Derivatives of human growth hormone (hGH) of increasing size were produced by reaction with the N-hydroxysuccinimide ester of polyethylene glycol-5000 (PEG5000), a 5-kDa reagent that selectively conjugates to primary amines. By adjusting the reaction conditions and purification procedure, it was possible to isolate hGH derivatives containing up to seven PEG moieties that altered the Stokes radius and thereby the effective molecular masses of the unmodified hormone from 22 to 300 kDa. Fortunately, the most reactive amines were ones that did not lie in either of the two sites important for receptor binding. Nonetheless, increasing the level of PEG modification linearly reduced the affinity of hGH for its receptor and increased the EC$_{50}$ in a cell-based assay up to 1500-fold. Most of the reduction in affinity was the result of slowing the association rate for the receptor. The clearance rate of hGH in rats was inversely proportional to effective molecular weight and closely fit a filtration model. We have tested the potency of these analogs by injecting them daily or every 6 days into hypophysectomized rats and determining the effects on body and organ growth. The efficacy of these analogs was optimal for hGH conjugated with 5 eq of PEG$_{5000}$ and the potency was increased by about 10-fold compared with unmodified hGH. Such PEG-hGH derivatives show promise as long-acting alternatives to daily injections of hGH. More generally these studies show that improving hormone clearance properties, even at the expense of reducing receptor binding affinity, can lead to dramatic increases in hormone efficacy.

The ability of a hormone to elicit a biological effect in vivo depends on many factors including the affinity for its receptor and the rate at which it is cleared from the circulation. Some hormones, like atrial natriuretic peptide, have a very high affinity for their receptor (10 pm) and are cleared very rapidly (t$_{1/2}$ ~ 0.5 min) by receptor and protease-mediated events (1). Other hormones, like human growth hormone (hGH), have lower affinity for their receptor (300 pm) but are cleared more slowly (t$_{1/2}$ ~ 30 min in rats), primarily via the kidney (2, 3).

Understanding the relationships between hormone affinity, clearance, and efficacy is important in optimizing hormone therapy. To study this systematically one would like to vary these parameters and evaluate their relative importance in regulating biopotency. hGH is a good model system in this regard as much is known about its structure and function (for review see Ref. 4). Simple receptor binding (5, 6), cell-based assays (7, 8), and growth parameters in rodents (9) can be used to determine biopotency in vitro and in vivo. The properties of proteins such as hGH that are cleared by kidney filtration can be modulated by attachment of polyethylene glycol (PEG) polymers, which increases the hydrodynamic volume of the hormone and thereby slows its clearance (10, for recent review see Ref. 11).

Here, we describe a set of hGH derivatives conjugated with increasing numbers of PEG$_{5000}$ polymers. The number and locations of modified amines were characterized as well as the effects on receptor binding kinetics and affinity. We also studied the circulating half-lives and in vivo potencies for PEG-hGH derivatives. We find that despite huge reductions in receptor on-rate and affinity, the efficacy of these analogs in vivo increases with increasing level of PEG modification and reaches an optimum at five PEG$_{5000}$ groups per hGH. Thus, to a point, increasing circulating half-life can overcome the deficits in receptor binding affinity. Such analogs may be useful as long-acting alternatives to daily injections of hGH for treating growth hormone deficiency in children and in adults.

**EXPERIMENTAL PROCEDURES**

Materials—Clinical grade recombinant hGH and hIGF-I were produced and provided by Genentech. The monomethyl ether (low dial) of PEG$_{5000}$ was from Union Carbide; DCC and NHS were from Aldrich. Human GH binding protein (hGHbp) was produced in Escherichia coli. Preparation and Purification of PEG-hGH Derivatives—PEG$_{5000}$-monocarboxylic acid was prepared from the PEG$_{5000}$-monomethyl ether by reaction with DCC and NHS in ethyl acetate to provide PEG$_{5000}$-NHS as described (12). Briefly, the acid of PEG$_{5000}$ was purified by dissolution in warm ethanol (1 g per 20 ml) and crystallized by cooling slowly to 4°C. The acid was filtered, washed three times with cold diethyl ether, and dried in vacuo. The pure acid (15 g, 3 mmol) was dissolved in ethyl acetate (150 ml) by warming, and NHS (0.86 g, 7.5 mmol) and DCC (1.55 g, 7.5 mmol) were added. The solution was stirred for 18 h at 30°C. Occasionally, the product precipitated during the reaction, in which case the white suspension was warmed until only the flocculent dicyclohexylurea remained undissolved. The latter was removed by filtration through Celite® and the solution cooled to 4°C for 20 h to precipitate the PEG$_{5000}$-NHS product. This was collected by filtration, washed three times with cold ethyl acetate, and dried in vacuo to give 14.7 g of PEG$_{5000}$-NHS.

