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Solid-Phase Synthesis of Crystalline Glucagon†

Svetlana Mojsov and R. B. Merrifield*

ABSTRACT: Mammalian glucagon was synthesized by a stepwise solid-phase method. The support was an alkoxybenzyl alcohol resin, and the biphenylylisopropyloxycarbonyl group was used for temporary α-amino protection. After purification by gel filtration and ion-exchange chromatography, the 29-residue hormone was readily crystallized from water at alkaline pH. The product was homogeneous and indistinguishable from natural bovine glucagon by gel electrophoresis, ion-exchange chromatography, reverse-phase high-pressure liquid chromatography, fluorescence spectroscopy, and amino acid analysis. The synthetic hormone was fully active in the rabbit hyperglycemia assay.

The bihormonal hypothesis of Unger (Unger & Orci, 1975) has created renewed interest in glucagon physiology and its role in diabetes mellitus. It is generally agreed that some of the remaining questions about the mechanism of action of glucagon can best be answered by total synthesis of the hormone and of appropriate analogues. The early synthetic efforts on this 29-residue peptide proved to be quite difficult because of its unusual chemical structure (Schröder, 1967; Wünsch, 1966). The first successful synthesis of mammalian glucagon was accomplished in 1968 (Wünsch & Wendelburger, 1968) and made use of the classical fragment condensation method. The crystalline product was indistinguishable from the native hormone and was shown to be of high purity and full activity in several systems. The second synthesis, several years later, was by the Protein Synthesis Group in Shanghai (1975) who used a solid-phase fragment approach. The protected peptide-resin was cleaved and fully deprotected with HF to give an active product. Recently, Yajima reported solution syntheses of mammalian (Fujino et al., 1978) and avian (Ogawa et al., 1978) glucagons by a scheme which differed from the first two in the size of the fragments, the protecting groups, and the coupling reagent.

The first stepwise solid-phase synthesis of glucagon, briefly reported by us in 1977 (Merrifield et al., 1977), is described here in detail. Because of the presence of an Asp-Ser sequence, in addition to Trp and Met residues, we decided to rely on a mild acidolytic system to avoid α–β rearrangement and excessive alkylation side reactions. Therefore, the C-terminal residue was anchored to *p*-alkoxybenzyl alcohol resin through an ester bond (Wang, 1973). This bond, which was cleavable by 50% trifluoroacetic acid in dichloromethane, required the use of a temporary N^{α}-protecting group such as the biphenylylisopropyloxycarbonyl group (Sieber & Iselin, 1968) that could be removed selectively at each cycle of the synthesis with very mild acid. Side-chain protection was based on the *tert*-butyl group, *tert*-butyl esters for the three aspartic acid residues, *tert*-butyl ethers for the nine hydroxyl-containing residues, and a *tert*-butyloxycarbonyl group for the lysine. In addition, the guanidino groups of the two arginine residues were protected with nitro groups, thus providing the option of their removal by catalytic hydrogenation or by HF or of their retention in the final product. Histidine was protected against racemization during coupling by a dinitrophenyl group on the imidazole nitrogen. Since this residue was N terminal, it was incorporated with an N^{α}-Boc group. Special precautions were taken against nitrile formation during coupling of asparagine and glutamine by using DCC/HOBt.[1] All other couplings were with DCC alone. The protection scheme is summarized in Figure 1. This new synthetic approach to glucagon has provided a rapid and efficient synthesis in moderate yield of homogeneous, fully active hormone that

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[1] Abbreviations used: DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DCHA, dicyclohexylamine; CHA, cyclohexylamine; Bpoc, biphenylylisopropyloxycarbonyl; DMF, dimethylformamide; Dnp, dinitrophenyl; high-pressure LC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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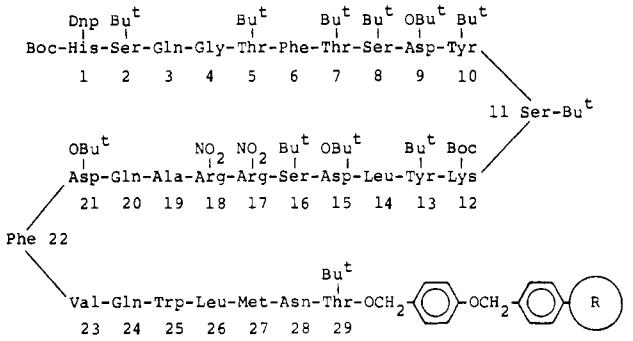

FIGURE 1: Protection scheme for the stepwise solid-phase synthesis of glucagon.

could be crystallized. It now allows the convenient synthesis of a series of analogues for studies on conformation and for use in a search for antagonists and superagonists.

