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Research Article

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ABSTRACT Most of the immunoreactive growth hormone (IRGH) in human plasma elutes from Sephadex G-75 as "little" GH (LGH), mol wt 22,000, but 14–39% elutes earlier ("big" GH, BGH). In saline extracts of human pituitary, 11–17% of IRGH eluted as BGH. On gel filtration of pituitary and plasma BGH in 8 M urea, 59–81% ran as LGH, but when the remaining BGH was refiltered in urea, all ran as BGH. Thus there is a "urea-stable" and a "urea-labile" form of BGH. Similarly, freezing and thawing converted over half of pituitary and plasma BGH to LGH, but when the "freeze-stable" BGH was again frozen, thawed, and refiltered, almost all ran as BGH. Urea-stable BGH was not dissociated by freezing, and most of the freeze-stable BGH was stable in urea, so the two forms are very similar or identical. Since 8 M urea and freezing dissociate peptides linked by noncovalent bonds, probably the BGH that is dissociated by urea and freezing consists of LGH bound noncovalently to another moiety, while in stable BGH the LGH is bound to another molecule by covalent or unusually strong noncovalent linkage. On centrifugation, the sedimentation of urea-stable BGH was consistent with a mol wt about twice that of LGH. Trypsinization of urea-stable BGH converted 36–59% to LGH, suggesting that some BGH may be a "prohormone" of LGH. On retrypsinization of the BGH that was not converted to LGH, only 13–24% converted, suggesting that there may be two forms of urea-stable BGH which vary in their response to trypsin.

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INTRODUCTION

In a previous study, we investigated the possibility that immunoreactive growth hormone (IRGH) [1] in human plasma is composed of more than one molecular species (1). The work was prompted in part by earlier studies on this subject by Bala, Ferguson, and Beck (2), and by Berson and Yalow (3). Plasma from acromegalic and normal subjects was fractionated on Sephadex G-75, and IRGH was measured in the effluent fractions. In every sample studied, almost all of the IRGH was found in two discrete peaks. In every instance the more retarded component ("little" IRGH, LGH) migrated almost identically with the major component of radioiodinated human pituitary GH (mol wt 22,000), while "big" IRGH (BGH) migrated at a rate consistent with a molecular size about twice that of LGH. BGH constituted 14–28% of total IRGH in the plasma of the acromegalics and 25–28% in the normal subjects. Two peaks of IRGH, indistinguishable from plasma BGH and LGH, were observed on gel filtration of "clinical grade" human pituitary GH. Similar results have been reported by Gorden, Lesniak, Hendricks, and Roth (4, 5).

In our study, on rechromatography of LGH and of BGH freshly isolated from plasma, there was no conversion of one form to the other (1). However, some conversion of BGH to LGH was noted in preparations of BGH stored at −20°C.

In the present investigation, we have extracted BGH from saline extracts of human pituitary as well as from normal and acromegalic plasma, and have attempted to define some of its characteristics. The results of the study indicate that there are at least two distinct

[1] *Abbreviations used in this paper:* B/F, bound/free; BGH, big IRGH; GH, growth hormone; HGH, human GH; IRGH, immunoreactive GH; LGH, little IRGH; p, pituitary.

forms of BGH in plasma and in pituitary extracts, and suggest the possibility that one of these forms may be a precursor or "prohormone" of LGH.

METHODS

Gel filtration. Gel filtration was carried out as previously described (1), with the following modifications. Human gamma globulin (Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.) was iodinated with ^{125}I by a modification of the method of Hunter and Greenwood (6), and added to the plasma and pituitary samples before gel filtration to serve as a column marker. The other column markers employed were [^{131}I]albumin and [^{131}I]sodium iodide obtained from E. R. Squibb & Sons, Princeton, N. J., and ^{125}I-labeled LGH. The latter was prepared from ^{125}I-labeled pituitary GH (pHGH) obtained from Cambridge Nuclear Corp., Cambridge, Mass., by filtering the material through a 2.5 × 80-cm Sephadex G-75 column as previously described (1), counting the ^{125}I in the effluent tubes, and pooling the fractions of the major peak, which eluted at a point consistent with a mol wt of approximately 22,000.

In the studies in which gel filtration was carried out in urea, urea (Fisher Scientific Co., Pittsburgh, Pa.) was dissolved in the filtration sample to a concentration of 8 M, and the sample was placed on a column containing Sephadex G-75 equilibrated with 8 M urea in 0.04 M barbital buffer, pH 8.2, containing 0.3% albumin. The sample was run through the column at 25°C at a rate of 10–20 ml/h, with the same solution.

In the preparation of BGH from the plasma of subject A. F. for gel filtration in urea, 10 ml of plasma was gel-filtered in barbital buffer on three separate occasions, the effluent fractions containing the BGH were pooled, and the pool was concentrated from an initial volume of 60 ml to 10 ml by pressure dialysis through an Amicon UM-2 filtration membrane (Amicon Corp., Lexington, Mass.). In experiments in which effluent fractions containing urea were subsequently gel-filtered in the absence of urea, the urea was removed from the pooled fractions by dialysis against the albumin-containing barbital buffer with a cellulose dialysis membrane with a pore diameter of 4.8 nm (Fischer Scientific Co.). The immunochemical grade pHGH used in this study was obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Md.

GH radioimmunoassay. The radioimmunoassay procedure was used as outlined previously (1), with the following modifications. The anti-HGH antibody employed was a guinea pig antibody prepared by Drs. S. Berson and R. Yalow and provided by the National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Md. Where rapid results were desired, the [^{125}I]HGH was added immediately to the reactions tubes, which were incubated for 16–24 h before charcoal separation and counting. Performed in this manner, the assay was sensitive to 0.125 ng/assay tube. Where greater sensitivity was required, the assay tubes were incubated without ^{125}I for 3–4 days, at which time the iodinated hormone was added, and the tubes were incubated an additional 1–2 days before separation. Under these conditions, the assay was usually sensitive to 0.015 ng/assay tube. As we have observed that urea affects the results of our radioimmunoassay for HGH, in assays of effluent samples containing 8 M urea, we added 0.2 ml of the effluent diluted appropriately in the urea-containing buffer to each assay tube, and 0.2 ml of the urea-containing buffer to the standard tubes.

The method we employed for investigation of immunochemical identity by serial dilution has been described previously (1). The sample was dialyzed free of urea before the serial dilutions were prepared.

