Glucagon Carboxyl-Terminal Derivatives: Preparation, Purification, and Characterization†

Richard D. England, Barry N. Jones,‡ Kathleen Corey Flanders, Sharon A. Coolican, T. Michael Rothgeb,§ and Ruth S. Gurd*

ABSTRACT: Chemical and enzymatic methods have been used to prepare the following series of seven glucagon derivatives modified in the carboxyl-terminal region important for hormone–receptor binding: [des-Asn^{28},Thr^{29}](homoserine lactone^{27})glucagon, [des-Asn^{28},Thr^{29}](homoserine^{27})glucagon, (*S*-methyl-Met^{27})glucagon, [des-Thr^{29}](*S*-methyl-Met^{27})-glucagon, [des-Thr^{29}]glucagon, [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})glucagon, and [des-Asn^{28},Thr^{29}]glucagon. The derivatives were isolated in high yield, extensively purified, and chemically characterized. All were found to be full agonists of native glucagon. Binding affinity was evaluated by displacement of mono[^{125}I]iodoglucagon prepared by new methods. Binding and biological activities closely correlated, indicating that most modifications affected the relative binding affinity and relative biological potency of glucagon to a comparable extent. Circular dichroism measured in dilute acid solution resembled that of native glucagon except for [des-Asn^{28},Thr^{29}]glucagon which displayed increased α helicity (25%). All derivatives formed helical structures in 2-chloroethanol, although the amount of helicity induced was not closely correlated with biological activity. Binding and biological activities were not affected by removal of Thr-29, though both were reduced 20-fold when Asn-28 was also removed, irrespective of whether homoserine or native methionine remained at the carboxyl terminus. Lactone formation was associated with a further 5-fold reduction in binding affinity but not in activity. Methylation of Met-27 had essentially the same effect as removing the two carboxyl-terminal residues, although the combined effect of both modifications was a greater than 100-fold reduction in binding and activity. These findings provide additional insight concerning glucagon structure–function relationships.

Glucagon is a 29 amino acid hormone known for its key role in the activation of adenylate cyclase (Rodbell, 1980) and the maintenance of fuel homeostasis and for its possible involvement in the pathogenesis of diabetes mellitus (Unger & Orci, 1981). Recently investigative interests have increasingly focused on the development of a wide variety of modified derivatives to probe the molecular mechanisms involved in glucagon action as well as to search for clinically useful antagonists of the hormone (Bregman et al., 1980). The general conclusion that can be reached from these numerous studies is that virtually the whole molecule participates in the receptor recognition process, and essentially the entire sequence is required for the full expression of hormonal activity (Epand et al., 1981). However, the lipophilic carboxyl-terminal region seems to serve no essential function in the mechanism of action and may be important only for the high receptor affinity of the hormone (Wright et al., 1978; Hruby et al., 1981). It has been suggested that receptor binding involves a helical conformer of glucagon induced or stabilized at the receptor by hydrophobic interactions involving residues in the carboxyl-terminal region of the molecule (Sasaki et al., 1975). The hydrophobic, helix-favoring methionine residue at position 27 has been considered to be particularly important for receptor binding since it appears to facilitate helix formation in glucagon, and the ability of glucagon derivatives to assume an α-helical conformation appears to correlate with their receptor affinity and biological activity (Bromer, 1976; Deranleau et al., 1978). A significant contribution to binding from the hydrophilic Asn-28 and Thr-29 residues and possibly the terminal carboxylate group has also been suggested on the basis of results obtained from studies of cyanogen bromide cleaved glucagon (Lin et al., 1975).

The methylation–demethylation technique of Rothgeb et al. (1977) provides a unique approach to the synthesis of carboxyl-terminal derivatives of glucagon useful for investigating structure–function relationships. In the present study, we have taken advantage of the methylation reaction not only to prepare methylated derivatives but also to restrict enzymatic cleavages and to provide a chromatographic handle for purification. Using this strategy along with the improved cyanogen bromide cleavage reaction (Jones & Gurd, 1981), we have prepared a series of seven derivatives in sufficient quantity for analysis of their physical properties and in sufficient purity for assessment of the role of bulk, charge, and hydrophobicity for their biological properties.