Experimental Procedures

Materials and Methods. Commercial reagents included the following: Bpoc amino acid DCHA salts (Chemical Dynamics), trifluoroacetic acid (Halocarbon Products), pyridine and diisopropylethylamine (Aldrich) both distilled over ninhydrin, dicyclohexylcarbodiimide (Schwarz/Mann), 1-hydroxybenzotriazole (Aldrich), dichloromethane (Eastman) distilled from Na_2CO_3, chloromethyl-copoly(styrene–1%-divinylbenzene)-resin (Bio-Rad), and [^{14}C]Leu (New England Nuclear). Bpoc-Trp·DCHA salt, Bpoc-Met·DCHA, and Bpoc-[^{14}C]Leu·CHA (specific activity 1.63×10^8 dpm/mmol) were prepared by the Triton B method from the amino acid and biphenylylisopropyl phenyl carbonate according to Feinberg & Merrifield (1972). Butyloxycarbonyl-N^{im}-dinitrophenyl-L-histidine (Chemical Dynamics) was recrystallized from 2-propanol; mp 180–181 °C. The purity of all the amino acid derivatives was assessed through their melting points and thin-layer chromatography.

Hydrolysates of peptide-resins were in 12 N HCl/phenol/HOAc (2:1:1) at 110 °C, 24 h, as described by Gutte & Merrifield (1971). Hydrolysates of natural and synthetic glucagon were in 6 N HCl according to Crestfield et al. (1963) and in base, for tryptophan determination, according to Hugli & Moore (1972) (P. Blackburn and S. Moore, personal communication). Peptide synthesis was on the automated Beckman 990 synthesizer. HF reactions were carried out in a Diaflon HF line from Toho Co., Osaka. Analytical high-pressure LC was on a thermostated reverse-phase μBondapak C-18 silica column (4 mm × 30 cm) in a Waters Associates instrument fitted with a Schoeffel variable wavelength UV photometer. Spectra were taken on a Perkin-Elmer Model MPF-2A fluorescence spectrophotometer. The Glucostat reagent was obtained from Worthington, and the glucose analyses were by the method described in their manual.

Results

Assembly of the Amino Acid Sequence of Glucagon. (*a*) *Preparation of the Resin.* 4-(Methoxycarbonyl)phenoxymethyl-resin was prepared from chloromethyl-copoly(styrene–1%-divinylbenzene)-resin beads and 4-hydroxybenzoic acid methyl ester according to Wang (1973). The IR spectrum showed the expected absorption bands at 1716 cm^{-1} for the ester and at 1245 cm^{-1} for the ether. Volhard determination for chlorine was negative. The product (21.5 g) contained 0.64 mmol/g of methoxy groups by microanalysis. The ester was reduced with $LiAlH_4$ (52.7 mmol) in dry ether (400 mL) under nitrogen for 20 h at 55 °C. The resin was filtered and washed with ether, ethyl acetate, MeOH, and CH_2Cl_2 to give a gray product. The color was removed by stirring in 2 L of a 1:1 mixture of 1 N H_2SO_4 and dioxane for 45 h at 25 °C. The resin was filtered, washed with dioxane, CH_2Cl_2, and MeOH, and dried under vacuum to give 19.5 g of white 4-(hydroxymethyl)phenoxymethyl-resin. This is often referred to as "alkoxybenzyl alcohol resin". The IR spectrum showed complete disappearance of the ester band at 1716 cm^{-1}, although microanalysis indicated 0.06 mmol of OCH_3 per g.

(*b*) *Bpoc-Thr(Bu^t)-OCH$_2$-resin.* The 4-(hydroxymethyl)-phenoxymethyl-resin (5.10 g, 2.9 mmol of hydroxyl sites) was esterified by stirring at 25 °C for 6 h with a solution containing Bpoc-Thr(Bu^t) (1.2 g, 2.9 mmol), DCC (0.60 g, 2.9 mmol), and 4-(dimethylamino)pyridine (1.1 g, 9 mmol) in 35 mL of CH_2Cl_2 according to Wang (1973). The resin was filtered and washed with CH_2Cl_2, DMF, MeOH, and CH_2Cl_2, and the excess hydroxymethyl sites were benzoylated by stirring the resin with a mixture of benzoyl chloride (5.9 mL) and pyridine (5 mL) in 50 mL of CH_2Cl_2 for 15 min at 0 °C. The resin was filtered, washed with CH_2Cl_2, 2-propanol, and CH_2Cl_2, and dried under vacuum. The substitution level of threonine was found to be 0.20 mmol/g by acid hydrolysis and amino acid analysis and 0.23 mmol/g by picric acid titration (Gisin, 1972) of the free amino group liberated by deprotection in 0.5% CF_3COOH/CH_2Cl_2.