Recombinant hGH (10 mg/ml, in 0.05 M sodium borate buffer (pH 8.5)) was reacted for 30–60 min at room temperature with 1-3 eq of PEG$_{5000}$-NHS per amino group on hGH (a total of 9 lysines plus the α-amine). After the reaction, buffer was added that contained 1.4 M sodium citrate, 0.05 M Tris (pH 7.5) to a final citrate concentration of
The mixture of PEG/hGH products was loaded onto a phenyl Sepharose high performance column (1.6 x 26 cm, Pharmacia) equilibrated in 25 mM sodium acetate (pH 4.0). The PEG5000 per hGH for each PEG-hGH derivative was analyzed by mass spectroscopy on a laser desorption ionization mass spectrometer (TOF). For trypsin digests, purified PEG-hGH samples (1 mg/ml in 1 mM sodium acetate, 10 mM Tris (pH 8.3)) were incubated with bovine trypsin (Worthington) at a protein weight ratio of 1:100 (trypsin:PEG-hGH) as described (13). The trypsin was added at time 0 and again at 2 h of digestion. After incubation for 6 h at 37 °C, digestion was stopped by addition of phosphoric acid to pH 2, and samples were stored at 4 °C. Digestive samples (100 μg) were loaded onto a 15 x 0.46 cm C-18 column (5-μm bead, 100-Å pore size) (Nucleosil) in 0.1% aqueous trifluoroacetic acid and eluted with a gradient from 0 to 60% acetonitrile over 120 min at a flow rate of 0.4 ml/min at 40 °C. The elution of tryptic peptides was monitored by absorbance at 214 nm.

The different PEG-hGH species were separated on a sulfopropyl-Sepharose high performance column (1.6 x 26 cm, Pharmacia) equilibrated in 25 mM sodium acetate (pH 4.0) at a concentration of 2.1 mg of bovine serum albumin, 0.05% polysorbate 20, and 0.01% thimerosal. Fractions containing PEG-hGH species were pooled and concentrated 5–10-fold by ultrafiltration using a PD-10 column (Pharmacia) or a Filtron 5K Omega 150-ml concentrator (Filtron). The concentrated protein was exchanged into 25 mM sodium phosphate, 18 mg/ml mannitol, and 0.68 mg/ml glycine (pH 7.4).

The purified proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue R-250 to determine the purity of the sample. Purity for four of the five peaks was further confirmed by analytical high performance liquid chromatography on a sulfopropyl TSK 5PW column (panel B).

Concentrations of hGH or PEG5000-hGH were computed using standard curves corresponding to the hormone analog of interest. Standard deviations of hormone concentrations between assays were less than 15%. Pharmacokinetic parameters were estimated by fitting values of hormone concentration versus time to compartmental models using a non-linear least-squares regression analysis (NONLIN 84, Version 1987, Statistical Consultants, Lexington, KY). Clearance values normalized to animal weight clearance rate per animal weight and terminal half-lives (t1/2) were calculated using the coefficients and exponents obtained from the intravenous bolus model fits.

Analysis of Potency in Rodents—Young female hypophysectomized rats (85 to 105 g, Tacorn Farms, Germantown, NY) were weighed every 2–3 days for 10 days; any animal gaining more than 7 g during this period was excluded from the study. Treatment was started at 8 weeks of age and 15 days following surgery. Animals were fed a standard diet of rodent pellets and water ad libitum and kept in a room of constant humidity and temperature with controlled lighting (12 h light followed by 12 h dark). The animals were randomized for both treatment group and cage to give groups of five with balanced equal mean initial body weights prior to treatment.

Body weights were recorded daily and organs weighed at the time of sacrificing the animals.
Different batches of PEG-hGH were prepared in which hGH was reacted with varying amounts of PEG-NHS as described under “Experimental Procedures.” The molecular weights of the various PEG-hGH species were determined by matrix-assisted laser desorption ionization mass spectrometry. Heterogeneity in molecular mass of the PEG5000-NHS starting material resulted in broad peaks generally varying by ± 300 Da for the different PEG-hGH derivatives. In reporting the molecular masses, we show the average molecular mass of the predominant PEG-hGH species, generally >85% pure. Some of the chromatographic species contained roughly equal mixtures of two forms of PEG-hGH, and these are indicated as n + (n + 1). Some PEG-hGH species (e.g. 3a and 3b) had the same number of PEG groups, but these were attached to different sites because the species eluted differently. The EC50 values for activation of the hGH receptor were determined by proliferation of FDC-P1 cells transfected with the hGH receptor (8). The EC50 for receptor activation by unmodified hGH is ∼20 pm (7, 8). See “Experimental Procedures” for additional details.

| PEG5000 per hGH | Molecular Mass (kDa) | EC50 (nM) | EC50(PEG)/EC50(hGH) |
|-----------------|----------------------|-----------|----------------------|
| 0               | 22                   | 0.13 (1)  |
| Preparation 1   |                      |           |                      |
| 2               | 33                   | 0.4       | 2.3                  |
| 2 + 3           | 33, 38               | 1.1       | 6.5                  |
| 3a              | 38                   | 0.98      | 5.7                  |
| 3b              | 38                   | 3.6       | 21                   |
| 4               | 43                   | 7.4       | 44                   |
| 5               | 48                   | 20        | 120                  |
| Preparation 2   |                      |           |                      |
| 3 + 4a          | 39, 42               | 5.1       | 48                   |
| 3 + 4b          | 39, 43               | 7.6       | 71                   |
| 4 + 5           | 43, 47               | 11        | 100                  |
| 5               | 48                   | 25        | 240                  |
| 5 + 6           | 48, 53               | 66        | 610                  |
| Preparation 3   |                      |           |                      |
| 4a              | 43                   | 8.8       | 68                   |
| 4b              | 43                   | 10        | 76                   |
| 4 + 5           | 45, 48               | 12        | 92                   |
| 5               | 48                   | 19        | 150                  |
| 5 + 6           | 49, 52               | 33        | 250                  |
| 6               | 53                   | 87        | 670                  |
| 7               | 57                   | 193       | 1500                 |

Results are expressed in terms of ng/ml recombinant human IGF-I.