Experimental Procedures

Materials

Crystalline porcine glucagon (Lot No. IGN70 QA097U) was provided through the courtesy of Eli Lilly and Co. Carboxypeptidase Y and 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (IODO-GEN)[1] were purchased from Pierce Chemical Co., and PMSF–carboxypeptidase A was obtained from Worthington Biochemical Corp. Dithiothreitol came

† From the Medical Sciences Program and the Department of Chemistry, Indiana University, Bloomington, Indiana 47405. *Received August 31, 1981.* This work was supported by U.S. Public Health Service Research Grant AM-21121. R.D.E. gratefully acknowledges support by American United Life through the Insurance Medical Scientist Scholarship Fund.

‡ Present address: The Roche Institute of Molecular Biology, Nutley, NJ 07110.

§ Present address: The Procter and Gamble Co., Cincinnati, OH 45217.

[1] Abbreviations: IODO-GEN, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril; PMSF, phenylmethanesulfonyl fluoride; HPLC, high-performance liquid chromatography; ODS, octadecylsilane; BSA, bovine serum albumin; CD, circular dichroism; SMM, *S*-methylmethionine; Hse, homoserine; other abbreviations are those recommended by IUPAC–IUB.

0006-2960/82/0421-0940$01.25/0

from Sigma Chemical Co., "ultrapure" grade Tris base from Schwarz/Mann, 2-chloroethanol from Eastman Kodak Co., and fluorescamine from Hoffmann-La Roche. Carrier-free [^{125}I]iodine in NaOH (>350 mCi/mL), [^{3}H]cAMP (30–50 Ci/mmol), and Aquasol scintillation cocktail were purchased from New England Nuclear Corp., and [α-^{32}P]ATP (10–25 Ci/mmol) was from ICN. Unlabeled cAMP and ATP came from Calbiochem, and GTP, creatine phosphate, and creatine phosphokinase were from Boehringer Mannheim Corp. Male Sprague-Dawley rats (140–160 g) were obtained from Laboratory Supply Co., Indianapolis.

The Sephadex G-25F and G-50SF gel filtration resins were obtained from Pharmacia and the CM-cellulose (CM52) and DEAE-cellulose (DE52) ion-exchange resins from Whatman. Dowex AG 50W-X4 (200–400 mesh, H^+ form), Dowex AG 1-X8 (100–200 mesh, Cl^- form), and the materials required for isoelectric focusing came from Bio-Rad laboratories. Woelm neutral alumina (activity grade 1) was obtained from ICN Nutritional Biochemicals. Oxoid "Nuflow" filters came from Medox Chemicals (Ottawa, Canada), and Spectrapor 6 dialysis tubing was from Cole-Parmer. All solutions of urea were freed of cyanate immediately before use by passage through a 2.5 × 50 cm column of Rexyn I-300 (Fisher Scientific Co.). The solvents used for HPLC were distilled from ninhydrin. All other chemicals and reagents were the best grade commercially available and were used without further purification. Distilled–deionized water was used throughout.

Methods

Amino Acid Analysis. Routine acid hydrolysates were prepared in 6 N HCl at 110 °C for 22 h and were run on a Beckman 120 amino acid analyzer equipped with Durrum DC-6A resin (Jones & Gurd, 1981) or analyzed by reverse-phase HPLC after precolumn derivatization with *o*-phthaldialdehyde (Jones et al., 1981). Tryptophan and *S*-methylmethionine were analyzed on a column (0.9 × 19 cm) of Beckman PA-35 resin following hydrolysis at 110 °C for 24 h in either 3 N *p*-toluenesulfonic acid containing 3-(2-aminoethyl)indole or 4 N methanesulfonic acid (Liu & Chang, 1971; Jones et al., 1976; Simpson et al., 1976). Homoserine was differentiated from homoserine lactone by its elution position on the DC-6A column as previously described (Jones & Gurd, 1981). No corrections were made for destruction of amino acids during acid hydrolysis. Total enzymatic digests were performed and analyzed according to established procedures (Rothgeb et al., 1977).