(*c*) *Automated Assembly of the Glucagon Chain.* The general methods of solid-phase peptide synthesis (Merrifield, 1963, 1969; Barany & Merrifield, 1979) were applied, but with a number of modifications. The synthesis was begun by loading 5.10 g of Bpoc-Thr(Bu^t)-OCH_2-resin into the reaction vessel of the Beckman Model 990 peptide synthesizer where all the operations of the chain assembly were carried out. All of the metered solvent volumes were 75 mL except for the amino acid reagents which were 20 mL and the DCC reagent which was 5 mL. One cycle of the synthesis consisted of the following operations: (1) CH_2Cl_2 wash and preswell (3 × 1 min); (2) deprotect with 0.5% CF_3COOH/CH_2Cl_2 (3 × 1 min prewashes + 1 × 20 min) (3) CH_2Cl_2 wash (5 × 1 min); (4) neutralize with 5% diisopropylethylamine in CH_2Cl_2 (2 × 5 min); (5) CH_2Cl_2 wash (5 × 1 min); (6) equilibrate with Bpoc amino acid (4 equiv, in 20 mL of CH_2Cl_2, 5 min); (7) without filtration, add DCC (4 equiv, in 5 mL of CH_2Cl_2), couple 120 min; (8) CH_2Cl_2 wash (5 × 1 min); (9) repeat steps 4–9 with 2 equiv each of Bpoc amino acid and DCC. For the first six cycles of the synthesis, any unreacted chains were terminated by benzoylation (Wang, 1973). After incorporation of tryptophan at position 25 (fourth coupling), 0.1% indole was added to the deprotection reagent to protect against alkylation of the indole ring of tryptophan (Wünsch et al., 1977) and the thioether of methionine. The reaction vessel was maintained under nitrogen throughout the synthesis to minimize air oxidation. For the coupling of radioactive leucine at position 26, only 2 equiv of Bpoc-[^{14}C]Leu (0.16 mCi) was used in the first coupling, and 4 equiv of unlabeled Bpoc-Leu was used in the second coupling to help ensure complete reaction. The total incorporation of Leu was determined to be 0.54 mmol, and the specific activity of [^{14}C]Leu in the peptide chain was 3.4×10^7 dpm/mmol. To avoid nitrile and amidine formation, Bpoc-Asn and Bpoc-Gln were coupled as 1-hydroxybenzotriazole esters (König & Geiger, 1970; Mojsov et al., 1980). For that purpose, HOBt (674 mg, 4.4 mmol) was dissolved in 2 mL of DMF and added to DCC (906 mg, 4.4 mmol) in 10 mL of CH_2Cl_2 at 0 °C. Bpoc-Asn or Bpoc-Gln (4.4 mmol) dissolved in 8 mL of CH_2Cl_2/DMF (5:3) was added, and after 10 min at 0 °C, the mixture was added to the neutralized

Table I: Radioactive Monitoring of Chain Loss during the Deprotection Step

glucagon residue deprotected	^{14}C in deprotection filtrate (cpm)	loss of chains[a] (%)
Gln-24	27 200	0.20
Val-23	13 600	0.10
Gln-20	30 300	0.22
Ala-19	37 700	0.27
Arg-18	34 900	0.25
Arg-17	34 900	0.25
Leu-14	50 900	0.37
Lys-12	22 200	0.16
Ser-11	30 900	0.22
Asp-9	23 700	0.17
Thr-7	24 700	0.18
Gln-3	13 600	0.10

[a] Based on the total [^{14}C]Leu^{26} counts found in the combined filtrate and washings from the 0.5% CF_3COOH/CH_2Cl_2 deprotection step. The counts were corrected for background, quench, and sample dilution. The total ^{14}C counts in the resin at the start of the synthesis was 1.36×10^7 cpm.

peptide-resin in the reaction vessel. Coupling was 120 min at room temperature. This was followed by a second coupling with 2 equiv of the reagents under the same conditions. It was important to avoid prolonged exposure of the Bpoc group to excess acidic HOBt.

Following the washing step after each coupling reaction, the machine was stopped, and an aliquot of the peptide-resin was monitored by treating with fluorescamine to test for the presence of any unreacted free amino groups (Felix & Jimenez, 1973). Under these conditions, using Bpoc-amino acids, a slight positive background was always observed. The best evidence for completion of the coupling reaction was the constancy of the color after successive couplings. In this particular synthesis, no difference in background fluorescence was ever observed between samples removed after the first and second couplings except for those following Bpoc-[^{14}C]Leu where only a small excess of reagent was used in the first coupling.

After the incorporation of the radiolabeled [^{14}C]Leu at position 26, the losses of peptide chain from the resin during the deprotection step could be quantitated. Filtrates from the 0.5% CF_3COOH reaction (prewash, deprotection, and wash steps) were combined and counted. As shown in Table I, the average value for the peptide chains lost was 0.2% per step.

Workup and Purification of Synthetic Glucagon. (*a*) *Deprotection of His(Dnp).* The dinitrophenyl group was removed from the imidazole nitrogen of histidine by thiolysis while the fully protected peptide was still anchored to the resin (Stewart et al., 1972; Lin et al., 1972). The peptide-resin (3.22 g) was placed in the reaction vessel of a manual shaker, thiophenol (19.5 mL of a 0.2 M solution in DMF, 19 equiv relative to His) was added, and the mixture was shaken for 60 min. The removal of the Dnp group was quantitated by measuring the absorbance of the product, 2,4-dinitrophenyl phenyl sulfide ($\epsilon_{337}^{1cm} = 10250$). The reaction was complete within 60 min, and 0.20 mmol of product was found, representing the total number of growing chains. The decrease in growing chains relative to the starting Bpoc-Thr(Bu^t)-resin is thought to be due to a termination in the early stages of the synthesis not detected by the monitoring procedure. These conclusions are consistent with the results of amino acid analyses.

