The Analysis and Fractionation of LH^{125}I by Gel Filtration (36124)

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Experienced immunoassayists are well aware of the variability in the immunoreactivity of different batches of radioiodinated LH. At the present time, there is no good method for assessing the quality of a preparation of labeled LH other than testing it in the radioimmunoassay itself (1, 2). Although this provides information about the quantity of immunoreactive LH which is present, it gives no indication of what qualitative differences exist between immunoreactive and damaged preparations. Hence, this type of analysis is of little use to anyone who wishes to study the cause of the variation in the immunoreactivity of the radioiodinated hormone. For that purpose, an analytical method of high resolution which provides qualitative as well as quantitative data is required.

The following is a report of how high resolution gel filtration can be used not only for the analysis, but for the fractionation of the heterogeneous product which is obtained upon the iodination of LH.

Materials and Methods. Iodination. Immunochemical grade LH (LER 960[1]) was iodinated with Na^{125}I by a modification of the method of Greenwood, Hunter and Glover (3). To 5 μg of LER 960 in 20 μl of 0.5 *M* phosphate buffer, pH 7.5 were added: 1) 2 mCi Na^{125}I in 5 μl of 0.1 *M* NaOH and 30 μg Chloramine T in 15 μl of 0.05 *M* phosphate buffer, pH 7.5. The solution was agitated for 30 sec, then 125 μg of sodium metabisulfite in 500 μl of the 0.05 *M* phosphate buffer was added. After stopping the reaction with sodium metabisulfite, 100 μl of a 0.6 *M* KI solution in 16% sucrose was added to aid the transfer of the iodination mixture to a Biogel P-2 desalting column. The vial was then rinsed with 70 μl of a 0.6 *M* solution of KI in 8% sucrose and the rinse solution added to the sample on the gel column.

Desalting gel filtration. The iodination mixture was freed to unreacted iodide by gel filtration on a 1 $\times$ 20 cm column of Biogel P-2 (Biorad Laboratories, Richmond, CA) which had been swollen in PBS–BSA (0.01 *M* phosphate buffer, pH 7.5, containing 0.15 *M* NaCl, 1% bovine serum albumin, and 0.1% NaN_3). The sample was eluted from the column with phosphate buffered saline, which contained no BSA. The eluate was collected in fractions of approximately 1 ml each. The radioactivity of each fraction was determined by counting the tubes over a Packard Manual Gamma Spectrometer well with sufficient shielding interposed to reduce the counts to 20,000 cpm in the peak tubes. All of the protein fractions which were free of unreacted iodide were pooled and labeled "P-2 protein."

Analytical gel filtration. Aliquots of the "P-2 protein" containing approximately 10^6 cpm were gel filtered at room temperature on a 2.5 $\times$ 45 cm column of Biogel P-60 (100–200 mesh) which had been swollen in PBS–BSA. The sample was eluted with the same buffer and was collected in forty-drop fractions. The radioactivity of each fraction was determined and was plotted as a function of the tube number. To demonstrate reproducibility of the elution characteristics, selected fractions of the eluate were rerun on the same column.

Immunoreactivity. To compare the immunoreactivity of the components obtained by gel filtration on the Biogel P-60, antigen inhibition curves were constructed for each, using LER 907[1] as the reference preparation in each case. Table I shows the composition of the reactant tubes. A one day incubation was allowed prior to addition of 0.1 ml of undiluted anti-rabbit gamma globulin (goat)

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[1] Obtained through the generosity of the National Pituitary Agency, NIAMD.

TABLE I. Composition of Antigen-Inhibition Tubes.

Tube designation	Contents					
	0.1 *M* EDTA (ml)	PBS–BSA (ml)	1:300 NRS (ml)	Anti-HCG[a] (ml)	LH^{125}I Component[b] (ml)	LH Standard[c] (ml)
"O"	0.1	0.7	0	0.1	0.1	0
LH Standard	0.1	0.6	0	0.1	0.1	0.1
"Total"	0.1	0.7	0.1	0	0.1	0

[a] Obtained from Miles Laboratory, diluted 1:3000 in 1:300 normal rabbit serum (NRS).
[b] Component I, II, III (obtained on P-60 gel filtration) diluted to contain 100,000 cpm/ml.
[c] LER 907, 31—2000 ng/ml.

serum. After an additional six days of incubation, the tubes were centrifuged at 750*g* for 30 min at 4°. The supernatants were decanted and counted in a Nuclear Chicago Auto Gamma counter. The number of counts bound in the tubes at each point on the standard curve was determined by subtracting the average supernatant count for each point from the average supernatant count of the "total" tubes. This technique proved more reproducible than counting the precipitates because the variations in geometry of the radioactive material were less. The slope of the line which best fit the function, logit $(100\ B/B_0) = a + b \log X$ (4) was determined by the method of Steel (5).

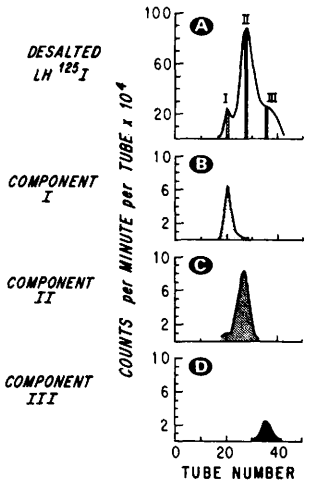

FIG. 1. Gel filtration patterns obtained on Biogel P-60, 100–200 mesh: a. LH^{125}I from desalting column; b. Reanalysis of sample from peak I; c. Reanalysis of sample from peak II; d. Reanalysis of sample from peak III.