Purification of Peptides. Reaction mixtures were desalted on a Sephadex G-25 or G-50 column (1.6 × 100 cm) eluted at 30 mL/h with 5% acetic acid. Purification of glucagon and derivatives by cation-exchange chromatography was performed on a CM-cellulose column (1.6 × 15 cm) equilibrated with a first buffer of 10 mM ammonium acetate in 6 M urea, pH 4.5. Peptides were eluted at 20 mL/h by using a 24-h linear gradient formed from equal volumes of the first buffer and a second buffer of 100 mM ammonium acetate in 6 M urea, pH 5.4 (Jones & Gurd, 1981). Urea and electrolytes were removed from the peptide fractions by gel filtration in 5% acetic acid as described above. Reverse-phase HPLC was performed on an Altex Ultrasphere ODS column (5-μm particle size; 0.46 × 25 cm). Formic acid (0.5 M)–pyridine (0.4 M) buffer, pH 4.0, was used with gradients of 1-propanol to elute the glucagon derivatives from the HPLC column at a flow rate of 20 mL/h (Lewis et al., 1979). An automated fluorescence detection system using fluorescamine was utilized for monitoring peptides in column effluents (Böhlen et al., 1975). All column fractions were lyophilized and stored at −20 °C until further characterized.

Preparation of [Des-Asn^{28},Thr^{29}](homoserine lactone^{27})-glucagon and [Des-Asn^{28},Thr^{29}](homoserine^{27})glucagon (Figure 1, Reactions I and II). Cyanogen bromide cleaved glucagon was prepared and purified as previously described by Jones & Gurd (1981). The methionine content of the product determined by overloading the amino acid analyzer was less than 0.2 nmol in 200 nmol of peptide hydrolysate, indicating a purity greater than 99.9%. The isolated lactone derivative was treated with triethylamine to open the lactone ring, and the homoserine derivative was recovered by lyophilization. Subsequent isoelectric focusing analysis of the homoserine derivative indicated that it was about 90% in the open ring form.

Preparation of (S-Methyl-Met^{27})Glucagon (Figure 1, Reaction III). The methylation of native glucagon to yield (*S*-methyl-Met^{27})glucagon and the regeneration of native glucagon by subsequent demethylation have been described in detail (Rothgeb et al., 1977, 1978). The *S*-methyl derivative was isolated by cation-exchange chromatography, and its purity was determined by amino acid analysis to be greater than 99.5% based upon a methionine content of less than 0.4 nmol/100 nmol of peptide hydrolysate and an *S*-methylmethionine content of at least 98 nmol/100 nmol of hydrolysate.

Preparation of [Des-Thr^{29}](S-methyl-Met^{27})glucagon and [Des-Thr^{29}]glucagon (Figure 1, Reactions IV and V). Threonine was removed from the carboxyl terminus of purified (*S*-methyl-Met^{27})glucagon by digestion with carboxypeptidase Y (Hiyashi, 1977). The substrate (2.0 mg, 570 μmol) was dissolved in 1.0 mL of 0.1 M sodium acetate (pH 5.0) containing 6 M urea and 1 mM EDTA. To this peptide solution was added 100 μL (50 μg) of the enzyme preparation, and the resulting mixture was stirred at room temperature. Aliquots of the enzyme were also added after 12 h (100 μL) and 25 h (50 μL). The reaction rate was monitored by quantitating the release of threonine. After 26 h of digestion, greater than 99.5% of the carboxyl-terminal threonine residue was removed with essentially no release of other amino acids. The reaction was terminated with an equal volume of glacial acetic acid and desalted on a G-25 column. The product was further purified by cation-exchange chromatography. Purity of the resulting derivative, [des-Thr^{29}](*S*-methyl-Met^{27})glucagon, as judged by the release of threonine and amino acid analysis of the peptide was greater than 99.5%. Virtually no native glucagon could have been present in the final product since any trace of the native hormone in the purified (*S*-methyl-Met^{27})glucagon starting material would have been removed by the carboxypeptidase Y treatment.