Studies on the effects of freezing and thawing on BGH. In experiments in which we studied the effects of repeated freezing and thawing on BGH, the following technique was employed: an 8–12-ml aliquot of BGH in barbital buffer containing 0.3% albumin was frozen rapidly in a dry ice-methanol mixture and thawed in tap water at room temperature, and this process was repeated six times in the course of about 45 min. After this, the sample was subjected to gel filtration.

Preparation of saline or barbital pituitary extracts. Pituitary glands were obtained from nonacromegalic patients at autopsy within 12 h of death. Portions of the anterior lobe tissue were homogenized in 0.9% saline (J. L. and H. L.) or iced barbital buffer (M. W.), the homogenates were centrifuged at 6,000 rpm for 30 min, and the supernate was decanted and immediately placed on the column.

Sucrose gradient centrifugation. The sedimentation properties of selected forms of IRGH were studied by centrifugation in a sucrose gradient. A linear 5–20% sucrose gradient was prepared in 4.7 ml of 0.3 M NaCl and 0.04 M barbital buffer, pH 8.2, by the method described by Gerhart (7). Over the top of the gradient, we layered selected specimens of IRGH in 0.2 ml of the NaCl-barbital solution, to which had been added [^{125}I]pHGH (mol wt 22,000) and [^{131}I]albumin (mol wt 69,000), which served as markers. The tubes were then centrifuged at 50,000 rpm for 17 h at 13°C in a Beckman L2-65 centrifuge with an SW-50 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). 30 fractions of approximately 0.16 ml each were then collected from the bottom of each gradient tube and aliquots of these fractions were assayed for IRGH, ^{131}I, and ^{125}I. It was found that the amounts of sucrose and NaCl carried over to the assay tubes in these studies had no effect on the immunoassay.

Trypsinization studies. Trypsinization of selected fractions of IRGH were performed by adding chymotrypsin-free trypsin (Sigma Chemical Co., St. Louis, Mo.) in varying concentrations to solutions of IRGH in the albumin-containing barbital buffer, and incubating for 30–45 min at 37°C. The trypsin was then inactivated by the addition of soy bean trypsin inhibitor (Sigma Chemical Co.) to a final concentration twice that of the trypsin.

RESULTS

Gel filtration of saline and barbital extracts of human pituitary. The gel filtration pattern of a saline extract of human pituitary (subject J. L.) is illustrated in Fig. 1A, and two discrete peaks of IRGH are apparent. The major peak, representing 79% of the total IRGH in the effluent, migrated identically with iodinated pHGH and thus was identical in its migration pattern to LGH in plasma (1). The minor peak, representing 17% of total IRGH, eluted between LGH and albumin and thus migrated like BGH in plasma (1). 4% of the IRGH appeared in a shoulder between the void volume and the BGH peak. Very similar results have been obtained on gel filtration of a saline extract of

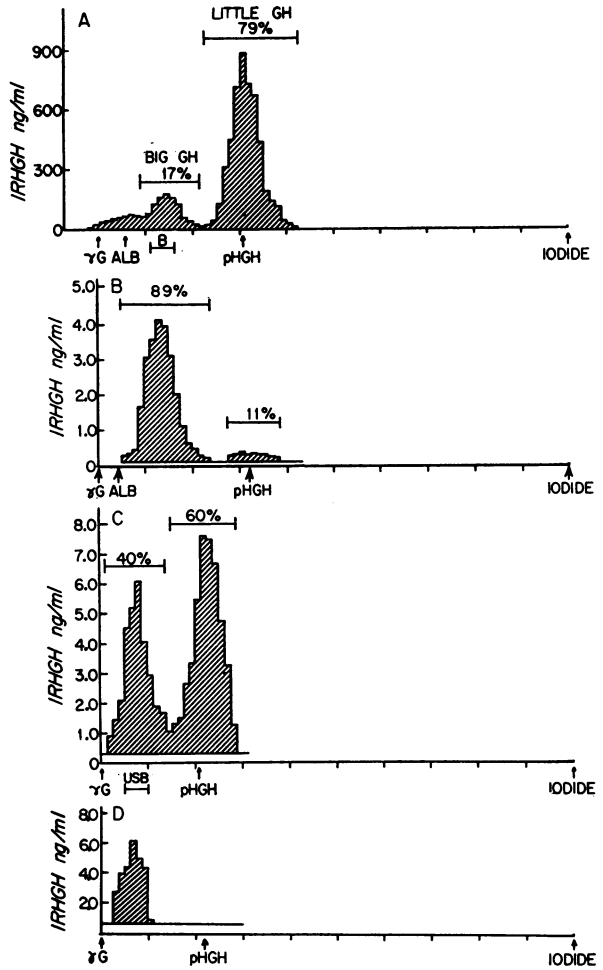

FIGURE 1 Gel filtration studies (Sephadex G-75) of a saline extract of human anterior pituitary from patient J. L. The arrows beneath the base line indicate the peaks of the radioiodinated column markers. The vertical lines beneath the base line indicate the percent of the effluent volume between the peaks of gamma globulin and iodide, with each line representing a tenth percentile. The base line indicates the lower limit of sensitivity of the immunoassay employed. The numbers over each peak indicate the percent of total effluent IRGH contained within the peak. (A) Gel filtration in barbital buffer of 5 ml of a saline extract of anterior pituitary. The brackets labeled "B" indicate the fractions pooled for the rechromatography studies shown in B and C. Recovery of filtered IRGH was 72%. (B) Gel filtration in barbital buffer of 1 ml of pooled BGH ("B" from Fig. 1A) after 70 h storage at 4°C. Recovery of filtered IRGH was 74%. (C) Gel filtration in 8 M urea of 5 ml of pooled BGH ("B" from Fig. 1A) after 70 h storage at 4°C. Recovery of filtered IRGH was 69%. The brackets labeled "USB" (for urea-stable BGH) denote the fractions pooled for rechromatography in study (D). (D) Gel filtration in 8 M urea of 12 ml of pooled USB from Fig. 1C. Recovery of filtered IRGH was 114%.

TABLE I

Summary of Data Concerning Big and Little IRGH in Plasma and in Pituitary Extracts

Subject*	Total plasma IRGH	Big IRGH	Little IRGH	Pre-big IRGH‡
	ng/ml		%	
Acromegalics				
G. C.	21	16	78	6
P. F.	27	28	68	4
E. M.	50	11	89	0
M. H.	129	13	82	5
B. L.	176	25	75	0
B. S.	88	28	70	2
T. G.	50	14	86	0
A. S.	125	14	83	3
Normals (post-hypoglycemia)				
K. T.	25	39	60	1
A. F.	26	24	70	6
T. M.	24	28	72	0
B. K.	37	25	67	8
Pituitary extracts				
J. L. (saline)		17	79	4
H. L. (saline)		12	85	3
M. W. (barbital)		11	85	4
N. I. H. immunochemical grade HGH		10	86	4
Clinical grade HGH		12	84	4

* Data concerning the last four acromegalics, the last two normal subjects, and the clinical grade HGH have been presented elsewhere (1).