Results. Iodination. Twenty five percent of the ^{125}I was eluted from the Biogel P-2 column in the protein peak. The specific activity of the preparation was 102 μCi/μg.

Analytical gel filtration. Three peaks were obtained when the protein from the P-2 column was gel filtered on the 2.5 × 45 cm Biogel P-60 column (Fig. 1, A). The molecular weights estimated from the elution volumes were approximately 60,000, 30,000, and 15,000 (6). Reanalysis of samples from each peak confirmed the reproducibility of the elution characteristics of each and indicated negligible interconversion of the components over the period of one week (Fig. 1, B–D).

Immunoreactivity. The curves of displacement of each of the radioiodinated components by unlabeled LER 907 are shown in Fig. 2. Component I, with the estimated molecular weight of 60,000 bound poorly (8% in the "O" tube) and had an extremely flat inhibition curve–if any at all. The slope of the logit function was not calculated because a statistically significant number of counts were not obtained during the counting period which was selected.

The second component, with the molecular weight estimated at 30,000, gave the most satisfactory inhibition curve (Fig. 2, curve II). Thirty-seven percent of the radioactivity of the "O" tube was precipitable by the dilution of anti-HCG which was used. The slope of the logit plot of the inhibition curve was —1.2. This value is higher than would be expected if the labeled and standard hormone had an identical affinity for the anti-HCG.

The 15,000 molecular weight component,

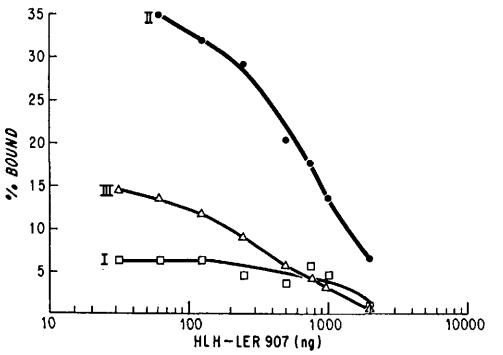

FIG. 2. Standard inhibition curves with unlabeled LER 907 and each of the radioactive LH components.

peak III, bound to anti-HCG rather poorly (18.6%), but did show an inhibition curve which was steeper than that obtained with component I.

Discussion. The results of gel filtration of the LH^{125}I on the Biogel P-60 are similar to the findings for unlabeled LH reported by de la Llosa (7). In his study of the gel filtration characteristics of recombined LH subunits, three components with estimated molecular weights of 60, 30, and 15 thousand were observed. His identification of these peaks as aggregate, monomeric LH and subunits seems appropriate for this study as well, since the molecular weights of the peaks isolated here are compatible with the values which others have reported for the three forms of LH (8, 9). Assuming this to be the correct identification, it is reasonable to ask whether the immunochemical grade hormone (LER 960) was originally in three forms, or whether the heterogeneity was the result of the iodination process. The latter seems the more likely, since markedly different gel filtration patterns are obtained with different batches of radioiodinated LER 960. The cause of the aggregation is not clear; however, it seems to vary with different batches of Na^{125}I. The size of the subunit peak appears to vary with the amount of excess sodium metabisulfite which is used in the reaction.

The reproducibility of the elution characteristics of the isolated components indicates that there is negligible tendency for interconversion. This is of particular importance since it indicates that monomeric LH^{125}I could be isolated by gel filtration and used in a radioimmunoassay with little concern that it would be converted to the inactive aggregate or subunit during the incubation of the assay tubes.

Finding that the most immunoreactive component was the middle component of the three explains the observation of Burger *et al.* (2) that the most immunoreactive fractions were those on the trailing side of the G-25 protein peak.

The slope of the logit plot for the 30,000 mol wt component (—1.2) deviates from the theoretical value of —2.303. It seems likely that this is due to the use of a relatively high concentration of anti-HCG in this experiment, which would lead to variations in the amount of hormone bound over the range of the standard curves (4).

The gel filtration technique reported here can be used for dual purposes. It provides an analysis of the quality of a new batch of LH^{125}I on the day of iodination. In addition, it serves as a fractionation procedure which allows separation of immunoreactive LH^{125}I from the heterogeneous iodination mixture.

If 1% bovine serum albumin is added to the solution of LH^{125}I to protect it from radiolysis during storage, aggregation of the monomer is retarded. Quantities of monomer sufficient to set up a radioimmunoassay can be isolated (by gel filtration) from aliquots of this stock solution at any time during a 7–8 week period.

Summary. Gel filtration can be used to fractionate LH^{125}I into three components: aggregate, monomer, and subunit. Only the monomer has satisfactory immunoreactivity. The plot of logit 100 B/B_0 vs nanograms unlabeled hormone is linear over the range of the standard curve. The slope deviated from the theoretical value of —2.303, probably because of the use of an excess amount of specific antiserum. The high resolution gel filtration used provides an assessment of the quality of the LH^{125}I on the day of preparation, and can also be used over a period of 7–8 weeks to isolate sufficient immunoreactive monomer from the heterogeneous stock solution to perform radioimmunoassays.

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