

## The Synthesis of a Protein Possessing Growth-Promoting and Lactogenic Activities

Sir:

The isolation<sup>1</sup> and structure<sup>2</sup> of human pituitary growth hormone (HGH) have previously been reported from this laboratory. We now wish to report our investigations on the total synthesis of a protein with HGH activity.

The HGH molecule consists<sup>2</sup> of a single polypeptide chain with 188 amino acid residues containing two disulfide bridges and one residue of tryptophan. In addition to growth-promoting activity, the molecule possesses<sup>3</sup> the biological activity of lactogenic hormone.

Synthesis of the protected polypeptide chain corresponding to the amino acid sequence<sup>2</sup> of HGH was carried out essentially according to the strategy employed for the synthesis<sup>4</sup> of ribonuclease by the solid-phase method.<sup>5</sup> The *tert*-butyloxycarbonyl (Boc) group<sup>6</sup> was used for protection of the  $\alpha$ -amino group of all amino acids including the following derivatives: Asp ( $\beta$ -OBzl), Thr (Bzl), Ser (Bzl), Glu ( $\gamma$ -OBzl), Cys (Bzl), Tyr (Bzl), Lys (Z), and Arg (NO<sub>2</sub>). N<sup>α</sup>-Boc (*im*-Boc)-L-histidine<sup>7</sup> was synthesized for the introduction of the histidine residue. Coupling was achieved with dicyclohexylcarbodiimide<sup>8</sup> (DCCI) with the exception that the asparagine and glutamine residues were coupled by means of their nitrophenyl esters.<sup>9</sup>

The COOH terminal phenylalanine of HGH served as starting point for synthesis by esterification of 0.52 mmol of Boc-phenylalanine to 1% cross-linked polystyrene resin (2.5 g total weight). Stepwise synthesis was then carried out through 187 cycles on an automated instrument.<sup>10</sup> Removal of the Boc group was effected by a 15-min treatment with 50% (v/v) trifluoroacetic acid in methylene chloride, and after the incorporation of the tryptophan residue in position 25 dithiothreitol<sup>11</sup> (DTT) was added (0.08 M) to this deblocking reagent. For DCCI couplings (5 hr), 4 equiv of the appropriate reagents was usually used for every equivalent of phenylalanine residue originally attached to the starting resin, but for the incorporation of the valine, isoleucine, and nitroarginine residues 6 equiv was employed. Nitrophenyl ester couplings were performed with 10 equiv of the active ester for 5 hr followed by an additional 5-hr treatment in the presence of 5 equiv of imidazole.<sup>12</sup> Efficiency of couplings was

(1) C. H. Li and H. Papkoff, *Science*, **124**, 1293 (1956); C. H. Li, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **16**, 775 (1957).

(2) C. H. Li, W.-K. Liu, and J. S. Dixon, *J. Amer. Chem. Soc.*, **88**, 2050 (1966); C. H. Li, J. S. Dixon, and W.-K. Liu, *Arch. Biochem. Biophys.*, **133**, 70 (1969).

(3) C. H. Li, *Excerpta Med. Found. Int. Congr. Ser.*, **158**, 3 (1968).

(4) B. Gutte and R. B. Merrifield, *J. Amer. Chem. Soc.*, **91**, 501 (1969).

(5) R. B. Merrifield, *ibid.*, **85**, 2149 (1963); *Biochemistry*, **3**, 1385 (1964).

(6) Symbols and abbreviations are in accordance with the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **241**, 2491 (1966); **242**, 555 (1967).

(7) This compound was crystallized as the dicyclohexylamine salt: mp 157–159° (uncorrected);  $[\alpha]_D^{25} +17.6^\circ$  (c 2, CHCl<sub>3</sub>). *Anal.* Calcd for C<sub>28</sub>H<sub>48</sub>N<sub>4</sub>O<sub>8</sub>: C, 62.66; H, 9.01; N, 10.4. Found: C, 62.71; H, 8.82; N, 10.1.

(8) J. C. Sheehan and G. P. Hess, *J. Amer. Chem. Soc.*, **77**, 1067 (1955).

(9) M. Bodanszky and V. duVigneaud, *ibid.*, **81**, 5688 (1959).