‡ This term refers to IRGH eluting between the BGH peak and the void volume.

the pituitary of a second patient (H. L.), and of a 0.04 M barbital extract of a third patient (M. W.), as well as on filtration of immunochemical grade pituitary HGH (Table I).

When the BGH obtained from the saline extract of patient J. L. was rechromatographed, 89% ran as BGH (Fig. 1B).

Filtration of BGH in urea. The effect of 8 M urea on BGH was studied because this material is known to have the capacity to dissociate peptide subunits held together by noncovalent bonds (8). When BGH extracted from the pituitary of J. L. (Fig. 1A) was refiltered in 8 M urea, 60% migrated as LGH, eluting at the same point as iodinated pHGH, while the remainder ran as BGH (Fig. 1C).[2] A portion of the material that ran as BGH in the presence of urea ("urea-stable" BGH) was then refiltered in 8 M urea and all of it ran as BGH (Fig. 1D). Very similar results were obtained in a study of the BGH fraction

[2] In the presence of urea, the albumin, BGH, LGH, and [^{125}I]pHGH all eluted earlier than in the absence of urea, and this effect of urea has been observed for other proteins as well (9). However, the relation of these fractions remained the same, in that LGH eluted at the same point as the [^{125}I]pHGH, and BGH appeared between LGH and [^{131}I]albumin.

from the saline pituitary extract of patient H. L.; on initial filtration of the BGH in 8 M urea, 41% ran as BGH and 59% ran as LGH, but when the urea-stable BGH fraction was refiltered in 8 M urea, over 99% of it ran as BGH. These observations indicate that there are at least two fractions of BGH in human pituitary: one form that dissociates in the presence of urea to yield LGH, and a second form that is stable in the presence of urea.

Similar results were observed in studies of BGH extracted from acromegalic plasma (Fig. 2). When plasma BGH was refiltered in barbital buffer 40 h after it had been extracted from plasma into the buffer, 30% ran as LGH (Fig. 2B), and when the same fraction of BGH was filtered in 8 M urea, 63% ran as LGH (Fig. 2C). However, when the urea-stable fraction of BGH in Fig. 2C was refiltered in 8 M urea, all of it maintained its identity as BGH (Fig. 2D). Thus, there appear to be "labile" and urea-stable fractions of BGH in acromegalic plasma as well as in pituitary.

In a study of BGH extracted from the plasma of another acromegalic subject, M. H., on filtration of the BGH fraction in 8 M urea, 20% ran as BGH, with the remainder eluting as LGH. Similarly, 18% of BGH extracted from the plasma of a normal subject, A. F., ran as BGH in urea, with the remainder running as LGH. When BGH extracted from immunochemical grade pituitary HGH was filtered in 8 M urea, 52% ran as BGH and 48% as LGH.

The LGH generated from pituitary by treatment with 8 M urea (Fig. 1C) was compared with the LGH present in an untreated saline extract of pituitary (Fig. 1A) with regard to its reactivity with the antibody employed in our immunoassay. In a serial dilution study, no significant difference between the bound/free (B/F) curves of these two LGH preparations and the N.I.H. pituitary HGH standard was found (Figs. 3A and 3B).

Effects of repeated freezing and thawing of BGH. Under appropriate conditions, freezing and thawing, like exposure to 8 M urea, appears to cause dissociation of peptide units linked by noncovalent bonds (10). To investigate further the possibility that there are two discrete forms of BGH, an aliquot of the BGH that had been extracted from the pituitary of J. L. (Fig. 1A) was subjected to repeated freezing and thawing and then refiltered in barbital buffer, and it was found that 57% of the IRGH migrated as LGH with 43% eluting as BGH (Fig. 4A). The fraction of BGH that proved to be freeze-stable was again subjected to freeze-thaw treatment and refiltered in barbital, and 82% of it maintained its identity as BGH (Fig. 4B). Similar studies were performed with BGH from acromegalic plasma (Fig. 5). When BGH from plasma was refiltered in barbital after 40 h of storage in barbital at 4°C, 48% ran as LGH (Fig. 5B), and when an aliquot of the same BGH was refiltered after repeated freezing and thawing, 71% ran as LGH (Fig. 5C). However, when the freeze-stable fraction of BGH was again subjected to repeated freezing and thawing, and refiltered in barbital, all of it ran as BGH (Fig. 5D). These observations indicate that the pituitary and plasma BGH examined in this study consist of two forms, one of which dissociates during storage and