(*b*) *Cleavage of the Peptide from the Resin and Simultaneous Deprotection of All tert-Butyl-Based Groups.* After removal of the Dnp group by thiolysis, the partially protected peptide-resin (3.2 g, 0.20 mmol of peptide) was washed with DMF and CH_2Cl_2. A solution (30 mL) of CF_3COOH/CH_2Cl_2 (1:1 v/v) containing 300 mg (2.56 mmol) of indole was added, and the suspension was shaken for 120 min at room temperature. The resin was filtered, washed with CF_3COOH/CH_2Cl_2 and CH_2Cl_2, and evaporated to dryness. The resin was further extracted with 50% aqueous acetic acid 3 times. The acetic acid extracts were combined with the dry CF_3COOH extract and lyophilized. The crude dinitroglucagon weighed 250 mg. The cleavage yield was 35% based on both amino acid analyses and [^{14}C]leucine. A 65-mg portion was dissolved in 50% acetic acid, placed on a Sephadex G-25 superfine column (2.5 × 90 cm), and eluted with 1% acetic acid at 17 mL/h. The column was monitored spectrophotometrically at 280 nm and by scintillation counting of 100-μL aliquots. The material eluting between 186 and 228 mL with a peak at 210 mL corresponded to the position of natural glucagon on the same column. Material eluting with peaks at 154 and 322 mL was discarded. The main peak accounted for 30.7 mg (47%); by radioactivity the recovery was 42%.

(*c*) *Removal of the Nitro Protecting Groups from Arginine.* An aliquot (11.3 mg) of synthetic [bis(Arg(NO_2))^{17,18}]-glucagon recovered from the Sephadex column was added to the Teflon reaction vessel of the HF cleavage apparatus. Anisole (0.5 mL) and indole (10 mg) were added, and 4.5 mL of HF was collected at −78 °C by distillation. The temperature was maintained at 0 °C for 30 min, and the HF was then removed by vacuum pump at 0 °C. The peptide was extracted into 30 mL of 1% HOAc, and anisole and its derivatives were extracted from the aqueous phase with ether (3 × 15 mL). The aqueous solution was lyophilized (11.3 mg).

As a control, performed before this synthetic strategy was undertaken, the above procedure was carried out on natural glucagon. No damage to the hormone was observable, as evidenced by ion-exchange chromatography on DEAE-cellulose or by bioassay based on the increase of blood sugar levels in the rabbit.

Alternatively, the nitro groups could be removed by catalytic hydrogenation in the presence of freshly prepared palladium black (Wieland, 1912). This procedure was based on our early work with Met-Lys-bradykinin (Merrifield, 1964) in which two nitroarginine residues were readily reduced in the presence of a methionine residue. However, recovery of glucagon from dinitroglucagon by hydrogenation has been less satisfactory.

(*d*) *Ion-Exchange Chromatography of Synthetic Glucagon.* A sample (6.5 mg) of the HF-treated synthetic material was dissolved in 7 mL of a pH 8.5 buffer that was 0.01 M in Tris, 0.001 M in EDTA, and 7 M in urea and placed on a DEAE-cellulose cation-exchange column (2.6 × 30 cm) (Bromer et al., 1972) and equilibrated in the same buffer. It was eluted in this buffer with a linear gradient of 0 to 0.4 N NaCl. The eluant was monitored by radioactivity as shown in Figure 2 (part b). The same elution pattern was obtained by UV monitoring at 280 nm. The main peak at 96 mL corresponded to 0.10 N NaCl and accounted for 57% of the radioactivity applied to the column. The two small, well-separated peaks at 122 mL (0.13 N NaCl) and 205 mL (0.22 N NaCl) accounted for an additional 40%.

The chromatographic run of synthetic glucagon was followed immediately on the same column under the same conditions by a run of commercial native glucagon. This time the eluant was monitored by absorbance at 280 nm. Glucagon, the main component, again eluted at 0.10 N NaCl (93 mL), and in addition, a second component was found at 0.25 N NaCl (Figure 2, part a). On the basis of the data of Bromer et al. (1972), this contaminating component and the slower