(10) R. B. Merrifield, J. M. Stewart, and N. Jernberg, *Anal. Chem.*, **38**, 1905 (1966). The authors wish to thank Drs. S. Farber and J. Meienhofer of the Children's Cancer Research Foundation, Boston, Mass., for the loan of their instrument.

(11) W. W. Cleland, *Biochemistry*, **3**, 480 (1964).

tested<sup>13</sup> in 43% of all cases, and in selected cases a re-treatment with the same reagents was performed. About 48% of the overall yield was sacrificed for this and other purposes. The yield of the protected 188-residue polypeptide resin was 4.35 g (about 47% based on weight and amino acid analysis).

For scission of the solid support and all protecting groups from the synthetic polypeptide, treatment with hydrogen fluoride<sup>14</sup> and then with sodium in liquid ammonia<sup>15a</sup> was employed. A portion (1.34 g) of the protected polypeptide resin was treated with liquid HF in the presence of anisole for 15 min at 0° and approximately 15 min at 0–20°. After a preliminary purification on Sephadex G-25 in 50% acetic acid, the product (330 mg) was treated in four batches with sodium in liquid ammonia<sup>15b</sup> and then oxidized in air, in the presence of DTT, at a concentration of about 0.25 mg/ml at pH 8.4 for 4–5 hr at 25° as previously described.<sup>16</sup> The oxidized material, isolated by lyophilization, was desalted on Sephadex G-25 in 50% acetic acid, a solvent in which native HGH retains full biological activity.<sup>17</sup> It was then subjected to repeated gel filtration on Sephadex G-100 in 20% acetic acid until a fraction was isolated which traveled in the column as a single peak with a maximum close to the position of native HGH. The yield of synthetic protein was 11.6 mg.

Spectrophotometric measurements<sup>18</sup> on the synthetic protein indicated a tyrosine:tryptophan ratio of 7.5 as compared to the known value<sup>2</sup> of 8. Amino acid analysis<sup>19</sup> of an acid hydrolysate gave: Lys<sub>12.8</sub>His<sub>1.8</sub>Arg<sub>9.7</sub>Asp<sub>25.5</sub>Thr<sub>9.2</sub>Ser<sub>17.8</sub>Glu<sub>22.0</sub>Pro<sub>5.9</sub>Gly<sub>9.9</sub>Ala<sub>7.8</sub>Cys<sub>4.3</sub>Val<sub>9.2</sub>Met<sub>1.5</sub>Ile<sub>7.2</sub>Leu<sub>30.1</sub>Tyr<sub>3.6</sub>Phe<sub>12.4</sub>. These values were comparable with the analysis of HGH treated with HF and Na-NH<sub>3</sub>: Lys<sub>9.9</sub>His<sub>2.5</sub>Arg<sub>10.0</sub>Asp<sub>23.3</sub>Thr<sub>9.9</sub>Ser<sub>17.1</sub>Glu<sub>28.7</sub>Pro<sub>8.1</sub>Gly<sub>9.3</sub>Ala<sub>7.1</sub>Cys<sub>3.1</sub>Val<sub>7.6</sub>Met<sub>1.2</sub>Ile<sub>6.7</sub>Leu<sub>24.7</sub>Tyr<sub>6.4</sub>Phe<sub>11.9</sub>.

The synthetic product was found to react immunologically with the rabbit antiserum to HGH as revealed by the agar diffusion test.<sup>20</sup> When the synthetic product was assayed by the rat tibia<sup>21</sup> and pigeon crop-sac<sup>22</sup> tests, it gave approximately 10% growth-promoting potency and 5% lactogenic activity in comparison with that of the native hormone. These values were higher when compared with the treated<sup>23</sup> HGH.

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(13) K. Esko, S. Karlsson, and J. Porath, *Acta Chem. Scand.*, **22**, 3342 (1968).

(14) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jap.*, **40**, 2164 (1967); J. Lenard and A. B. Robinson, *J. Amer. Chem. Soc.*, **89**, 181 (1967).