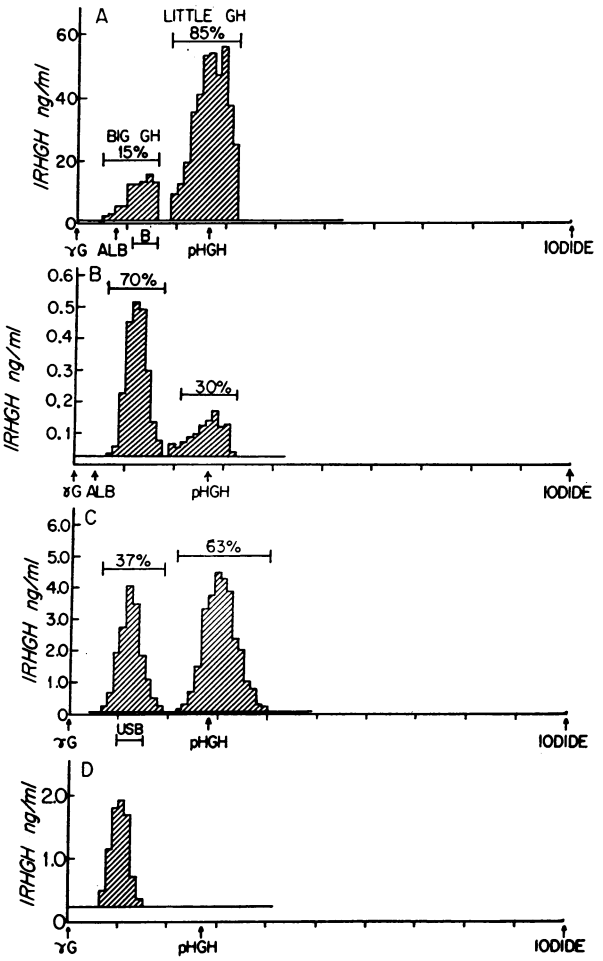

FIGURE 2 Gel filtration studies (Sephadex G-75) of plasma from acromegalic patient B. L. (A) Gel filtration in barbital buffer of 10 ml of frozen plasma from an acromegalic patient. Recovery of filtered IRGH was 100%. (B) Gel filtration in barbital buffer of 1 ml of the fraction of BGH labeled "B" in Fig. 2A, after 40 h storage at 4°C. Recovery of filtered IRGH was 102%. (C) Gel filtration in 8 M urea of 12 ml of fraction "B" from Fig. 2A. Recovery of filtered IRGH was 116%. (D) Gel filtration in 8 M urea of 12 ml of urea-stable BGH (USB) from Fig. 2C. Recovery of filtered IRGH was 90%.

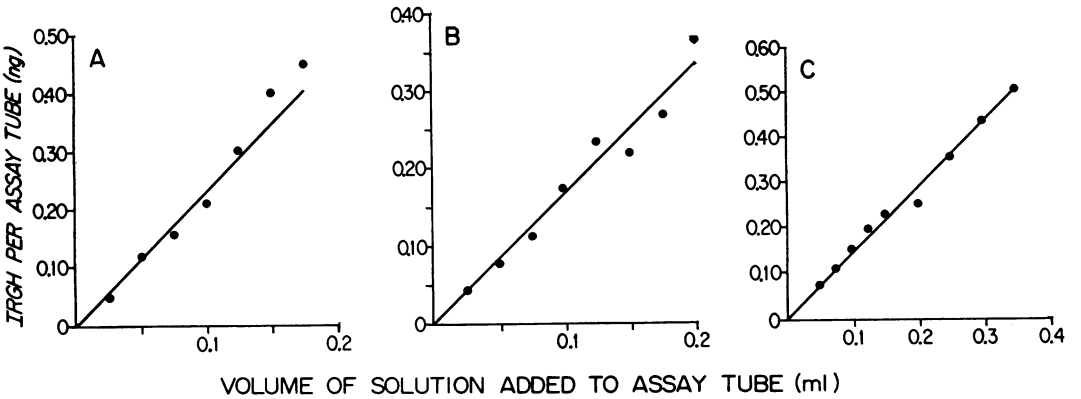

FIGURE 3 Serial dilution studies of the immunoreactivity of three forms of LGH prepared from a saline extract of human pituitary, in an assay system in which N.I.H. immunochemical grade pituitary HGH was employed as the standard. In each study, if the B/F curve of the material being assayed was identical with that of the HGH standard, all of the points would fall along the straight line. (A) Study of LGH extracted from untreated saline extract of pituitary. (B) Study of LGH generated by exposure of pituitary BGH to 8 M urea. (C) Study of LGH generated by treatment of urea-stable BGH with trypsin.

during freezing and thawing with consequent liberation of LGH, and one of which is not dissociated under these conditions.

Relation of urea-stable BGH to freeze-stable BGH. Since both 8 M urea and freeze-thaw treatment appear to reveal the presence of a labile and a stable form of BGH, studies were performed to determine if the urea-stable fraction of BGH is identical to the freeze-stable fraction. To investigate this possibility, a sample of urea-stable BGH was dialyzed free of urea, subjected to repeated freezing and thawing, and refiltered in barbital buffer (Fig. 6A). It was found that all of the material eluted as BGH, indicating that the urea-stable BGH was entirely freeze-stable. Similarly, an aliquot of freeze-stable BGH was subjected to gel filtration in 8 M urea and most of this (86%) ran as BGH, indicating that most of the freeze-stable BGH was urea-stable (Fig. 6B). These results suggest that most, if not all, of the freeze-stable BGH appears to be identical with the urea-stable fraction.

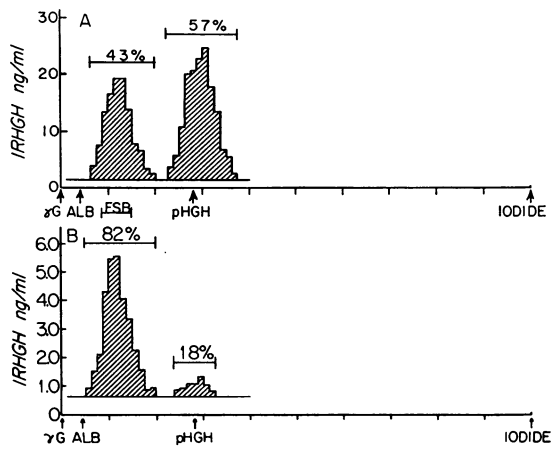

FIGURE 4 Gel filtration studies (Sephadex G-75) of the effect of freezing and thawing on pituitary BGH. (A) Gel filtration in barbital buffer of 11 ml of the fraction of pituitary BGH designated "B" in Fig. 1A, after repeated freezing and thawing. Recovery of filtered IRGH was 117%. (B) Gel filtration in barbital buffer of 10 ml of the fraction designated "FSB (freeze-stable BGH)" in Fig. 4A, after repeated freezing and thawing. Recovery of filtered IRGH was 73%.

The urea-stable and freeze-stable form of BGH does not appear to dissociate on standing in barbital buffer, for on many occasions we have rechromatographed samples of this form of BGH after they had stood in barbital buffer at 4°C for several days, and noted less than 5% conversion to LGH (Figs. 1D, 2D, 5D, 6A, and 8B). Thus the fraction of BGH that tends to dissociate on prolonged standing in barbital buffer (Figs. 2B and 5B) presumably represents a portion of the fraction of BGH subject to dissociation by 8 M urea and freezing.

Sucrose density centrifugation of urea-stable BGH. To assess the molecular weight of pituitary urea-stable BGH by a technique other than gel filtration, an aliquot of this material was subjected to centrifugation in a 5–20% sucrose density gradient. As seen in Fig. 7A, after centrifugation at 50,000 rpm for 17 h, urea-stable BGH appeared as a discrete peak between [^{125}I]-albumin and [^{125}I]pHGH, at a position indicative of a mol wt of approximately 40,500 (11). This corresponds closely to the molecular weight of BGH as estimated by gel filtration (1). When an aliquot of

pituitary LGH was centrifuged, it migrated identically with [^{125}I]pHGH (Fig. 7B).