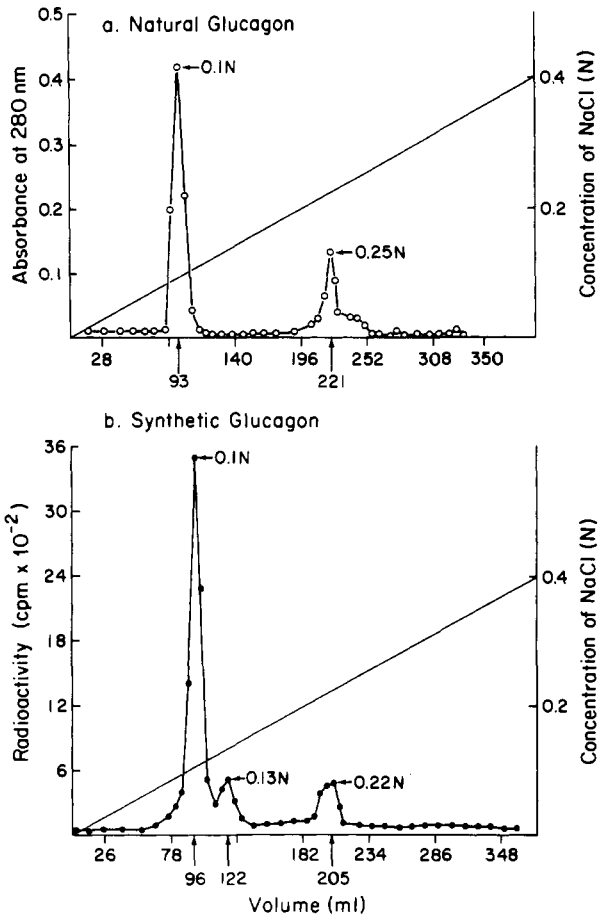

FIGURE 2: Ion-exchange chromatography of (a) natural and (b) synthetic glucagon on a DEAE-cellulose column (2.6 × 30 cm) in 0.01 N Tris buffer, pH 8.5, containing 7 M urea and 0.001 M EDTA. Peptides were eluted in this buffer with a 0 to 0.4 N NaCl linear gradient at 18 mL/h.

moving material at 0.22 N NaCl from the synthetic product are presumed to be deamidated glucagon.

The lyophilized synthetic fraction from peak I of the DEAE-cellulose column was dissolved in 1% acetic acid and desalted on a Sephadex G-25 column (2.5 × 90 cm) in 1% acetic acid. The peak of radioactivity, which eluted at 210 mL, was collected and lyophilized (96% of the counts applied to the column were recovered).

(*e*) *Rechromatography.* The homogeneity of the synthetic glucagon in this system was checked by reapplication of 2 mg of sample to the DEAE-cellulose column. The conditions were exactly the same as before, and the eluate was monitored by radioactivity. This time a single symmetrical peak was obtained at 0.10 N NaCl (95 mL), and none of the slower moving components were detectable.

(*f*) *Cochromatography.* Finally, the chromatographic identity of the synthetic and natural glucagons was tested by cochromatographing a mixture of the two on the same column. For that purpose, a small (1.5 mg) sample of purified synthetic material, which could be detected by radioactivity, was mixed with a larger amount (10 mg) of natural glucagon, which could be detected by absorbance at 280 nm. They were applied together on the DEAE-cellulose column (2.6 × 30 cm) and eluted as before in the pH 8.5 Tris–urea buffer with a linear 0–0.4 N NaCl gradient. The curves for both radioactivity and UV absorbance were symmetrical and superimposable, and the peak was at 0.10 N NaCl (95 mL). The expected small UV peak at 0.25 N NaCl derived from the natural glucagon sample was also detected.

Table II: Amino Acid Composition of Synthetic and Natural Glucagon

amino acid	expected	found: synthetic purified peptide	found: natural
Trp[a]	1	0.98	1.05
Lys	1	0.97	0.95
His	1	0.95	0.95
Arg	2	2.05	1.91
Asp + Asn	4	4.10	4.20
Thr	3	2.96	3.03
Ser[b]	4	3.75	3.90
Glu (Gln)	3	3.02	3.03
Gly	1	1.05	1.07
Ala	1	1.00	1.00
Val	1	0.95	0.97
Met	1	0.98	1.07
Leu	2	2.01	2.05
Tyr	2	1.95	2.00
Phe	2	1.95	1.98

[a] The value for tryptophan was determined by alkaline hydrolysis with 4 N NaOH. [b] Values for serine are corrected 15% for partial destruction during the acid hydrolysis.

Characterization of Synthetic Glucagon. All further characterization of synthetic glucagon was performed on the material recovered from peak I of the DEAE-cellulose column and desalted on Sephadex G-25. The natural glucagon standard was a commercial sample that had also been chromatographed on DEAE-cellulose and desalted on Sephadex G-25.

(*a*) *Amino Acid Analysis.* Two samples of synthetic glucagon (0.90 and 1.05 μmol) and two of natural glucagon (0.80 and 1.20 μmol) were hydrolyzed in 6 N HCl, and the composition was determined on the amino acid analyzer. The values for tryptophan were determined on other samples after alkaline hydrolysis (Table II). The amino acid composition of synthetic glucagon was in good agreement with that of the natural control and with that expected from the known sequence. The mean deviation of the 29 amino acid residues from equimolar ratios was ±3% for both analyses. Note that the molar ratio of Tyr/Trp was 1.99 (theory 2).

(*b*) *Spectrophotometric Characterization.* This method of analysis was used to show that no significant modifications of the indole ring of tryptophan had occurred. The spectra were taken on samples (300 μg/mL) in 1% acetic acid. The excitation maxima of both synthetic and natural glucagon were at 292 nm. The broad emission spectra had maxima between 350 and 360 nm and were indistinguishable.

