The major desamido form of human growth hormone (hGH) results from deamidation of asparagine 152. Peptide mapping and amino acid sequencing were used in the identification. This desamido form (hGH<sub>Asp152</sub>) could be produced by incubation of the deamidated hormone in an alkaline medium. Another minor desamido form which contained glutamic acid at 137 (hGH<sub>Glu137</sub>) also was identified in preparations of hGH. This form was not produced by alkaline treatment of hGH.

Limited hydrolysis of hGH with subtilisin produced two cleaved forms, one with cleavages at positions 139 and 149 and another with cleavages at 139 and 146. hGH<sub>Asp152</sub> underwent only one type of modification, cleavage at positions 139 and 146. Presence of hGH<sub>Glu137</sub> resulted in cleavages in the region of 129 to 149 identical with those noted with hGH, but in addition, proteolysis had occurred in the region of 95 to 127, an area where hGH was not attacked by subtilisin. That Glu at 137 modified cleavage points was also indicated by the greater resistance of hGH<sub>Glu137</sub> to hydrolysis by subtilisin as compared to hGH.

The results demonstrate that deamidation can alter points of proteolytic cleavage. If proteolytic processing of hGH is found to be of physiological significance, deamidation may be a way of directing specific cleavages.

All preparations of human growth hormone contain a component that has been assumed to be a deamidated form of the hormone. Structure analysis has never confirmed this assumption and in fact the notion that hGH deamidates at the same electrophoretic mobility as this presumed deamido form in extracts of pituitary tissue taken at surgery, presence in fresh tissue would suggest a physiological significance, deamidation may be a way of directing specific cleavages.

**EXPERIMENTAL PROCEDURES**

**Electrophoretic Techniques**

Two disc electrophoresis systems were used to follow the purification of the deamidated proteins and to assess homogeneity: the method of Davis (2) where separation takes place near pH 10 and a procedure of Rodbard and Chrambach (3) where the separation occurs at pH 7.8. Staining was done by the procedure of Dieret al. (4).

Electrophoresis in sodium dodecyl sulfate (5) with and without reduction of the test material with 2-mercaptoethanol was useful in detecting proteolytically cleaved forms (6). Staining was done for 16 h in 0.1% Coomassie brilliant blue R-250 dissolved in methanol/glacial acetic acid/water (5:1:5). Destaining was done by diffusion in isopropanol/glacial acetic acid/water (10:7:83). Molecular weights were estimated by comparing mobilities with a commercially available (Gallard-Schlesinger) molecular weight marker made up of polymeric units of lysozyme.

**Isolation Procedures**

**Purification of hGH<sub>Asp152</sub>**—The starting material for isolation of this substance was a partially purified preparation of hGH (7). Briefly, the procedure for preparation of this type of hGH involves extraction of fresh frozen pituitary glands with 0.9% saline, precipitation of the hormone with 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and chromatography of the precipitated material on Sephadex G-100 with development with 0.05 M NH<sub>4</sub>HCO<sub>3</sub>. The monomer peak contained the hGH<sub>Asp152</sub> and the material was rechromatographed on Sephadex G-50 in 0.05 M NH<sub>4</sub>HCO<sub>3</sub> to remove low molecular weight components that failed to separate during chromatography on Sephadex G-100. Since recovery of the monomer material by Sephadex chromatography has been described (7), the elution patterns are not shown. Disc electrophoresis (pH 7.8 system) was used to locate the monomer material.