Effect of trypsinization of urea-stable BGH. To investigate the possibility that urea-stable BGH might consist of LGH bound to another moiety by a peptide

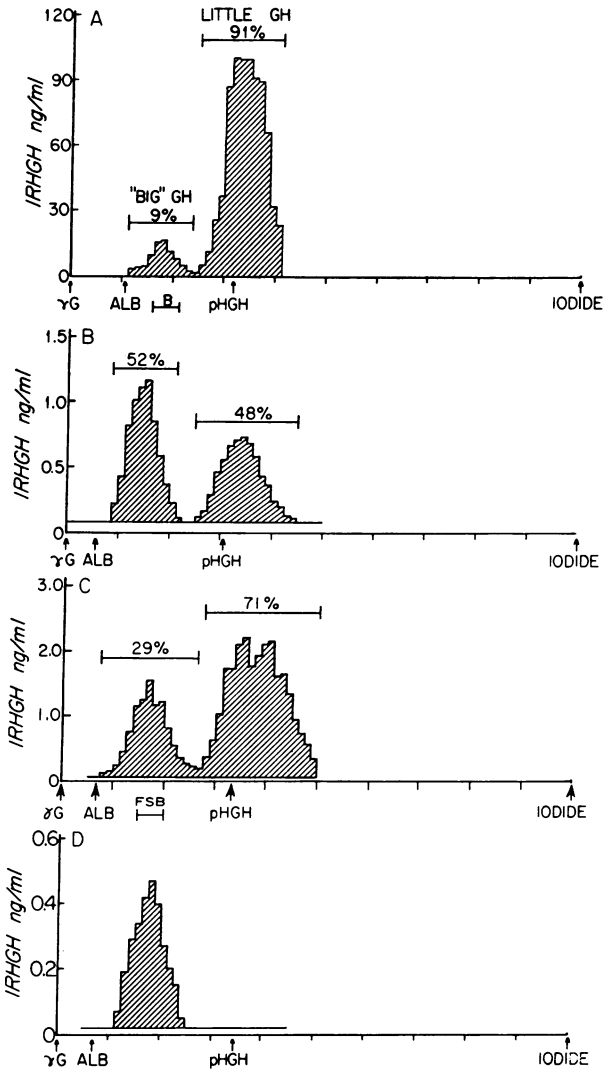

FIGURE 5 Gel filtration studies (Sephadex G-75) on the effect of freezing and thawing on BGH from plasma. (A) Gel filtration in barbital buffer of 10 ml of acromegalic plasma from patient B. L. Recovery of filtered IRGH was 169%. (B) Gel filtration in barbital buffer of 2.5 ml of the fraction of BGH designated "B" in Fig. 5A, after 40 h storage at 4°C. Recovery of filtered IRGH was 140%. (C) Gel filtration in barbital buffer of 12 ml of the fraction of BGH designated "B" in Fig. 5A, after repeated freezing and thawing. Recovery of filtered IRGH was 91%. (D) Gel filtration in barbital buffer of 12 ml of the fraction designated freeze-stable BGH (FSB) in Fig. 5C, after repeated freezing and thawing. Recovery of filtered IRGH was 68%.

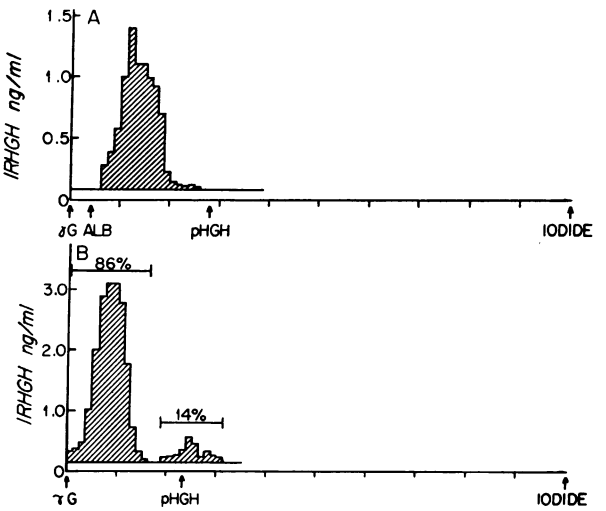

FIGURE 6 Study of the relation between urea-stable BGH and freeze-stable BGH. (A) Gel filtration in barbital buffer of 12 ml of urea-stable BGH, dialyzed free of urea, and subjected to repeated freezing and thawing. Recovery of filtered IRGH was 64%. (B) Gel filtration in 8 M urea of 12 ml of freeze-stable BGH. Recovery of filtered IRGH was 95%.

bond, we studied the effects of trypsin on this fraction. In this study we decided to employ a concentration of trypsin that would not cause significant destruction of LGH itself. To determine the appropriate concentration, in preliminary studies an aliquot of pituitary LGH was exposed to varying concentrations of trypsin for 30 min at 37°C, the reaction stopped by the addition of soy bean trypsin inhibitor, and the IRGH concentration measured and compared with that of an untrypsinized aliquot. It was found that there was a 64% decrease in IRGH after incubation with 200 μg/ml trypsin, a 35% decrease with 100 μg/ml, and an 18% decrease with 20 μg/ml, but there was no diminution in IRGH after incubation with 2 μg/ml. Incubation of pituitary urea-stable BGH for 30 min with 2 μg/ml of trypsin also caused no decrease in total IRGH concentration. Subsequently, we performed our studies on the conversion of urea-stable BGH to LGH with either 1.0 or 1.5 μg/ml trypsin.