Because of residual endopeptidase activity, in subsequent preparations of this derivative, the digestion was performed as stated above except that the reaction was terminated after 12 h. In most cases, 60–70% of the carboxyl-terminal threonine had been released after the 12-h reaction time. In each case, the reaction mixture was desalted on Sephadex G-25 and then lyophilized. In order to remove any uncleaved substrate from the desired product, the peptide mixture was dissolved in 4 M urea–0.4 M pyridine formate (pH 4.0) and purified by reverse-phase HPLC. The purity of the [des-Thr^{29}](*S*-methyl-Met^{27})glucagon obtained in 50% yield by this procedure was greater than 99% as judged by the chromatographic resolution of the derivative by HPLC and its amino acid composition.

[Des-Thr^{29}](*S*-methyl-Met^{27})glucagon was demethylated by a slight modification of the published demethylation pro-

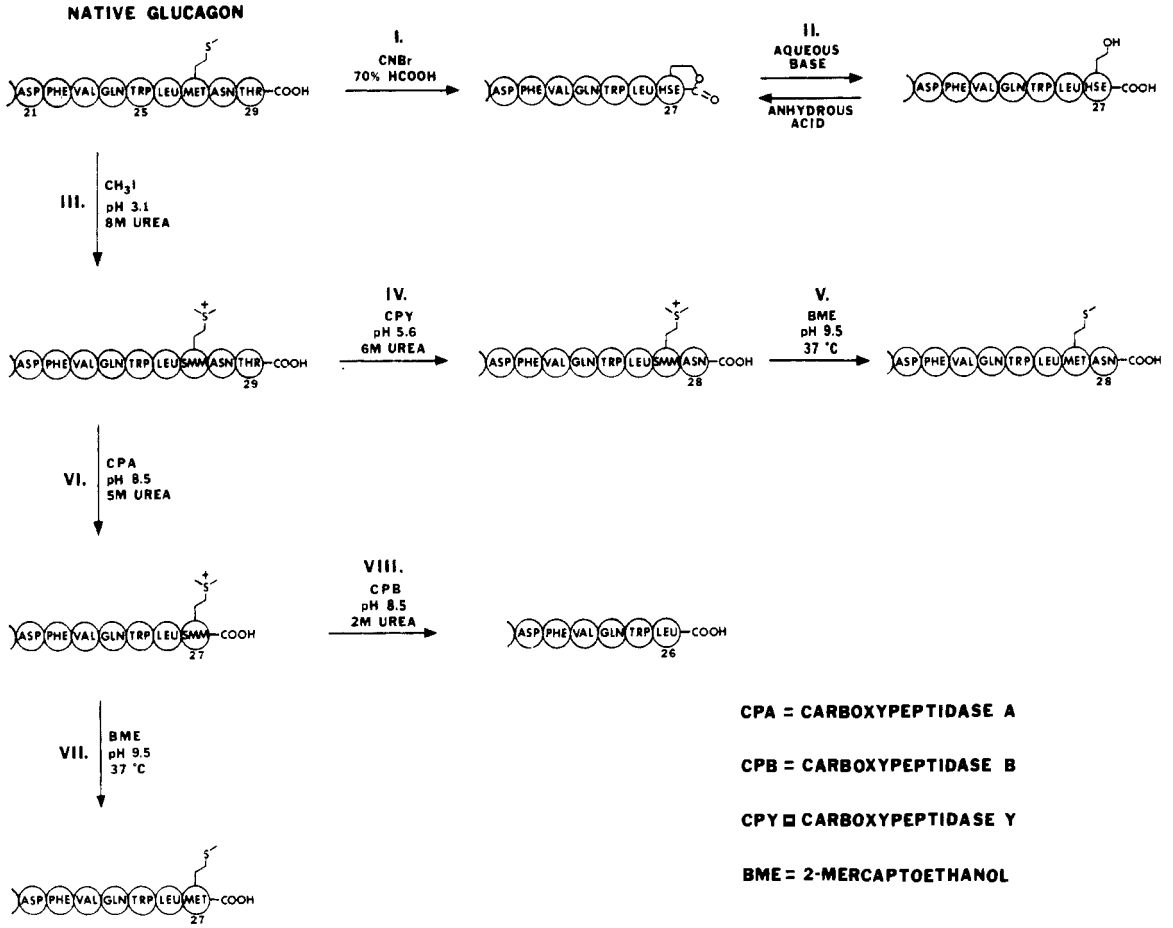

FIGURE 1: Summary of the reaction schemes employed in the generation of carboxyl-terminal derivatives of glucagon.