The monomer peak from the second chromatography on Sephadex G-50 was next chromatographed on DEAE-cellulose (Whatman D32). Fig. 1a shows the elution pattern for the chromatography. The column was packed with exchanger, previously equilibrated with 0.01 M NH<sub>4</sub>HCO<sub>3</sub>, pH 8 (conductivity = 1.0 mmho). A flow rate of 1 ml/min for a column (2 x 17 cm) was used. The sample, 800 mg of monomer peak, was dissolved in 0.01 M NH<sub>4</sub>HCO<sub>3</sub>, pH 8, and water was added if the conductivity was much higher than 1.0 mmho. Once the sample had been introduced, a gradient was begun (G-1, Fig. 1a). The gradient was made by mixing 200 ml each of solutions of NH<sub>4</sub>HCO<sub>3</sub> with conductivities of 1.0 and 9 mmho. A series of minor peaks was eluted with this gradient but none had an electrophoretic mobility near that of hGH. This part of the elution pattern, therefore, is not shown in Fig. 1a. After this gradient, the column was eluted with NH<sub>4</sub>HCO<sub>3</sub> with a conductivity of 9 mmho. This eluted hGH and a deamidated form (hGH<sub>Glu137</sub> described below) which was different from hGH<sub>Asp152</sub>. At the position indicated as G-2 of Fig. 1a, a second gradient was begun. This was made by mixing 200 ml each of solutions of NH<sub>4</sub>HCO<sub>3</sub> with conductivities of 9 and 27 mmho. Shortly after...
Purification of hGHhp152 from Intentionally Deamidated hGH.

As indicated in the next section, incubation of hGH in 0.05 M NH4HCO3, pH 8.5, produced a component with the same electrophoretic mobility as hGHhp152 isolated from a preparation of hGH. To isolate this component from the incubation mixture, chromatography on DEAE-cellulose was used. The column and conditions were the same as those used for purification of hGHhp152 (Fig. 1b).

Intentional Deamidation

Samples of hGH that were essentially free of faster migrating electrophoretic components were dissolved in 0.05 M NH4HCO3 whose pH was adjusted to pH 8.0 with NH4OH. The solution of hormone (3 mg/ml) was made 1 mM in both DFP and NaN3 and incubated at 37 °C. Aliquots were removed every 24 h to determine the degree of conversion of the major component to more acidic components. Electrophoresis at pH 10 was used to monitor the conversion. The incubation was stopped after 66 h when the major portion of the hGH had been converted to acidic forms. Electrophoresis in SDS under reducing conditions indicated that no proteolytic conversion had occurred to convert the hGH to a two-chain structure (6). An incubation mixture such as this was used also as starting material for isolation of the major acidic form produced during the incubation (see “Purification of hGHhp152 from Intentionally Deamidated hGH”).

The procedure used by Skyrle et al. (1) in an attempt to produce deamidation was also used. Here, a 5 mM DFP solution of hGH was made in 0.1 M KOH and incubated 8 h at 50 °C. The solution was analyzed directly by electrophoresis. The study was done with and without DFP (1 mM).

Peptide mapping was done on thin layer plates coated with Avicel 400 microcrystalline cellulose, 0.1 mm (Birckmann). Plates were eluted first with 5% pyridine and then with 1.5% formic acid. After applying the digest, chromatography was done in the first direction with n-butyl alcohol/glacial acetic acid/pyridine/water (15:3:12:10). When dry, the plate was subjected to electrophoresis at pH 4.4 in a buffer of water/acetic/glacial acetic acid/pyridine (40:8:2:1) for 2 h at 250 v. Ethanesulfonate was done on a supporting plate cooled to 15 °C.

When peptides were to be eluted for sequencing and amino acid analysis, detection was done with fluorescamine as described by Fleer et al. (9). Staining for detection only was done with a cadmium-methyl green spray (10).

Microsequencing—For sequencing, the fluorescamine-positive spot was outlined in pencil, and the cellulose was scraped from the plate and eluted with 50% pyridine on a small sintered glass funnel. The eluate was collected in the tubes to be used for microsequencing (11) and evaporated to dryness in a vacuum desiccator. This sequencing procedure makes use of 4-N,N-dimethylaminoazobenzene-4'-sulfonic acid and can easily distinguish Glu from Gln and Asp from Asn.

