistent finding of LH activity in the outer dialysate after dialysis of individual and pooled serum samples ("mini" LH). The fact, that on several occasions (five different sera) LH activity has been found in a low molecular weight area of approximately 1000 daltons is in contradiction to all what is known about molecular weights of molecules with LH like activity. It should be pointed out here, that the hormonal activity was proven by RRA, TPA and RIA as well. This is, however, not the first observation of LH activity in the outer dialysate after dialysis of gonadotrophin fractions. Such observations have been made by Reiss & Haurowitz (1929) and by Wettstein & Benz (1951, 1956), as well as by von Eickstedt (1956). Although the origine and the physicochemical properties of "mini" LH are unknown yet, its immunoreactivity suggests peptide nature. There is no doubt that the various new aspects of LH heterogeneity demonstrated here and the development of sensitive LH-assays with different endpoints make the quantitation of LH in serum even more problematic and complex than it has already been before using radioimmunoassays. Some of the problems one is faced with are quite obvious:

1. None of the reference preparations available fulfils the criterion being identical to the biological material to be studied.

2. All molecular forms of LH activity derived from serum can be quantitated against the pituitary reference preparation in all three assays and their relative quantities are comparable to each other. However, it is unknown how much these forms contribute to the LH activity when measurement is made with the serum still in the native state.

3. Because of either relatively low sensitivity of the assay used or method interfering substances, pre-treatment of serum is necessary for some assays (TPA, RRA) for LH measurement. Comparison of "bioassayable" and "immunoassayable" LH values become, however, questionable, when one LH determination is done in native serum, the other in pre-treated serum.

While such pre-treatment is frequently associated with loss of activity, few instances of increased activity after pre-treatment have been observed (Karg et al. 1969; Qazi et al. 1974; Leidenberger et al. 1976).

As indicated in Results, we have in analogy to Qazi et al. (1974) found, that immunoreactive LH activity eluted from Sephadex columns exceeded by far (3–4 fold) that applied to the column. The nature of this "unmasking" effect is poorly understood, but it should be noted that the calculation of recovery of immunoreactive LH is based on LH-determination in native serum,
while for LH quantitation in the RRA, serum has to be pre-treated with ethanol or ether. That ethanol treatment of individual sera causes “unmasking” of immunoreactive LH has also been shown by our group (unpublished results). The demonstration of “unmasking” of LH immunoactivities after various pre-treatments and of the loss of immuno- and \textit{in vitro}-bioassayable LH activity into the outer dialysate after dialysis of serum serves as a caution against oversimplified interpretation of data from sera which have to be pre-treated prior to the assay. Of course, the same scepticism should be applied to measurements in native serum or plasma, whatever assay is being used, at least until the biological significance of method interfering substances in native serum and of the various forms of LH in blood is understood.

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