**RESULTS**

Preparation and Biochemical Characterization of PEG5000-hGH Derivatives—hGH contains 10 primary amines that can theoretically react with PEG5000-NHS including the α-amino of Phe-1 and ε-amino groups of nine lysine side chains. These amines were modified to varying extents with PEG5000-NHS by adjustment of the reagent excess, protein concentration, and pH. The different hGH derivatives were isolated by hydrophobic interaction and cation-exchange chromatography as described in Fig. 1 and the “Experimental Procedures.” The stoichiometries of PEG5000 per hGH were assessed by mass spectrometry (Table I). In this way it was possible to isolate hGH derivatives containing up to seven PEG moieties. While many of these derivatives were >85% pure, other chromatographic species were mixtures differing by one PEG moiety.

To survey the effect of the PEG modification on the bioactivity of the hGH, we analyzed the ability of each derivative (or mixture) to stimulate the proliferation of FDC-P1 cells that were stably transfected with the hGH receptor (8). The concentration of hormone required for 50% maximal stimulation of cell proliferation (EC50) systematically increased with the extraction of hormone required for 50% maximal stimulation of one PEG-hGH species (preparations 1–3) were prepared in which hGH was reacted with varying amounts of PEG-NHS as described under “Experimental Procedures.” The molecular weights of the various PEG-hGH species were determined by matrix-assisted laser desorption ionization mass spectrometry. Heterogeneity in molecular mass of the PEG5000-NHS starting material resulted in broad peaks generally varying by ± 300 Da for the different PEG-hGH derivatives. In reporting the molecular masses, we show the average molecular mass of the predominant PEG-hGH species, generally >85% pure. Some of the chromatographic species contained roughly equal mixtures of two forms of PEG-hGH, and these are indicated as n + (n + 1). Some PEG-hGH species (e.g. 3a and 3b) had the same number of PEG groups, but these were attached to different sites because the species eluted differently. The EC50 values for activation of the hGH receptor were determined by proliferation of FDC-P1 cells transfected with the hGH receptor (8). The EC50 for receptor activation by unmodified hGH is ∼20 pm (7, 8). See “Experimental Procedures” for additional details.

**TABLE I**

| PEG5000 per hGH | Molecular Mass (kDa) | EC50 (nM) | EC50(PEG)/EC50(hGH) |
|-----------------|----------------------|-----------|----------------------|
| 0               | 22                   | 0.13 (1)  |
| Preparation 1   |                      |           |                      |
| 2               | 33                   | 0.4       | 2.3                  |
| 2 + 3           | 33, 38               | 1.1       | 6.5                  |
| 3a              | 38                   | 0.98      | 5.7                  |
| 3b              | 38                   | 3.6       | 21                   |
| 4               | 43                   | 7.4       | 44                   |
| 5               | 48                   | 20        | 120                  |
| Preparation 2   |                      |           |                      |
| 3 + 4a          | 39, 42               | 5.1       | 48                   |
| 3 + 4b          | 39, 43               | 7.6       | 71                   |
| 4 + 5           | 43, 47               | 11        | 100                  |
| 5               | 48                   | 25        | 240                  |
| 5 + 6           | 48, 53               | 66        | 610                  |
| Preparation 3   |                      |           |                      |
| 4a              | 43                   | 8.8       | 68                   |
| 4b              | 43                   | 10        | 76                   |
| 4 + 5           | 45, 48               | 12        | 92                   |
| 5               | 48                   | 19        | 150                  |
| 5 + 6           | 49, 52               | 33        | 250                  |
| 6               | 53                   | 87        | 670                  |
| 7               | 57                   | 193       | 1500                 |

**Fig. 2.** Relationship between the number of PEG5000 groups attached to hGH and the reduction in the log of bioactivity. The reduction in bioactivity is expressed as the EC50 for activating cell proliferation for PEG-hGH derivatives divided by that for unmodified hGH. This is presented in log form because it is proportional to the reduction in the free energy of the interaction. For mixtures of PEG-hGH containing different numbers of PEG’s per hGH, the average number of PEG’s per hGH is plotted. Data are taken from Table I for the different preparations of PEG-hGH (circles, preparation 1; triangles, preparation 2; and squares, preparation 3).

**Fig. 3.** Stimulation of thymidine incorporation into FDC-P1 cells stably transfected with the hGH receptor. Cells were treated with increasing amounts of hGH or PEG5000-hGH analogs. See “Experimental Procedures” for details.
reductions in affinity resulted from decreases in the association constant (\(k_{\text{on}}\)). There was a good correlation between the increase in EC50 and increase in \(K_d\) for binding at Site 1 (Fig. 4) suggesting that most of the reduction in bioactivity results from an inability to react at Site 1. The fact that the slope of this line is less than unity could be a consequence of the observation that only a fraction of the receptors need to be dimerized for maximal cell proliferation (16). (The EC50 is about 10 times lower than the \(K_d\) (7)).