Total Amino Acid Composition—For total amino acid analysis, the fluorescamine-positive spot was eluted on a small sintered glass funnel with 6 N HCl containing 0.02% 2-mercaptoethanol and a small crystal of phenol. Elution was made directly into the tube to be evacuated and sealed for the hydrolysis. Hydrolysis was for 20 h at 110 °C. Amino acid analysis was done by single-column methodology.

Digestion with Subtilisin

The growth hormone was treated with crystalline subtilisin (Novo) sold under the name of Protease (Kabi-Vitrum) purchased from Cahico-Behring. The hormone was dissolved in 0.05 M NH4HCO3 to give a 2-mg/ml solution and proteolysis was carried out with an enzyme/substrate ratio of 1.7000 for 3 h at room temperature. The reaction was stopped by making the solution 1 mM in DFP.

To remove low molecular weight peptides from the cleaved hGH, the digest was immediately chromatographed on a column of Sephadex G-100 in 0.05 M NH4HCO3 (Fig. 2). For 2 mg of digest, a column bed (0.9 × 56 cm) was used; 2.5 ml of effluent was collected per tube. The major peak which contained the cleaved hGH was recovered by lyophilization.

RESULTS

Purification of the Desamido Forms—For the sake of clarity, we use here the designations of hGHhp152 and hGHhp137 for the two deamidated forms even though evidence for this is not given until later on in this paper. Fig. 3 compares the electrophoresis patterns (pH 7.8) of hGH, hGHhp152, and hGHhp137; Rf values were 0.42, 0.49, and 0.52, respectively. hGHhp137 is the more acidic of the two desamido forms, yet it elutes from DEAE-cellulose before the major portion of hGHhp152. There was always some hGHhp152 mixed with the hGHhp137 isolated by the first DEAE-cellulose chromatography step (Fig. 1c), so a second DEAE-cellulose column was required to remove this small amount of hGHhp152 (Fig. 1c). The hGHhp152-rich fraction from the first DEAE-cellulose column always contained other more acidic forms of hGHhp137.
The cleaved forms are designated by an hGH, hGHAsplS2, and hGHcUla7, respectively. Patterns of the subtilisin digests of hGH, hGHA.plSP and hGHcUla7, respectively. digestion with subtilisin. Patterns hGH to faster migrating components during incubation in a condition (patterns not shown) the two desamido modifications migrated to the column and developed with 0.05 M NH4HCO3. Peak 1 was aggregated material and was not used; peak 2 contained the cleaved hormone and this fraction was used for peptide mapping. Peak 3 was made up of low molecular weight fragments produced by the cleavage with subtilisin.

FIG. 2. Separation of low molecular weight fragments from cleaved forms of hGH by chromatography on Sephadex G-100. The sample of subtilisin-digested hormone was applied to the column and developed with 0.05 M NH4HCO3. Peak 1 was aggregated material and was not used; peak 2 contained the cleaved hormone and this fraction was used for peptide mapping. Peak 3 was made up of low molecular weight fragments produced by the cleavage with subtilisin.

FIG. 3. Electrophoresis patterns (pH 7.8 system) obtained with hGH and two desamido forms before and after limited digestion with subtilisin. Patterns a, b, and c are for untreated hGH, hGHAsplS2 and hGHcUla7, respectively. Patterns d, e, and f are of the subtilisin digests of hGH, hGHAsplS2 and hGHcUla7, respectively. The cleaved forms are designated by an S. In pattern f, I represents undigested hGHGlu137.

hGH, so here again a second column chromatography (Fig. 1b) was required to obtained hGHAsplS2 of the purity shown in Fig. 3.

The patterns of Fig. 3 show that the hGH (Pattern a) contained about 5% of hGHAsplS2 and about the same amount of a component with a mobility of what we have denoted as “Fast-hGH” (12). We found it extremely difficult to free hGH of the Fast-hGH and there was always a spontaneous formation of hGHAsplS2 during work-up of column fractions. The patterns of hGHAsplS2 and hGHGlu137 (patterns b and c) also show that each of these contained a component which could be a Fast-form and a band which could have been formed by deamidation of a second residue of Gln or Asn.