(*c*) *Polyacrylamide Gel Electrophoresis.* Synthetic and natural glucagon were electrophoresed on a 20% polyacrylamide slab gel (1 mm thick, 170 mm long) by a modification of the method of Laemmli (1970) in which sodium dodecyl sulfate was omitted. Samples (10 μg/10 μL) were applied in a solution of 0.05 M Tris-HCl, pH 6.7, 6 M urea, and 0.001% bromphenol blue. The chamber buffer was a mixture of 28.8 g of glycine and 6.0 g of Tris-HCl per L of water. Running time was 14 h at 10 mA at 25 °C. Peptides were visualized by staining with 0.2% Coomassie Blue R in 35% methanol and 10% acetic acid and destaining in 10% acetic acid. This sytem allows detection of small differences in charge and is quite sensitive in testing for homogeneity. Both synthetic and natural glucagon gave sharp bands of identical appearance at 18 mm from the origin.

(*d*) *Analytical High-Pressure Liquid Chromatography.* Samples of synthetic and natural glucagon (10 μg) were run separately on a reverse-phase silica C-18 column (4 mm × 30

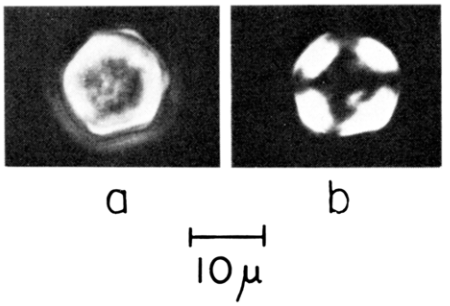

FIGURE 3: Crystals of synthetic glucagon.

cm) (Burgus & Rivier, 1976). Elution with a 0–15% linear gradient of 33% acetonitrile in 0.01 M ammonium acetate buffer, pH 4.00, as the first solvent and acetonitrile as the second gave a retention time of 7.0 min for both synthetic and natural glucagon.

(*e*) *Crystallization.* The purified, synthetic glucagon was crystallized twice, using two separate conditions. (a) Synthetic material (1 mg) was suspended in 0.4 mL of water in a 6 × 50 mm culture tube and dissolved by the addition of 0.1 N NaOH (60 μL) until the pH reached 10.2. The pH was then lowered to 8.8 with 0.1 N HCl (~50 μL), and the tube was placed in the cold room at 4 °C. Crystals appeared after 24 h. They were visualized with Nomarski optics under the microscope at 256 magnification (Figure 3a) and had the typical hexagonal appearance corresponding to the rhombic dodecahedra structure of glucagon (Staub et al., 1955). (b) Synthetic glucagon (3 mg) was dissolved in 1 mL of 0.2 N potassium phosphate buffer, pH 9.2, by slowly warming the solution to 50 °C. The test tube was then suspended in a Dewar flask containing water at 50 °C and allowed to cool to room temperature. After 24 h, small crystals appeared. They were birefringent under a polarized microscope at 256 magnification (Figure 3b).

Biological Activity. Hyperglycemic Activity. Rabbits (3 kg) were fasted for 18 h and then injected intravenously with 1.5 mL of synthetic glucagon in saline at doses of 0.5–4.0 μg/kg of body weight. Blood samples (1–2 mL) were withdrawn immediately before and at intervals of 3–5 min following injection for a period of 40 min. The samples were centrifuged, and the concentration of glucose in the serum was determined by a colorimetric glucose assay by using the coupled enzyme system of glucose oxidase and peroxidase (Glucostat reagent). Saline controls and natural glucagon standards were run on the same animals for comparison. One week was allowed between each test for the animal to recover. All samples and controls were assayed 3 times. The blood glucose level was plotted as a function of time, as illustrated in Figure 4. The average time to reach a maximum response for each of the samples tested was 20 ± 5 min. In addition, the maximum increase of blood glucose caused by the various glucagon concentrations per kilogram of body weight was plotted for the natural and synthetic hormones (Figure 5). It is evident that within the limits of our bioassay the hyperglycemic response to the synthetic and natural glucagon was the same.

Discussion

The purpose of this work was to develop a synthesis of glucagon that would be simple and rapid enough to allow the efficient preparation of a large series of analogues of the hormone. These were needed for studies on the conformation of the molecule and in a search for inhibitors and superagonists of glucagon. Although the classical syntheses already reported

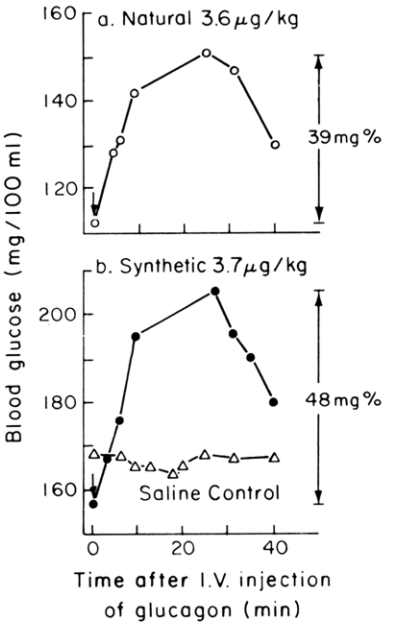

FIGURE 4: Blood glucose response to iv injection of (a) natural (3.6 μg/kg) and (b) synthetic (3.7 μg/kg) glucagon and saline into rabbits.