Sites of PEG Modification—The sites of PEG modification were analyzed by tryptic mapping including mass spectral analysis (Fig. 5) for derivatives containing an average of two, four, or seven PEG groups. In this way it was possible to estimate the reactivities for the different amines on hGH because the more reactive amines would be modified in forms of PEG-hGH containing few PEG groups, whereas less reactive (or unreactive) amines may not be modified (or only partially so) in the more heavily modified derivatives. From these studies there appeared four general classes of primary amine based on reactivity (Table III). The most reactive ones included the \(\alpha\)-amine of Phe-1 (T1) and the \(\varepsilon\)-amino group of Lys-140 (T13), followed by Lys-145 (T14), Lys-38 (T4), Lys-70 (T7), Lys-41 (T5), Lys-158 (T15), Lys-168 (T17), Lys-172 (T18), Lys-115 (T10). The unreactivity of Lys-115 was based upon the fact that the T10 tryptic fragment was intact even for PEG-8-hGH (data not shown). Except for the \(\alpha\)-amine, we found poor correlation with the reactivity of the amino group and its surface accessibility to a large (8 Å) or small (1.4 Å) probe or whether or not the amine appeared to be involved in intramolecular interactions (Table III).

Four of the nine lysine groups in hGH become buried to some degree upon binding of the two receptors (Fig. 6). Fortunately, the three that are buried in Site 1 (Lys-41, Lys-168, and Lys-172, Fig. 6A) are not very reactive with PEG-5000-NHS, and the one that is near Site 2 (Lys-115, Fig. 6B) is unreactive.

Clearance and Bioactivity in Rats—We analyzed the rate at which PEG-hGH analogs were cleared from the circulation. Serum levels of each analog were measured as a function of time after a single intravenous or subcutaneous injection into normal rats (Fig. 7). PEG modification dramatically slowed the clearance of hGH.
Long-acting Growth Hormones Produced by Conjugation with PEG

The reactivities of amines on hGH with PEG<sub>5000</sub>-NHS and their surface accessibilities to probes of 1.4 or 8 Å, as well as the presence or absence of intramolecular side chain contacts

| Amine | Accessibility | Intramolecular contacts<sup>a</sup> |
|-------|---------------|-------------------------------------|
|       | 1.4 Å probe   | 8 Å probe      |                               |
| Highly reactive |                     |                     |                               |
| F1 (α-amine)    | 53             | 260               | None                         |
| Lys-140         | ND             | ND                | ND                           |
| Moderately reactive |                 |                     |                               |
| Lys-145         | 21             | 26                | H                            |
| Lys-38          | 27             | 12                | s                            |
| Lys-70          | 36             | 33                | None                         |
| Poorly reactive |                 |                     |                               |
| Lys-172         | 9              | 0                 | h                            |
| Lys-41          | 16             | 0                 | h                            |
| Lys-158         | 39             | 45                | None                         |
| Lys-168         | 23             | 0                 | H,S                          |
| Unreactive      |                 |                     |                               |
| Lys-115         | 53             | 154               | H                            |

<sup>a</sup> None, no contacts; H, short hydrogen bond (<3 Å); S, short salt bridge (<3 Å); S, long salt bridge (3–3.5 Å); ND, not ordered in the structure so accessibility cannot be formally calculated.

clearance irrespective of the route of administration. Moreover, the PEG modification also increased the time to reach peak blood levels after subcutaneous administration (Fig. 7B).

The clearance rates decreased systematically with increasing level of PEG modification (Table IV). Moreover, a plot of clearance rate versus effective molecular mass of the PEG-hGH derivatives (assessed by gel filtration chromatography) could be fit closely to a filtration model having a molecular mass cut-off of about 70 kDa (Fig. 8). Complexing hGH with 2 eq of the hGHbp also slowed the clearance of hGH (17), and the clearance rate for the complex also fell on this curve (Fig. 8). This result suggests that clearance of hGH is determined by its effective molecular weight, not by the nature of the modification.

Efficacy Studies in Hypophysectomized Rats—We analyzed the abilities of PEG<sub>5000</sub>-hGH analogs to promote weight gain in hypophysectomized rats (9). In the first experiment (Fig. 9A), five different PEG-hGH derivatives (containing 4, 5, 5 + 6, 6 or 7 PEG moieties per hGH) were injected subcutaneously. The growth rates were compared with those produced by unmodified hGH or an excipient buffer control. The excipient-treated rats showed the expected minimal weight gain over the 12 days while those receiving hGH every 6 days showed a small but significant weight gain. All the PEG-hGH derivatives gave much larger weight gains after both the first and second injections. The largest weight gains were caused by the analogs containing 4, 5, or 5 + 6 PEG moieties; smaller weight gains resulted from the administration of analogs with 6 or 7 PEG moieties.

The PEG-hGH analog containing five PEG’s per hGH (PEG-5-hGH) appeared most effective, and we therefore compared its ability to promote growth when given infrequently at two doses to that of hGH given daily (Fig. 9B). Rats given excipient alone failed to gain weight, and daily injections of hGH caused the expected dose-related steady increase in body weight. In a dose-related manner, infrequent injections of PEG-5-hGH every 6 days caused greater weight gain after 6 or 12 days than did daily injections of unmodified hGH. The wet organ weights of the heart, liver, kidney, spleen, and thymus were increased by all treatments with the liver, spleen, and thymus growing at a faster rate than overall body weight gain (data not shown).