When analyzed by SDS electrophoresis under reducing conditions (patterns not shown) the two desamido modifications migrated as single components with no evidence of cleaved forms (6). Under reducing conditions, proteolytically altered forms, ones where the peptide chain has been cleaved in the large disulfide loop, dissociate to fragments with molecular weights near 17,000 and 5,000.

Intentional Deamidation of hGH—Fig. 4, a and b, show the disc electrophoresis patterns (pH 10) of the conversion of hGH to faster migrating components during incubation in a 0.05 M NH4HCO3 buffer, pH 8.5. Pattern a is of untreated hGH; pattern b is of the same preparation after incubation at 37 °C for 66 h. The figure also shows the SDS electrophoresis patterns of the incubated material analyzed without (c) and with (d) 2-mercaptoethanol. Evidence of a small amount (<3%) of enzymatically altered material can be seen but the sample of hGH used as starting material for the incubation showed this quantity of cleaved hGH.

To isolate the first deamidated form (the major band of pattern b in Fig. 4) the incubated sample was chromatographed on a DEAE-cellulose column under conditions identical with those used to purify hGHAsplS2. The material that was isolated had essentially the same degree of purity as the hGHAsplS2 shown in Fig. 3. This type of material was used for peptide mapping. We were unable to isolate sufficient quantities of the second deamidated form seen in pattern of Fig. 4b to carry out unambiguous peptide mapping experiments.

We repeated the incubation conditions used by Skyler et al. (1) where they were unable to detect formation of more acidic components. Here the hGH was heated for 8 h at 50 °C in 0.01 M KOH. When we used highly purified hGH, material comparable to that obtained by chromatography on DEAE-cellulose (Fig. 1a), we noted the same type of conversion to more acidic components as we did when the pH 8.5 buffer was used (Fig. 4). Quite different results were obtained if we used a less highly purified sample of hGH; for example, the starting material used for DEAE-cellulose chromatography and if we omitted DFP during the incubation. The results are given in Fig. 5 where the patterns obtained with three different electrophoresis systems are shown. Patterns a and b were done with the pH 10 system, c and d with the pH 7.8 system, and e and f in SDS. As reported by Skyler et al. (1), no discrete new components were seen by disc electrophoresis in the incubated samples (patterns b and d) but instead a smear was seen. Patterns a and c are of the unincubated sample of hGH. Electrophoresis in SDS (patterns e and f) indicated production of at least four new, low molecular weight fragments in

FIG. 4. Electrophoresis patterns of hGH before and after incubation at pH 8.5, 37 °C for 66 h. Patterns a and b were obtained by the pH 10 electrophoresis system; a is hGH before incubation and b is the same preparation at the end of the incubation. Patterns c and d were from electrophoresis in SDS; c is the incubated sample analyzed without 2-mercaptoethanol and d is the same material run under reducing conditions.
Deamidation of Growth Hormone

Fig. 5. Electrophoresis patterns obtained with a partially purified sample of hGH before and after incubation for 8 h, 50 °C, in 0.01 M KOH. Patterns a and b were obtained with the pH 10 electrophoresis system; c is the unincubated hGH, and d is the incubated sample. Patterns e and f were obtained with the pH 7.8 electrophoresis system; c is the unincubated hGH, and d is the incubated sample. Patterns e and f were done with the SDS electrophoresis system with reducing conditions; e is the unincubated sample, and f is the incubated material.

the incubated sample pattern (f). Pattern e is of the unincubated hGH. When DFP was added to the incubation mixture, smearing was absent and discrete bands were seen in the disc electrophoresis patterns and there was no indication of new components in the SDS gels. An explanation for the results is that the partially purified sample contained a proteinase that degraded the hGH during the incubation. Contamination of preparations of growth hormone with proteinases has been known for some years (13).