(15) (a) R. H. Sifferd and V. duVigneaud, *J. Biol. Chem.*, **108**, 753 (1935). (b) Each batch (ca. 80 mg) was stirred near the boiling point for 2 hr in 350 ml of liquid ammonia (freshly distilled from sodium) and then treated at the boiling point with sodium until a light blue color was maintained for about 1 hr.

(16) T. A. Bewley and C. H. Li, *Arch. Biochem. Biophys.*, **138**, 338 (1970).

(17) J. Brovetto-Cruz and C. H. Li, *Biochemistry*, **8**, 4695 (1969).

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(19) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(20) O. Ouchterlony, *Acta Pathol. Microbiol. Scand.*, **26**, 507 (1949); **32**, 231 (1953).

(21) I. I. Geschwind and C. H. Li in "The Hypophyseal Growth Hormone, Nature and Actions," R. W. Smith, Jr., O. H. Gaebler, and C. N. H. Long, Eds., Blakiston, New York, N. Y., 1955, p 28.

(22) W. R. Lyons, *Proc. Soc. Exptl. Biol.*, **35**, 645 (1937); C. S. Nicoll, *Endocrinology*, **80**, 641 (1967).

(23) The treated HGH had only 35% growth-promoting potency and 20% lactogenic activity in comparison with that of the native hormone.

Although the homogeneity of the synthetic product has not yet been completely established, it exhibits to a significant degree those biological activities associated with HGH. These data provide evidence that the hormone does possess intrinsic<sup>3</sup> lactogenic activity.

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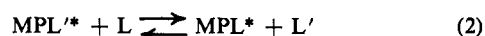
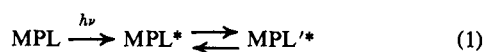
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### Reversible Energy Transfer in Metalloporphyrin Complexes. A Mechanism for Photocatalysis

Sir:

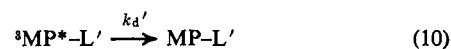
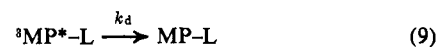
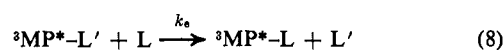
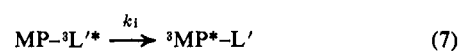
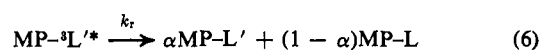
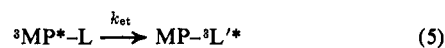
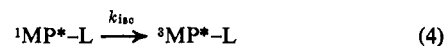
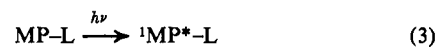
Previously we reported the unusually efficient *cis*-*trans* photoisomerization of stilbene-like olefins such as 1-(1-naphthyl)-2-(4-pyridyl)ethylene (NPE) and 4-stilbazole attached as ligands to zinc and magnesium etioporphyrin I.<sup>1</sup> For example, irradiation of degassed benzene solutions  $5 \times 10^{-5}$  M in zinc etioporphyrin I and  $5 \times 10^{-3}$  M in *cis*-NPE at 25–30° with light absorbed only by the near-ultraviolet or visible bands of the metalloporphyrin caused *cis*-to-*trans* isomerization of NPE with  $\varphi_{c \rightarrow t} = 7$ . The isomerization was efficient only in the thermodynamically favored *cis*-to-*trans* process; stationary states contained *ca.* 96% *trans*. Flash experiments revealed no transients other than the porphyrin triplet; the porphyrin triplet lifetime and absorption spectrum were unchanged by addition of NPE. The results were consistent with a photocatalysis described by eq 1 and 2, where MP = metalloporphyrin, L = *cis*-olefin, and L' = *trans*-olefin.



In the present communication we describe results of experiments which allow determination of the mechanism of the photocatalysis. While the observed results cannot be explained by porphyrin-to-ligand energy transfer and decay of a ligand excited state with isomerization, we suggest that reversible energy transfer with isomerization is a likely path.