To compare further the growth promoting effects of PEG-5-hGH to that of unmodified hGH, we measured serum IGF-I and epiphyseal growth plate widths as a function of time (Fig. 10). Over a 10-day period rats were given either excipient, hGH daily (30 μg/rat/day), or a single injection of hGH (300 μg) or PEG-5-hGH (180 μg). Excipient-treated rats did not gain weight, and daily injections of 30 μg of hGH maintained an expected linear increase in weight gain (Fig. 10A). One bolus injection of hGH (300 μg) produced only a small and transitory response, whereas a single bolus of PEG-5-hGH (180 μg) gave a much larger and more sustained response. In fact, for the weight gain from daily injections of hGH to be equal that from a single injection of PEG-5-hGH required 9 days, and 50% more hormone equivalents over that time (a total of 270 μg for daily hGH versus 180 μg for PEG-5-hGH).

There was a small but significant (<0.05) increase in epiphyseal plate width after one injection of hGH as tested on days 2, 4, and 7; the effect dissipated by day 10 (Fig. 10B). On days 4 and 7 epiphyseal plate width was greater after PEG-5-hGH than after daily injections of hGH, but this was reversed by day 10. In contrast the serum IGF-I levels were only increased by PEG-5-hGH on days 2 and 4 of treatment and
We evaluated the immunological reactivity and bioactivity of the circulating PEG-hGH as a function of time (Fig. 11). The level of PEG-5-hGH, as assayed either by ELISA or cell proliferation, decreased in parallel following a single injection over the 10-day experiment. The constant difference in hGH concentration estimated by bioactivity versus immunoreactivity reflects the 100-fold reduction in EC50 caused by the PEG modification (Table I). These data suggest that PEG-5-hGH is bioactive and that this molecular conjugate remains stable over the time course of the experiment. These data are inconsistent with the growth promoting activity being caused by the presence of unmodified hGH in the PEG-5-hGH preparation.

**DISCUSSION**

Characterization of PEG-hGH Derivatives—Of the 10 primary amines on hGH, some are more reactive than others with PEG5000-NHS (Table III). It is not surprising to find the α-amine to be highly reactive; it has very high surface accessibility and a pK<sub>a</sub> that is typically 2 to 3 units below any of the ε-amines (18). However, the basis for reactivity among the ε-amines is not so clear; reactivity does not directly correlate

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**TABLE IV**

Pharmacokinetic parameters in rats given a single subcutaneous injection of hGH, a complex of hGH with 2 eq of the hGHbp, or PEG5000-hGH derivatives

| Molecule         | N  | Body weight | PEG/ hGH | Effective size | CL/W  | t<sub>1/2</sub> |
|------------------|----|-------------|----------|----------------|-------|---------------|
| hGH              | 6  | 0.32 ± 0.03 | 0        | 22             | 496 ± 66 | 1.35 ± 0.2   |
| hGH(hGHbp)<sub>2</sub> | 6  | 0.31 ± 0.01 | 0        | 80             | 32 ± 42  | 8.4 ± 3      |
| PEG-hGH          | 3  | 0.31 ± 0.03 | 1        | 35             | 130 ± 7  | 1.3 ± 0.1    |
|                  | 3  | 0.32 ± 0.04 | 2        | 32             | 70 ± 4   | 5.8 ± 0.1    |
|                  | 3  | 0.32 ± 0.04 | 5        | 250            | 7.5 ± 1  | 15 ± 4       |
|                  | 4  | 0.32 ± 0.01 | 7        | 300            | 2.3 ± 0.2 | 24 ± 7       |

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**FIG. 7.** Time course of clearance from serum of hGH, PEG<sub>5000</sub>-hGH derivatives, or hGH in complex with 2 eq of the hGHbp after intravenous (panel A) or subcutaneous (panel B) injection into rats. Each group of rats (three to six in a group) was given a single bolus dose of 0.1 mg of protein/kg. Serum samples were taken over intervals extending to 200 h depending upon the analog. Serum samples were analyzed at indicated times for hGH or PEG-hGH by an ELISA as described under “Experimental Procedures.”

**FIG. 8.** Correlation of effective molecular size and clearance rates (CL) for PEG<sub>5000</sub>-hGH derivatives. Data from Table IV were plotted and fit to a filtration model that assumes a molecular mass cut-off of 70 kDa, as is typical for kidney filtration (26). See “Experimental Procedures” for details.
with the surface accessibility of the \( \epsilon \)-amine to either a small or large probe. For example, the \( \epsilon \)-amine from Lys-115 is one of the most surface-accessible but is the least reactive, whereas \( \epsilon \)-amines from 145 and Lys-38 are much less accessible yet moderately reactive. 

The reactivities cannot be solely accounted for by whether or not the \( \epsilon \)-amine is involved in an intramolecular hydrogen bonding or electrostatic interaction. For instance, Lys-158 is very accessible and makes no obvious intramolecular interactions in the structure yet is very poorly reactive; in contrast Lys-145 is less accessible and makes a good intramolecular hydrogen bond in the structure yet is moderately reactive. Application of an algorithm (Delphi) predictive of the rank order of \( pK_a \) 's of these amines did not correlate well with the reactivities of the amines either.\(^2\) Other factors such as weak binding of the PEG\(_{5000}\)-NHS to hGH, desolvation energies of the \( \epsilon \)-amines, and protein dynamics should also affect reactivity. Our data suggest that reactivity toward the large polymeric acylating agent, PEG\(_{5000}\)-NHS, depends on many factors that are not easily deconvoluted from simple inspection of the static structure of hGH.