Peptide Mapping

hGH—Fig. 6a shows a photograph and Fig. 6b a tracing of a peptide map obtained with a tryptic digest of intact hGH. Reduction and alkylation were not carried out because there was concern that during these manipulations dehydration could occur as suggested by earlier work (14). The numbering of the tryptic peptides is the same as we used in a previous report (14) where characterization data were presented.

hGHAsp150—Fig. 6c shows a tracing of a map obtained with the acidic component, hGHAsp150, isolated from a preparation of hGH (Fig. 1b). The map was identical with the one obtained with hGH except that peptide 15 had shifted its position to 15d as indicated in the drawing. Amino acid analysis showed no difference between peptides 15 and 15d but amino acid sequencing by the method of Chang et al. (11) indicated an Asp at position 152 in peptide 15d instead of Asn as was found in peptide 15.

The same type of mapping was done for the major acidic component produced during incubation of hGH at pH 8.5 (see Fig. 4). Again the only difference was that peptide 15 had shifted to the more acidic peptide 15d.

hGHDha137—The tryptic map shown in Fig. 6d was obtained with another acidic form of hGH, denoted hGDha137, that was isolated from partially purified hGH (Fig. 1c). Peptide 15 was in its proper location but peptide 13 had shifted to a more acidic peptide, 13d. Amino acid analysis confirmed that peptide 13d had the composition of peptide 13 and sequencing indicated a Glu instead of Gln at position 137.

When hGH was intentionally deamidated by incubation at pH 8.5 and the major altered component was mapped, no 13d was noted even though peptide 15 had shifted to 15d. This indicates that alkaline treatment causes deamidation of Asp152 but not Gln137.

Subtilisin Digestion—We reported earlier (15) that when hGH is subjected to limited digestion with subtilisin, three modified forms are produced (S1, S2, and S3). Fig. 3d shows the disc electrophoresis pattern for such a digest. Note that about equal amounts of S1 and S2 were produced whereas only a small amount of S3 was formed. When hGHAsp150 was treated with subtilisin, only one major form was produced (Fig. 3c) and this corresponded to the S1 form. The S1 produced during digestion of hGH probably came, therefore, from the small amount of hGHAsp150 in the starting material (Fig. 3a). Digestion of hGDha137 resulted in the production of two principal bands as seen in Fig. 3f. An explanation for these electrophoretic patterns was obtained by peptide mapping of tryptic digests of hGH, hGHAsp150, and hGDha137.

Fig. 7a shows the peptide map obtained with subtilisin-treated hGH. Peptides 13, 14, and 15 were missing and instead 13a, 15a, and 15, were seen. Amino acid and sequence analyses showed the new peptides to have the sequences shown below. Subtilisin had cleaved hGH at 139 so that tryptic digestion gave rise to 13, which is 13 without the COOH-Lys. Peptide 14 was removed completely by the subtilisin digestion and subsequent Sephadex chromatography (Fig. 2). Peptides 15a, and 15, were a result of subtilisin cleavage of hGH at positions 146 and 149 and then tryptic cleavage at 159. In a previous study (15) where we had made peptide maps of the purified S1 and S2 forms (not the mixture as reported here), we showed...
The question arises whether the formation of desamido hGH has physiologic relevance or whether the deamidation is a result of the manipulations used in the isolation procedure. This is a difficult question to answer and at this time only indirect evidence can be cited to support a physiologic role. It was important to know whether such forms exist in the functioning pituitary gland. The nearest we have been able to come to answering this question is examination of pituitary tissue removed by surgery. A neutral saline extract of the tissue was applied to a disc electrophoresis column within 1 h of removal of the gland from the patient. A component, amounting to about 10% of the hGH, was observed that had the electrophoretic mobility of hGH_{Asp152} in both the pH 10 and 7.6 electrophoresis systems. Almost identical results were obtained (16) when extracts of hGH-containing granules, prepared from pituitary glands removed at autopsy, were examined by electrophoresis. Because hGH has been found to deamidate only slowly (17), it seems unlikely that deamidation occurred during the extraction. It would appear, therefore, that the hGH_{Asp152} was indeed a component of the functioning gland. Robinson and Rudd (18) cite evidence for in vivo existence of desamido forms of various proteins.