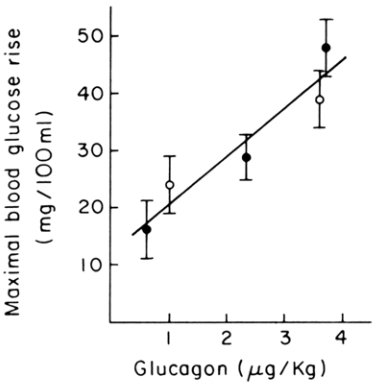

FIGURE 5: Dose–response curve of glucagon activity in the blood glucose assay. (●) Synthetic glucagon; (○) natural glucagon. Average of three separate determinations.

for glucagon were elegant and gave pure material, we believe their application to these analogue problems would require excessively large amounts of time and effort. A successful stepwise solid-phase synthesis should be more useful for our purposes. The synthesis described here using protecting groups that are removed under mild acid conditions has avoided most of the known problems associated with this molecule and has given a homogeneous, crystalline product with the physical, chemical, and biological properties of natural mammalian glucagon.

The ease of purification of the synthetic glucagon reflects to a major degree the suitability of the synthetic strategy employed. The overall strategy was to use *p*-alkoxybenzyl alcohol resin in combination with N-terminal and side-chain protecting groups that would allow the stepwise assembly of the peptide chain and the cleavage step, together with removal of all carbonium ion sources, to be carried out in the absence of strong acids. In that way, several of the known side reactions, especially aspartimide formation at the Asp^{15}–Ser^{16} bond and destruction of tryptophan, would be avoided. The final residues to be deprotected were the two ω-nitroarginines at positions 17 and 18. The nitro group was selected for the synthesis so that it might be removed either by catalytic hydrogenation or by anhydrous HF or could be retained in the final product. The use of HF to remove the nitro groups at

the end of the synthesis, after cleavage and removal of all the carbonium ion sources, was not expected to be deleterious. We had shown earlier (Yang & Merrifield, 1976) that aspartic acid residues with free β-carboxyl groups have little tendency to form imides in acid. The deprotection in HF in the presence of anisole and indole was found to be entirely satisfactory, and the generation of arginine was quantitative. A control to show the suitability of HF was performed by treating native glucagon under the same conditions and demonstrating unchanged chromatographic behavior and retention of full hormonal activity.

Even though glucagon contains a methionine residue, there was precedent for easy catalytic removal of nitro groups from two arginine residues in the presence of a methionine as in the case of methionyllysyl-bradykinin (Merrifield, 1964). However, this reaction so far has led to very low yields of free glucagon and requires more study.

To avoid alkylation of tryptophan (Wünsch et al., 1977), indole was added during the acid deprotection of the N^{α}-Bpoc groups and during the 50% CF_3COOH cleavage step where all the *tert*-butyl groups were removed. Both the fluorescence spectroscopy and the good recovery of Trp following alkaline hydrolysis indicated that this residue was unaltered in the purified product. The addition of 1-hydroxybenzotriazole to a DCC coupling according to König & Geiger (1970) was shown (Mojsov et al., 1980) to reduce the levels of nitrile and amidine formation from asparagine to less than 0.2%, and therefore this method was used with confidence to couple Asn and Gln.

Histidine-1 was protected against racemization during coupling (Windridge & Jorgenson, 1971) with an N^{im}-Dnp group (Shaltiel, 1967; Lin et al., 1972). The group was conveniently removed while the peptide was still attached to the resin by treatment with thiophenol and quantitated spectrophotometrically. This provided a check on the amount of growing peptide chains at the end of the synthesis.

The peptide ester bond to *p*-alkoxybenzyl alcohol resin of Wang, which has been used successfully in the past for the synthesis of a number of smaller peptides, showed satisfactory acid stability during the 28 steps of removal of the N^{α}-biphenylylisopropyloxycarbonyl protecting group in 0.5% trifluoroacetic acid in dichloromethane. The cumulative loss of chains from the resin by acidolysis was estimated to be only 5.6%, based on the measurement of radioactivity in the filtrates. The cleavage of the des-Dnp-peptide-resin with CF_3COOH/CH_2Cl_2 (1:1) for 2 h at 25 °C gave dinitroglucagon in only 35% yield. The starting resin in this particular synthesis was a commercial chloromethyl-resin that was later found to contain appreciable amounts of hydroxymethyl groups (Kent et al., 1979). Esterification at these sites would give rise to peptide chains that could not be cleaved by CF_3COOH and may explain in part the low cleavage yields.

Purification of the synthetic glucagon by ion-exchange chromatography on DEAE-cellulose was simple and quite effective. Two small byproducts were well separated, and 57% of the applied peptide was recovered in a single main peak. Rechromatography showed only a single peak in the same position as before which corresponded exactly with natural glucagon run separately on this same column. Furthermore, cochromatography of the natural and synthetic glucagons was not able to resolve them into more than one component.