An aliquot of urea-stable BGH was prepared from a saline extract of human pituitary (subject H. L.) by the methods described previously, and the material was dialyzed against barbital buffer to free it of urea. Trypsin was added to one portion in a concentration of 1 μg/ml and it was incubated for 30 min at 37°C, after which 2 μg/ml soy bean trypsin inhibitor was added. To a second portion, we added the trypsin inhibitor and trypsin simultaneously, before the 30-min incubation, and hence this portion was not exposed to effective trypsinization. Both aliquots were then gel-

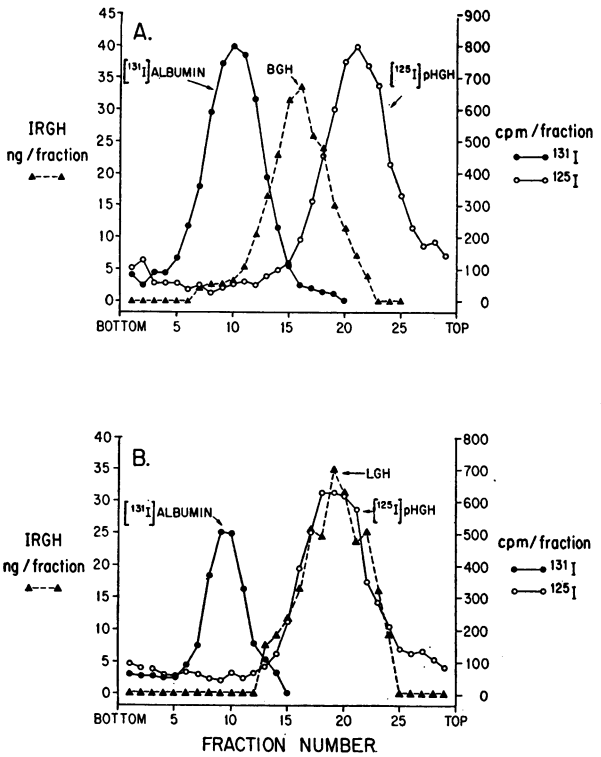

FIGURE 7 Sucrose gradient centrifugation of urea-stable BGH and of LGH. (A) Centrifugation of pituitary urea-stable BGH, [^{131}I]albumin and [^{125}I]pHGH in a 5–20% sucrose gradient. Fractions in which the IRGH content is indicated as zero contained less than 2 ng. (B) Centrifugation of pituitary LGH, [^{131}I]albumin and [^{125}I]pHGH in a 5–20% sucrose gradient. Fractions in which the IRGH content is indicated as zero contained less than 7 ng.

filtered in barbital buffer. In the sample of trypsinized BGH, 36% ran as LGH, indicating that trypsin had converted some of the BGH to LGH (Fig. 8A), whereas in the untrypsinized sample, almost all of the BGH retained its identity (Fig. 8B). Similarly, trypsinization of urea-stable BGH from a saline extract of the pituitary of another subject (J. L.) converted 48% of the BGH to LGH; trypsinization of urea-stable BGH from immunochemical grade HGH converted 44% to LGH; and trypsinization of urea-stable BGH from the plasma of an acromegalic patient (B. L.) converted 59% to LGH.

Since a large fraction of urea-stable BGH was not converted to LGH by trypsinization (Fig. 8A), the possibility was considered that urea-stable BGH may consist of two separate components, which differ in their response to trypsin. To investigate this possibility, an aliquot of the BGH that retained its identity after trypsinization ("TSB" in Fig. 8A) was again exposed to trypsin and only 13% was converted to LGH (Fig. 8C). For comparison, in a simultaneous experiment an aliquot of BGH, not exposed to effective trypsinization previously, was treated with trypsin under identical conditions, and 42% was converted to LGH (Fig. 8D). In two other experiments in which initial treatment with trypsin converted 48–52% of urea-stable pituitary BGH to LGH, on re-exposure of the trypsin-stable BGH to trypsin, there was only 14–24% conversion to LGH.

To further study this problem, we performed an additional study, depicted in Fig. 9. An aliquot of urea-stable BGH was trypsinized with conversion of 51% to LGH (Fig. 9A). An aliquot of the BGH that maintained its identity despite trypsinization ("TSB" in Fig. 9A) was then retrypsinized and only 16% was converted to LGH (Fig. 9B). The BGH that maintained its identity after this second trypsinization ("TSB" in Fig. 9B) was again exposed to trypsin and essentially the same proportion, 17%, was converted to LGH (Fig. 9C). These observations suggest that urea-stable BGH may consist of at least two components, one readily convertible by trypsin to LGH, and a second less readily convertible.

We next attempted to determine if trypsinization converted some of the urea-stable BGH to a urea-labile form. A portion of urea-stable BGH was trypsinized and on gel filtration, 40% ran as LGH. The portion that maintained its identity as BGH after trypsinization was then run on the column in 8 M urea and only 4% of it eluted as LGH. Thus trypsin does not appear to transform urea-stable BGH to a form of BGH that dissociates in urea.

LGH generated by trypsinization of urea-stable BGH from pituitary was compared with LGH found in an untreated saline extract of pituitary, and in a dilution study it was found to have a similar B/F curve (Fig. 3C).

DISCUSSION

As illustrated in Table I, we have found a major component of LGH and minor component of BGH in every sample of human plasma and pituitary we have studied. These include plasma samples from eight acromegalic and four normal subjects, one barbital and two saline extracts of pituitary, and clinical grade and immunochemical grade preparations of pituitary GH.

In the present study, it was observed that over half of the BGH complex found in the pituitary and plasma migrated as LGH after exposure to 8 M urea (Figs. 1C and 2C) or after repeated freezing and thawing (Figs. 4A and 5C). The fraction of BGH not susceptible to dissociation by urea appears to be very similar to or identical with the fraction not converted to LGH by freezing, for all of a sample of urea-stable BGH was found to be freeze-stable, and most of an aliquot of