The greater growth-promoting activity of S_{3} compared to S_{1} and S_{2} (15) indicated that cleavage of a deamidated form produces hGH with enhanced bioactivity. At the time those studies were done, we were unaware of two desamido forms because the pH 10 electrophoresis system did not resolve them. Since S_{1} from hGH_{Asp152} and S_{2} from hGH_{Asp157} have the same electrophoretic mobilities in the pH 10 system, we cannot say which of the two was in the preparation we called “S_{1}”. When additional S_{3} becomes available, we must repeat the titlable assays to determine if it is S_{2} or S_{3} that has enhanced growth-promoting activity.

There is the possibility that deamidation of Gln_{157} may occur in the pituitary gland by means of a glutamine deamidase since we had no evidence of formation of a Glu at position 157 during incubation of hGH at pH 8.5. A pH near 8.5 was used throughout our isolation procedure of hGH_{Asp157}, so artificial production of Gln_{157} must have been minimal. The absence of Asp_{152} from hGH_{Asp157} also suggests that Gln_{157} deamidates by a different mechanism. Robinson and Rudd report (18) that in general deamidation of Gln-containing peptides occurs at a much slower rate than that noted for peptides having an Asn residue.

In the past it has been assumed that asparagine and glutamine have a static function similar to the other amino acids but Robinson and Rudd (18) suggest that these amides may play a dynamic role in the biological activities of proteins. Through their instability, the amides confer an ability of change to the molecule and such changes may alter function of the protein. It was suggested that deamidations are molecular times for development, turnover, and aging, i.e. molecular clocks. The behavior in vivo of cytochrome c, aldolase, and lysozyme seem to support this theory.
Our studies suggest still another role for the deamidation process, that of directing the point of proteolytic attack on a peptide chain of a prohormone. A number of observations indicate that post-translational proteolytic cleavage of growth hormone may have physiologic meaning, for example, production of the modifications and fragments with enhanced growth promoting and lactogenic activities (6, 19), increased hyperglycemic and insulin secretagogue activities (15, 20), and generation of somatomedin-like properties (21). That prolactin, as well as growth hormone, may serve as a precursor of proteolytic modifications is indicated by the work of Mittra (22). A cleaved form of rat prolactin was noted to have mitogenic effects whereas the intact molecule was without activity in this respect.

If hGH serves as a prohormone, a mechanism that permits specific cleavages must be found. A double basic amino acid sequence is the structure involved in the proteolytic processing of numerous proteins, yet hGH has only one double basic sequence (Arg167-Lys168). We propose as a hypothesis that deamidation directs points of attack by proteinases so that a variety of different fragments with varying biological activities can be produced from hGH. It must be more than coincidence that, in both desamido forms of hGH, the deamidated residues are in the region where hGH is most susceptible to proteolytic attack.

Deamidation may not be the only means of controlling cleavage of growth hormone and prolactin. Increased hydrophobicity caused by acylation of the NH2 terminus, which we believe occurs in Fast-hGH (23), could alter folding of the NH2-terminal chain and expose new areas to proteolytic attack. Amino acid substitution to introduce additional basic groups may have occurred in Slow- and Slow-slow-hGH (12) and this could not only alter the tertiary structure but also provide double basic sequences as loci for proteolytic cleavage. The 20,000-dalton variant of hGH has a 15-amino acid deletion (24) which may alter folding of the peptide chain and expose new areas to proteolytic attack. Such a hypothesis would then provide an explanation for the multiple forms of not only growth hormone but also of prolactin (25, 26) that are being uncovered.

The multiple electrophoretic components of hGH, including those produced by cleavage with plasmin, have been referred to as isohormones (1). We think that the term should be reserved for specific gene products and not for post-translational modifications. This would be in line with the use of the terms “isoenzyme” and “isoenzyme” as suggested by the IUPAC-IUB Commission on Biochemical Nomenclature (27).

REFERENCES