The differential reactivities among these amines made it possible to isolate forms of PEG-hGH with discrete numbers of PEG moieties attached. Although the number of PEG groups attached is the same for many of these chromatographic species, it is very likely that there is heterogeneity among the amines modified. For example, the heterogeneity of sites modified is probably why some forms of PEG-5-hGH could be individually isolated while other forms remained mixed with PEG-4 or PEG-6-hGH (Fig. 1, A and B). In addition, the PEG\(_{5000}\)-NHS-modifying reagent is heterogeneous in polymer length and varies by about ±300 daltons around the average molecular mass (data not shown). It is important to appreciate the nature of the heterogeneity in composition and reactivity with PEG\(_{5000}\)-NHS as it relates to these derivatives as potential therapeutics.

PEG Modification Affects Binding Receptor Affinity and Bioactivity—Our data suggest that modification with PEG\(_{5000}\)-NHS causes a general weakening of binding affinity and reduction in bioactivity by indirectly interfering with access to the first bound receptor at Site 1. The most reactive amines are away from either of the two receptor binding sites, and Lys-115 near Site 2 is virtually unreactive (Fig. 6). There is a linear correlation between the log of the reduction in the \( EC_{50} \) for receptor activation and the number of PEG groups attached (Fig. 2). (The change in binding free energy is related to the log of the change in binding constant.) This indicates that each additional PEG moiety causes the same reduction in bioactivity. This is inconsistent with modification of a few crucial lysines at the receptor binding site.

\(^2\) B. McDowell, personal communication.

FIG. 9. Weight gain in hypophysectomized rats given subcutaneous injections of hGH or PEG\(_{5000}\)-hGH analogs once every 6 days in two experiments. In the upper panel the hormones (60 \( \mu \)g/rat) or excipient buffer were injected subcutaneously in 0.1 ml on days 0 and 6. The analogs contained an average of 4, 5, 5 + 6, 6, or 7 PEG\(_{5000}\) moieties hGH. In the lower panel two doses of hGH (60 or 180 \( \mu \)g/6 days) were given by subcutaneous injection either every 6 days (for PEG5-hGH) or daily (for hGH; 10 or 30 \( \mu \)g).

FIG. 10. Comparison of a single injection of hGH or PEG-5-hGH to daily injections of hGH over a 10-day period. On day 0 rats (20 to a group) were given a single injection of either PEG-5-hGH (180 \( \mu \)g, filled circles) or hGH (300 \( \mu \)g, filled squares) or given 10 daily injections of hGH (30 \( \mu \)g/rat, 0.3 mg/kg/day, open squares). A fourth group of rats received daily injections of excipient buffer (open circles). Five animals from each of the four groups were killed on days 2, 4, 7, and 10, and we measured body weight gains (panel A), epiphyseal plate widths (panel B), and total serum IGF-I (panel C). See “Experimental Procedures” for further details.
The dose-response curves for PEG-hGH to induce proliferation of FDC-P1 cells transfected with the hGH receptor are bell-shaped as they are for unmodified hGH (Fig. 3). Although the EC50 values increase for PEG-hGH, there is little or no change in the maximal level of cell proliferation. This suggests that once the hormone has bound to the receptor, its ability to activate it (by dimerization) is not different from wild-type hGH. The dose-response curve for hGH shows there is roughly a 10,000-fold difference between the EC50 and IC50 for stimulation and self-antagonism, respectively (7). This difference is maintained for PEG-hGH derivatives. Mathematical models for the sequential dimerization mechanism predict that mutants affecting Site 1 should shift the bell-shaped dose-response curve whereas mutants in Site 2 should affect the height and width of the bell (16). Thus, these data suggest PEG modification primarily affects initial binding at Site 1.

Modification with PEG5000 reduces affinity largely by reducing the association rate at Site 1 (Table II). Previous studies have shown that when direct-contact side chains are mutated to alanine primarily the off-rate is affected and not the on-rate (6). Taken together these data suggest that the reduction in affinity caused by PEG modification is not the result of direct modification of the receptor binding sites but rather from indirect effects; the long and floppy PEG5000 groups lower diffusion and reduce access to the receptor binding sites.

PEG Modification of hGH Systematically Slows Clearance—Incremental modification with PEG5000 caused a systematic increase in the serum half-life of the hormone whether given by intravenous or subcutaneous injection (Fig. 7). Not only was PEG-hGH cleared more slowly, it also was adsorbed more slowly from the injection site. Thus, the time to reach maximal serum levels of hormone after subcutaneous administration increased with the extent of PEG modification. Furthermore, clearance (after both the intravenous and subcutaneous administration) was slowed for the modified hormones. For wild-type hGH, clearance is slowed by binding to the hGHbp in serum. Because the PEG modification reduces binding to the hGHbp, the hGHbp cannot assist in slowing clearance for the PEG-hGH derivatives. Despite this, the PEG-hGH derivatives are cleared much slower that wild-type hGH and is further testimony to the PEG modification dominating the clearance properties of these molecules.