Stilbene triplets can be quenched selectively to the *trans* ground state by substances having lower triplet energies, such as azulene;<sup>2,3</sup> we have observed similar phenomena with the stilbazoles.<sup>4</sup> The "azulene effect" has been interpreted in terms of a triplet surface having energy minima near both twisted and *trans* geometries. The metalloporphyrin has  $E_T = 40\text{--}42$  kcal/mol;<sup>1</sup> the spectroscopic triplet of *trans*-NPE is estimated as

$\sim 50$  kcal/mol from the onset of weak singlet-triplet absorption in *o*-iodotoluene.  $E_T$  for *cis*-NPE is probably somewhat higher, based on analogy with similar systems.<sup>2,4-6</sup> Triplet energy transfer from the metalloporphyrin to *cis*-NPE should therefore require some thermal activation,<sup>7</sup> but the possibility of excitation to a distorted NPE triplet may well make the barrier easily surmountable at the temperatures studied (25–30°).<sup>2,5,12,13</sup> Triplet energy transfer to the ligand olefin, followed by rapid equilibration of the olefin triplet between *trans* and twisted forms, and subsequent energy transfer from *trans* triplet back to the porphyrin provide a path for isomerization without excited-state deactivation. The proposed sequence is described by



Making the usual steady-state approximations for intermediates and assuming  $k_d = k_d'$ , we obtain the following expression for  $\varphi_{c \rightarrow t}$ .

$$\varphi_{c \rightarrow t} = \frac{k_{\text{et}}(k_d + k_d[\text{L}])(k_i + \alpha k_r)\varphi_{\text{isc}}}{(k_d + k_d[\text{L}])(k_i + k_r)(k_{\text{et}} + k_d) - k_d[\text{L}]k_i k_{\text{et}}} \quad (11)$$

If we assume  $k_{\text{et}}[\text{L}] \gg k_d$ <sup>14</sup> and  $k_i \gg k_r$ , the expression reduces to<sup>16</sup>

$$\varphi_{c \rightarrow t} = \frac{k_{\text{et}}\varphi_{\text{isc}}}{k_d} \quad (12)$$

We have previously reported<sup>1</sup>  $k_d \approx 2 \times 10^3 \text{ sec}^{-1}$  for zinc etioporphyrin I-NPE in benzene solution at 25°.<sup>17</sup>

(5) W. G. Herkstroeter and G. S. Hammond, *ibid.*, **88**, 4769 (1966).

(6) The triplet energy of porphyrin-bound NPE is unknown; implicit in this discussion is the assumption that  $E_T$  of the bound ligand is nearly unchanged.

(7) Several cases<sup>8-11</sup> of activated energy transfer to higher energy excited states have recently been reported.

(8) J. Saltiel, *et al.*, *J. Amer. Chem. Soc.*, **92**, 410 (1970).

(9) A. A. Lamola, *ibid.*, **92**, 5045 (1970).

(10) P. J. Wagner, M. J. May, A. Haug, and D. R. Graber, *ibid.*, **92**, 5269 (1970).

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(13) A. A. Lamola, *Tech. Org. Chem.*, **14**, 17 (1969).

(14) Results of earlier experiments<sup>15</sup> indicate that both ground and excited states of zinc etioporphyrin undergo very rapid ligand exchange.

(15) D. G. Whitten, I. G. Lopp, and P. D. Wildes, *J. Amer. Chem. Soc.*, **90**, 7196 (1968).

(16) At very low ligand concentrations,  $\varphi_{c \rightarrow t}$  increases with increasing (NPE), but if the concentration is high enough to ensure complete coordination in the ground state, further increase in (NPE) has no effect on  $\varphi_{c \rightarrow t}$ .

(17) Although metalloporphyrin triplets have been shown to decay according to the equation  $-dT/dt = k_1(T) + k_2(T)^2 + k_3(T)(G)$ , where T = triplet concentration and G = ground-state concentration,<sup>18</sup> the relatively weak exciting light used in our flash experiments and the much weaker light used for steady irradiation result in such low concentra-

(1) D. G. Whitten, P. D. Wildes, and I. G. Lopp, *J. Amer. Chem. Soc.*, **91**, 3393 (1969).

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(3) J. Saltiel and E. D. Megarity, *ibid.*, **91**, 1265 (1969); J. Saltiel, *ibid.*, **90**, 6394 (1968); **89**, 1036 (1967).

(4) D. G. Whitten and M. T. McCall, *ibid.*, **91**, 5097 (1969).