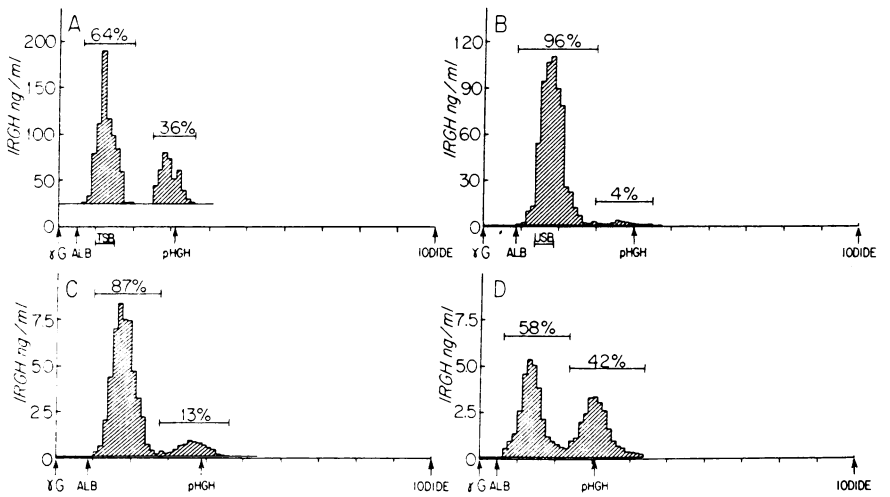

FIGURE 8 Study of the effect of trypsin on urea-stable BGH previously prepared from a saline extract of pituitary. (A) Gel filtration in barbital buffer of 6 ml of urea-stable BGH, immediately after a 30-min incubation with trypsin (1 μg/ml) and subsequent addition of trypsin inhibitor (2 μg/ml). Recovery of filtered IRGH was 112%. (B) Gel filtration in barbital buffer of 3 ml of urea-stable BGH immediately after a 30-min incubation with inactivated trypsin. (Trypsin inhibitor, 2 μg/ml, was added to the sample along with trypsin 1 μg/ml, before the incubation.) Recovery of filtered IRGH was 102%. (C) Gel filtration in barbital buffer of 2 ml of trypsin-stable BGH (TSB) from Fig. 8A, immediately after the same treatment employed in study A. The recovery of filtered IRGH was 100%. (D) Gel filtration in barbital buffer of 4 ml of the fraction designated urea-stable BGH (USB) in Fig. 8B, immediately after the same treatment as that employed in study A. Recovery of filtered IRGH was 102%.

freeze-stable BGH was found to be stable in urea (Fig. 6). Since urea and freezing can dissociate subunits linked by noncovalent bonds (8, 10), but are not known to disrupt covalent bonds, it is likely that the fraction of BGH that is convertible to LGH by urea or freezing consists of LGH bound to another subunit by noncovalent bonds, and that the stable fraction of BGH consists of LGH bound to another moiety by a covalent bond or by relatively strong noncovalent bonds.

Both forms of the BGH complex elute from Sephadex at a point consistent with a molecular size about twice that of LGH. However, factors other than size can affect the migration of proteins on gel filtration (11), and therefore we turned to centrifugation as an alternative means of molecular sizing. In the study illustrated in Fig. 7A, we centrifuged urea-stable BGH in a sucrose gradient and found its sedimentation rate to be consistent with a mol wt of 40,500, almost twice that of LGH, consistent with the results obtained on Sephadex chromatography.

It is possible that the form of BGH dissociated by urea and by freezing may consist at least in part of the dimer form of LGH, for it is known that LGH dimerizes to some extent when allowed to stand in concentrations over 5 mg/ml (12), and the intracellular concentration of GH in the human pituitary probably exceeds this level (13). Since the dimer form of LGH dissociates slowly when allowed to stand in low concentration (12), this cound account for the tendency of extracted BGH to undergo partial conversion to LGH on prolonged standing in barbital buffer. It is also possible that some of the urea-labile form of BGH may consist of LGH bound by noncovalent bonds to some other peptide, in which case this form of BGH would then be analogous to thyroid-stimulating hormone, luteinizing hormone, and chorionic gonadotropin (14).

To investigate the nature of the urea-stable BGH extracted from pituitary, we exposed a portion of this fraction to trypsin and it was observed that mild trypsinization converted 36–52% of it to LGH. Similarly, 59% of urea-stable BGH from a sample of acromegalic plasma was converted to LGH by trypsin. The LGH generated by trypsinization of pituitary BGH probably is very similar to or identical with the LGH present in untreated pituitary extracts, for in addition to having the same filtration characteristics, both forms have the same B/F curves (Fig. 3). Since trypsin specifically cleaves peptide bonds involving a lysine or arginine carboxyl group (15), our results suggest that much of urea-stable BGH may consist of LGH linked at its amino terminus to lysine or arginine in a second

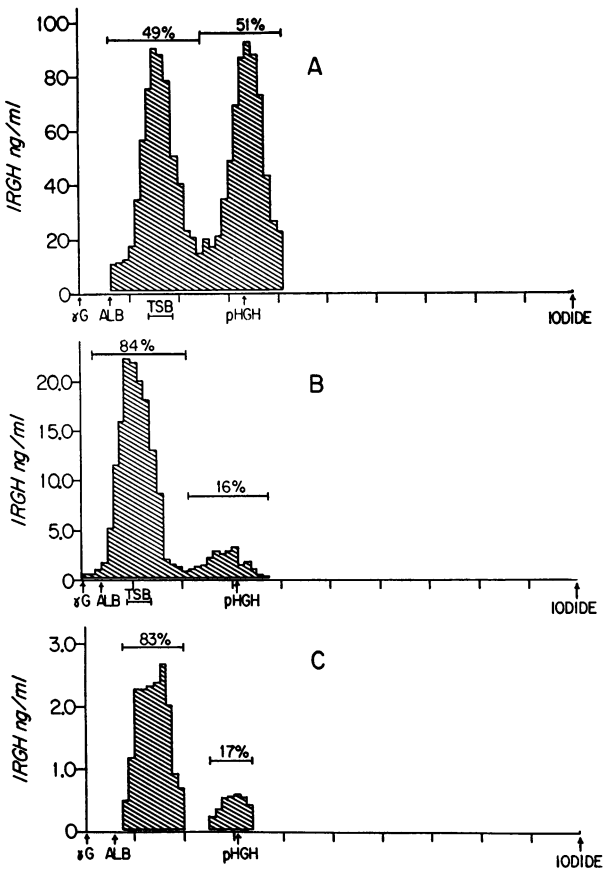

FIGURE 9 Study of the effects of repeated trypsinization on urea-stable BGH from pituitary. (A) Gel filtration in barbital buffer of 4 ml of urea-stable BGH immediately after a 45-min incubation with trypsin (1.5 μg/ml). Recovery of filtered IRGH was 80%. The same preparation of urea-stable BGH was used as in the study depicted in Fig. 8. (B) Gel filtration in barbital buffer of 10 ml of trypsin-stable BGH (TSB) from A immediately after the same treatment employed in A. Recovery of filtered IRGH was 116%. (C) Gel filtration in barbital buffer of 10 ml of fraction TSB from Fig. 8B, immediately after the same treatment as in A. Recovery of filtered IRGH was 93%.

peptide. The portion of urea-stable BGH that can be cleaved to LGH by trypsin is analogous to proinsulin (16), big gastrin (17), and big ACTH (18), and it is possible that this form of BGH is a precursor or prohormone of LGH in the pituitary, which is converted to LGH through the action of a trypsinlike enzyme. Alternatively, it is possible that the BGH dissociated by trypsin consists of a tightly bonded LGH dimer, and that trypsin renders it unstable by cleaving off a small portion of the monomeric components, thereby causing dissociation of the dimer. BGH itself may be derived from a larger hormone precursor, such as the very large IRGH demonstrated by Frohman, Burek, and Stachura in human pituitary, which appears to have a molecular weight of over 200,000 (19, 20).