PEG has been extensively used to modify the clearance properties of proteins (10, 11). It is believed that a predominant effect of PEG modification is to reduce kidney filtration. In one of the most thoroughly studied examples, Katre and co-workers (19, 20) showed that the half-life of PEG-interleukin-2 systematically increased with effective molecular weight and closely fit a kidney filtration model.

The fact that elimination half-lives are proportional to the molecular weight of the growth hormone species is consistent with elimination being mediated by a filtration process. However, the contribution of the kidney to the clearance of growth hormone has been estimated to be 25–53% in normal humans (3) and to be 67% in rats (2). Thus, if the effect of the PEG modification or binding to the hGHbp were only to slow elimination via the kidney, then we could only expect the elimination of these derivatives to be slowed by a factor of 2 at most. Other mechanisms, such as proteolysis in serum or uptake in tissues, are also involved in clearance of hGH. The fact that the PEG modifications extend elimination lifetimes much longer indicates that mechanisms other than kidney filtration are similarly slowed by PEG modification. In fact, it is well-known that PEG modification inhibits not only kidney filtration but also rates of proteolysis (11).

PEG-hGH Is Long Acting and More Potent Than Unmodified hGH—Hormone efficacy in vivo is a complex property that depends on affinity and persistence, among others. PEG modification of hGH has counter-acting effects; it reduces receptor binding affinity yet increases serum half-life. The uncertainties in predicting the relative importance of these effects required that we test the ability of a variety of PEG-hGH derivatives in vivo to determine which were most active.

The PEG-hGH derivative having an average of 5 PEG’s per hGH appeared the most effective long-acting molecule (Fig. 9). In fact, injection of PEG-5-hGH every 6 days over a 12-day period was even more effective than hGH given daily. However, there were some differences in the weight gain curves for these two regimens. Administration of PEG-5-hGH caused a burst of weight gain that waned on days 4–6, whereas daily hGH produced linear weight gain throughout. Part of this is due to clearance of the PEG-5-hGH by day 4 to a level that may be below its EC50 for activating the receptor. For example, a 60-ng injection of PEG-5-hGH into a rat could produce a maximal circulating level of 300 nm, which by day 4 would be reduced to about 10 nm (five half-lives, Table IV). On the human GH receptor the EC50 for PEG-5-hGH is 20 nM (Table I). Thus, readministration of PEG-5-hGH on day 6 would restore hormone concentrations above the EC50 and thereby produce a similar growth response as the initial injection. However, in other experiments (not shown) we gave PEG-5-hGH on a daily basis, and to our surprise found it less effective than when given in this 6-day regimen. The basis for this effect is unclear but could be that maximal receptor stimulation leads to receptor down-regulation that would require a recovery period to reset the system. Katre and co-workers (19) have reported similar findings for PEG-1L-2 and referred to this as a need for a “hormone holiday.”

There is considerable literature showing that in rodents the pattern of GH delivery or exposure can modify GH responses (21). Administering injections of PEG-hGH will tend to give a more continuous exposure to GH than giving injections of unmodified GH. To compare in more detail the results of daily injections of hGH versus infrequent injections of PEG-5-hGH, we analyzed other growth parameters such as the tibial plate widths and serum IGF-I levels throughout the growth experiment (Fig. 10). The tibial growth plate widths correlated
fairly well with the overall weight gain. Plate width initially was greater for PEG-5-hGH, but by day 7 the daily hGH-treated animals had caught up. By day 10, the PEG-5-hGH group had decreased showing that in the absence of continued treatment the tibial growth plate returned toward widths seen for animals given the excipient alone or a single injection of hGH.

We attempted to correlate serum levels of IGF-I with these growth parameters but found that IGF-1 levels did not change for the excipient, daily hGH, or single hGH groups. We did, however, see a large increase in the IGF-1 concentrations after day 2 to day 4 for the PEG-5-hGH-treated animals. These data suggest IGF-1 levels are not very sensitive to moderate or weak growth-promoting effects but did reflect the large initial burst of growth induced by PEG-5-hGH.

Although PEG-5-hGH caused a rapid increase in growth, our data indicate the nature of the growth was similar to the sustained growth seen for animals given daily hGH. For example, at day 7 when both the PEG-5-hGH and daily hGH groups had the same weight gained, their tibial growth plate widths were comparable (Fig. 10B). In recent studies we have shown that intermittent and continuous GH exposure can produce differential organ growth in rats. With PEG-5-hGH we observed some disproportionate growth of some internal organs including the liver, thymus, and spleen (data not shown). With appropriate intermittent injections of PEG-hGH, we believe that this difference between PEG-hGH and daily hGH injections would be minimized.

A number of pieces of data argue that the growth-promoting effects for the PEG-hGH preparations cannot be due to residual hGH contamination. First, the PEG-hGH molecules are long-acting and cleared more slowly, a property not possessed by unmodified hGH. The samples are purified by hydrophobic interaction and cation-exchange chromatography and have properties that are different from wild-type hGH. In addition, SDS-polyacrylamide gel electrophoresis on the PEG-hGH samples (data not shown) shows no detectable unmodified hGH. The detection limits for these experiments would indicate that a contamination could not be higher than about 2%. Finally the circulating bioactivity and immunoreactivity of the circulating PEG-hGH decreases in parallel after a single injection. If the bioactive component in the PEG-5-hGH preparation were unmodified hGH it would have been cleared rapidly and would not have persisted over the 10-day period as we observe. Thus, the growth-promoting and clearance activities of the PEG-hGH preparations, as well as their physical properties, are clearly different from unmodified hGH.