Chromatography, based on a different principle, on a reverse-phase μBondapak C-18 column on a high-pressure LC apparatus also showed identical behavior. Finally, polyacrylamide gel electrophoresis, a method based on net charge, gave sharp bands of identical appearance at the same distance from the origin. These two high-resolution systems provide good evidence for the similarity of the natural and synthetic peptides.

The chromatographically purified glucagon was further characterized by hydrolyses and amino acid anlyses. All residues, including tryptophan, were in good agreement with theory.

Glucagon is one of the few naturally occurring small peptides that is readily crystallized in the free form. Classically, crystallization has been viewed as the best way to purify an organic compound, and to show that the final product is pure, and although we do not believe that crystalline peptides must necessarily be pure, the fact that our synthetic glucagon was obtained as crystals is satisfying. It is taken as a further indication of its purity and identity with the natural hormone. Under the microscope, the crystals were birefringent and appeared primarily as hexagons.

Glucagon was discovered as a glucose-elevating substance in the pancreas (Kimball & Murlin, 1923). Therefore, for the assay of our synthetic glucagon, we used the hyperglycemic response of fasted rabbits to intravenous glucagon. In dose–response experiments, both the time required to reach a maximum blood glucose level and the average rise in blood glucose were found to be equivalent for the natural and synthetic hormone, and we conclude that our preparation has the biological activity of natural glucagon.

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Increased Translatable Messenger Ribonucleic Acid for Argininosuccinate Synthetase in Canavanine-Resistant Human Cells†

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ABSTRACT: The level of argininosuccinate synthetase activity in the human tissue culture cell line RPMI 2650 was 6-fold higher when citrulline was substituted for arginine in the culture medium. Canavanine-resistant (Can^r) variants were isolated and had enzyme activity up to 25 nmol min^{-1} (mg of protein)$^{-1}$ or 180-fold higher than that of the wild-type cells grown in arginine. The differences in enzyme activity were paralleled by differences in the amount of enzyme determined immunologically. The micrograms of enzyme per milligrams of protein, determined by complement fixation, were 0.03 for wild-type cells grown in arginine, 0.29 for wild-type cells grown in citrulline, and 6.73 for a Can^r variant. In vivo labeling studies suggested increased synthesis of argininosuccinate synthetase in Can^r cells, and in vitro translation of poly(adenylic acid) [poly(A)] messenger ribonucleic acid (mRNA) from wild-type and Can^r cells confirmed a quantitatively compatible increase in translatable poly(A) mRNA for the enzyme in Can^r cells. No precursor for the enzyme was recognized by using in vitro translation, and the poly(A) mRNA for the enzyme had a sedimentation value of 16 S by sucrose-gradient analysis. The levels of argininosuccinate synthetase activity in the Can^r cells were similar to those found in normal liver.

Argininosuccinate synthetase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1) are important enzymes in the urea cycle, and genetic deficiencies can cause hyperammonemia, mental retardation, and death. Through the reactions shown in eq 1 and 2, these enzymes can synthesize arginine from citrulline + aspartate +

$$\text{ATP} \underset{\text{Mg}^+}{\overset{\text{synthetase}}{\rightleftharpoons}} \text{argininosuccinate} + \text{AMP} + \text{PP}_i \quad (1)$$

$$\text{argininosuccinate} \overset{\text{lyase}}{\rightleftharpoons} \text{fumarate} + \text{arginine} \quad (2)$$

citrulline, a function which may be important in nonhepatic tissues and in cultured cell experiments. The activity of the urea-cycle enzymes in mammalian liver is increased with increased dietary protein (Schimke, 1962; Nuzum & Snodgrass, 1971). Argininosuccinate synthetase and lyase are subject to metabolite control in cultured mammalian cells. Schimke (1964) observed that argininosuccinate synthetase and argininosuccinate lyase activities were repressed coordinately by the presence of arginine in the growth medium of HeLa, KB, and L cells. Conversely, when arginine was removed and replaced by citrulline in the growth medium, the argininosuccinate synthetase and argininosuccinate lyase activities increased 4–20-fold in these cell lines. Irr & Jacoby (1978) found that argininosuccinate synthetase in cultured human lymphoblasts was increased when citrulline replaced arginine in the medium, but that argininosuccinate lyase activity remained constant.

Variation in argininosuccinate synthetase also is observed in certain cell variants. Jacoby (1978) described the isolation of human lymphoblast cell lines which were resistant to the arginine analogue canavanine. Canavanine is not a specific inhibitor of argininosuccinate synthetase but rather is toxic when incorporated into protein in place of arginine. The canavanine-resistant cells had increased argininosuccinate synthetase activity, and presumably were resistant to canavanine because of an increased conversion of citrulline to arginine. The canavanine-resistant lymphoblasts isolated by Jacoby (1978) had lost the ability to respond to changes in arginine concentration.

We have isolated canavanine-resistant variants of the human cell line RPMI 2650. The amount of argininosuccinate synthetase protein and its messenger ribonucleic acid (mRNA) were increased in the variant cell lines.

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