In repeated experiments, trypsin converted 36–52% of urea-stable pituitary BGH to LGH, but when the BGH not converted by initial trypsinization was retrypsinized under the same conditions, only 13–24% was converted to LGH. In one experiment, a portion of BGH that had been exposed to trypsin twice was exposed a third time, and again 17% was converted to LGH (Fig. 8). These observations suggest that there may be two forms of urea-stable BGH, one of which is less readily dissociated by trypsin than the other.

In the present study, we have directed our attention to the fraction of IRGH eluting from Sephadex between albumin and LGH, which we have designated BGH. However, there is also a fraction of IRGH in plasma and in pituitary extracts that elutes with or before albumin, noted by us as well as by Berson and Yalow (3), Bala, Ferguson, and Beck (2), and Gorden, Hendricks, and Roth (4). In our study this material, which generally appears as a broad "shoulder" rather than a peak, has comprised on the average only 3% of total IRGH in plasma and 4% in the pituitary extracts. It may represent aggregates of LGH or BGH, or consist of LGH bound to some other moiety. Some of it may be identical with the very large IRGH identified by Frohman, Burek, and Stachura (19, 20).

We do not currently know the biological and clinical significance of the existence of multiple forms of BGH. Gorden et al. have recently found that the BGH complex of human pituitary and plasma is much less effective than LGH in displacing ^{125}I-labeled pituitary LGH from the GH receptors of cultured human lymphocytes (5). In unpublished work, we have performed receptor assay studies on preparations of BGH complex containing 40–51% urea-stable BGH, derived from saline extracts of two pituitaries and from immunochemical grade HGH (NIH).[8] These three BGH complex preparations were found to be 31–40% as effective as LGH in displacing [^{125}I]LGH from cell membrane receptors of pregnant rabbit liver, by the method of Tsushima and Friesen (21). In contrast, the urea-stable BGH derived from these BGH complex preparations was 62–65% as effective as LGH. Similarly, in receptor assay studies employing cell membranes from pregnant rabbit mammary gland (22) and from rat liver (21), the BGH complex preparations were only 20–30% as effective as LGH in displacing the [^{125}I]-LGH, whereas the urea-stable BGH fractions were 55–67% as effective as LGH. These data are consistent with the idea that there are at least two forms

[8] V. Soman and A. D. Goodman. Unpublished observations.

of BGH, and raise the possibility that they may differ in biologic activity.

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REFERENCES

1. Goodman, A. D., R. Tanenbaum, and D. Rabinowitz. 1972. Existence of two forms of immunoreactive growth hormone in human plasma. *J. Clin. Endocrinol. Metab.* **35**: 868–878.
2. Bala, R. M., K. A. Ferguson, and J. C. Beck. 1970. Plasma biological and immunoreactive human growth hormone-like activity. *Endocrinology.* **87**: 506–516.
3. Berson, S. A., and R. S. Yalow. 1971. Heterogeneity of peptide hormones in plasma as revealed by radioimmunoassay. Proceedings of the XI Reunion of French-Speaking Endocrinologists, Masson et Cie., Paris, 239–269.
4. Gorden, P., C. M. Hendricks, and J. Roth. 1973. Evidence for "big" and "little" components of human plasma and pituitary growth hormone. *J. Clin. Endocrinol. Metab.* **36**: 178–184.
5. Gorden, P., M. A. Lesniak, C. M. Hendricks, and J. Roth. 1973. "Big" growth hormone components from human plasma: decreased reactivity demonstrated by radioreceptor assay. *Science (Wash. D. C.).* **182**: 829–831.
6. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature. (Lond.).* **194**: 495–496.
7. Gerhart, J. C. 1967. Separation of subunits of proteins by zone centrifugation in a density gradient. *Methods Enzymol.* **11**: 187–194.
8. Kauzmann, W. 1959. Some factors in the interpretation of protein denaturation. *Adv. Protein Chem.* **14**: 1–63.
9. Olesen, H., and P. O. Pedersen. 1968. Gel filtration of albumin on Sephadex G-200 in urea. *Acta Chem. Scand.* **22**: 1386–1394.
10. Chilson, O. E., L. A. Costello, and N. O. Kaplan. 1965. Studies on the mechanism of hybridization of lactic dehydrogenases *in vitro*. *Biochemistry.* **4**: 271–281.
11. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* **236**: 1372–1379.
12. Squire, P. G., and K. D. Pedersen. 1961. The sedimentation behavior of human pituitary growth hormone. *J. Am. Chem. Soc.* **83**: 476–481.
13. Li, C. H., W. K. Liu, and J. S. Dixon. 1962. Human pituitary growth hormone. VI. Modified procedure of isolation and NH_2-terminal amino acid sequence. *Arch. Biochem. Biophys.* Suppl. **1**: 327–332.
14. Pierce, J. G. 1971. The subunits of pituitary thyrotropin —their relationships to other glycoprotein hormones. *Endocrinology.* **89**: 1331–1344.
15. Bergmann, M., and J. Fruton. 1941. The specificity of proteinases. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1**: 63–98.
16. Steiner, D. F., and P. E. Oyer. 1967. The biosynthesis of insulin and a probable precursor of insulin by a human islet cell adenoma. *Proc. Natl. Acad. Sci. U. S. A.* **57**: 473–480.
17. Yalow, R. S., and S. A. Berson. 1971. Further studies on the nature of immunoreactive gastrin in human plasma. *Gastroenterology.* **60**: 203–214.
18. Yalow, R. S., and S. A. Berson. 1973. Characteristics of "big ACTH" in human plasma and pituitary extracts. *J. Clin. Endocrinol. Metab.* **36**: 415–423.
19. Frohman, L. A., L. Burek, and M. E. Stachura. 1972. Characterization of growth hormone of different molecular weights in rat, dog, and human pituitaries. *Endocrinology.* **91**: 262–269.
20. Frohman, L. A., and M. E. Stachura. 1973. Evidence for possible precursors in the synthesis of growth hormones by rat and human fetal anterior pituitary *in vitro*. *Mt. Sinai J. Med.* **40**: 414–421.
21. Tsushima, T., and H. G. Friesen. 1973. Radioreceptor assay for growth hormone. *J. Clin. Endocrinol. Metab.* **37**: 334–337.
22. Shiu, R. P. C., P. A. Kelly, and H. G. Friesen. 1973. Radioreceptor assay for prolactin and other lactogenic hormones. *Science (Wash. D. C.).* **180**: 968–971.