PEGylation has been extensively used to modify proteins both to increase serum half-life and reduce immunogenicity (11). Some of these are now approved as pharmaceuticals. For example, preparations of PEG-adenosine deaminase are efficacious when given weekly, compared with the daily injections of unmodified adenosine deaminase for the treatment of severe combined immune deficiency disease in children (22). Because proteins are cleared faster in rodents than in humans (23), it is possible that PEG-5-hGH given at bi-weekly or even monthly intervals could have efficacy comparable with daily injections of hGH in humans.

Conclusions—Our studies show that systematic modification of hGH with PEG leads to systematic changes in physical and biological properties. Despite the large reductions in binding affinity and bioactivity, the improved clearance properties can more than compensate. These studies support the use of PEGylation to extend the activity of protein hormones that are normally cleared by filtration and provide promising long-acting alternatives to daily hGH injections.

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REFERENCES

1. Chinkers, M. & Garbers, D. L. (1991) Annu. Rev. Biochem. 60, 553–575
2. Johnson, V. & Maack, T. (1977) Am. J. Physiol. 233, 185–196
3. Haffner, D., Schaefier, F., Girard, J., Ritz, E. & Mehi, O. (1994) J. Clin. Invest. 93, 1163–1171
4. Wells, J. A., Cunningham, B. C., Fuh, G., Lowman, H. B., Bass, S. H., Mulkerrin, M. G., Ullsch, M. & deVos, A. M. (1993) Recent Prog. Horm. Res. 48, 253–275
5. Spencer, S. A., Hammonds, R. G., Herzel, W. J., Rodriguez, H., Waters, M. J. & Wood, W. I. (1988) J. Biol. Chem. 263, 7862–7867
6. Cunningham, B. C., Wells, J. A. (1993) J. Mol. Biol. 234, 554–563
7. Fuh, G., Cunningham, B. C., Fukunaga, R., Nagata, S., Goodell, D. V., Wells, J. A. (1992) Science 256, 1677–1680
8. Colosi, P., Wong, K., Leong, S. & Wood, W. I. (1993) J. Biol. Chem. 268, 12617–12623
9. Groesbeck, M. D., Parlow, A. F. & Daughaday, W. H. (1987) Endocrinology 120, 1963–1975
10. Abuchowski, A., McCoy, J. R., Palczuk, N. C., van Es, T. & Davis, F. F. (1977) J. Biol. Chem. 252, 3582–3586
11. Zalkin, A., & De Meyts, P. (1994) in PEG Chemistry: Biotechnological and Biomedical Applications (Harriss, J. M., ed) pp. 347–370, Plenum Publishing Corp., New York
12. Budmann, A. F. & More, M. (1981) Makronan. Chem. 182, 1379–1384
13. Körn, W. J., Keck, R. & Harkins, R. N. (1982) Anal. Biochem. 122, 348–359
14. Albini, C., Sotos, J., Sherman, B., Jhanoven, A., Celniker, A., Hopwood, N., Quattin, T., Mills B. J. & MacGillivray, M. (1991) Pediatr. Res. 29, 619–622
15. Liebersman, S. A., Bukar, J., Chen, S. A., Celniker, A., Compton, P. G., Cook, J., Albu, J., Perlman, A. J. & Hoffman, A. R. (1992) J. Biol. Chem. 267, 30–36
16. Inoue, M. D., Darnbult, A. B., Cunningham, B. C., Wells, J. A., Shymko, R. M. & De Meyts, P. (1994) Endocrinology 134, 2397–2403
17. Baumann, G., Amburn, K. D. & Buchanan, T. A. (1987) J. Endocrinol. & Metab. 56, 657–660
18. Perrin, D. D. (1965) in International Union of Pure and Applied Chemistry, pp. 353–391, Butterworths Publishing, London
19. Knauf, M. J., Bell, D. P., Hirtzer, P., Luo, Z. F., Young, P. D. & Katre, N. V. (1988) J. Biol. Chem. 263, 15064–15070
20. Goodson, R. J. & Katre, N. V. (1990) J. Biol. Chem. 265, 15064–15070
21. Robinson, I. C. A. F. & Clark, R. G. (1987) in Clinical Aspects of Growth Hormone: Basic and Clinical Aspects (Isaksson, O., Binder, C., Wallin, G., & Hokfeld, E., eds) pp. 109–127, Elsevier Science Publishers B.V., Amsterdam
22. Hershfield, M. S., Buckley, R. H., Greenberg, M. L., Meton, A. L., Schiff, R., Harten, C., Kurtzhel, J., Markert, M. L., Kiyobashi, R. H., Kobayashi, A. L. & Abuchowski, A. (1987) N. Engl. J. Med. 316, 589–596
23. Mordenti, J., Chen, S. A., Moore, J. A., Ferraiolo, B. L. & Green, J. D. (1991) Pharmacol. Res. 8, 1351–1359
24. deVos, A. M., Uttsch, M. & Kossiakoff, A. A. (1992) Science 255, 306–312
25. Lee, B. & Richards, F. M. (1977) J. Biol. Chem. 255, 379–400
26. Venkataraman, M. A. & Rennke, H. G. (1978) Circ. Res. 43, 337–347