cedure (Rothgeb et al., 1977). The peptide (0.1 mg, 30 nmol) was dissolved in 200 μL of a 1.0 M solution of 2-mercaptoethanol (pH 9.5), and the mixture was incubated at 37 °C for 24 h. The reaction was terminated by the addition of 4 M urea–0.4 M pyridine formate (pH 4.0) (800 μL). The desired derivative, [des-Thr^{29}]glucagon, was then obtained by reverse-phase HPLC in 60% yield. The purity of this derivative was judged to be greater than 95% based on its chromatographic resolution and amino acid composition, although the contamination of this final product with uncleaved glucagon could not exceed the maximum 1% impurity present in the [des-Thr^{29}](*S*-methyl-Met^{27})glucagon previously purified by HPLC.

Preparation of [Des-Asn^{28},Thr^{29}](S-methyl-Met^{27})glucagon and [Des-Asn^{28},Thr^{29}]glucagon (Figure 1, Reactions VI and VII). The preparation of these two derivatives also utilizes (*S*-methyl-Met^{27})glucagon as the starting material. Removal of the carboxyl-terminal asparagine and threonine residues was accomplished by carboxypeptidase A (0.2 mL, 10 mg) with (*S*-methyl-Met^{27})glucagon (10 mg, 2.9 μmol) as the substrate. Digestion was allowed to proceed at room temperature in 5 mL of 0.25 M Tris-HCl (pH 8.5) containing 5 M urea, necessary for maintaining the mutual solubility of the enzyme and the substrate. The course of the reaction was monitored by quantitating the release of amino acids. After a reaction time of 8 h, quantitative release of the carboxyl-terminal asparagine and threonine residues had been achieved with less than 2% of the *S*-methylmethionine being released. The reaction was terminated by the addition of 8 M urea (pH 3.1). The truncated derivative, [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})-glucagon, was separated from the enzyme, urea, and buffer salts by gel filtration on Sephadex G-50 and then purified by cation-exchange chromatography on CM-cellulose. The yield of this derivative was 82%, and the purity was greater than 99% based upon its asparagine content. Again any trace contamination could not have represented native glucagon but rather could only have been in the form of sulfonium analogues of the native hormone.

Demethylation of the [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})-glucagon was accomplished as outlined above by using 1 M mercaptoethanol (pH 9.5) at 37 °C for 24 h. The reaction was terminated by the addition of glacial acetic acid and the product desalted on Sephadex G-25. The peptide fraction was lyophilized and then treated with 1 mL of anhydrous trifluoroacetic acid for 1 h in the dark. After lyophilization, final purification of the demethylated derivative, [des-Asn^{28},Thr^{29}]glucagon, was by cation-exchange chromatography. The yield of this derivative was 50%, and its purity was judged to be greater than 99% based on its chromatographic resolution and its amino acid composition. A well-separated secondary peak was also obtained in the final chromatographic profile of this derivative. From its amino acid composition and chromatographic behavior, this second peak was identified as [des-Asn^{28},Thr^{29}](homoserine lactone^{27})glucagon.

Purification of Radiolabeled Nucleotides. Preparations of [α-^{32}P]ATP and [^{3}H]cAMP were chromatographically purified to remove any radiochemical contaminants which might interfere with the adenylate cyclase assays (Counis & Mongongu, 1978; White & Karr, 1978). [α-^{32}P]ATP was purified on a 1-mL column of Bio-Rad AG 50W-X4 resin packed in a disposable Pasteur pipet. The column was rinsed with water and then loaded with a 1.0 mL solution containing 500 μCi of [α-^{32}P]ATP adjusted to a specific activity of 4.2 Ci/mmol. An additional 0.8 mL of water was used to rinse the ATP from

the column, and the total eluate (1.8 mL) was collected in a vial containing 0.2 mL of 25 mM Tris-HCl, pH 7.6, and stored at −20 °C.

[^{3}H]cAMP was purified on a 1-mL column of Bio-Rad AG 1-X8 resin (Schwartzel et al., 1977). The resin was converted to its formate form and then loaded with 250 μCi of [^{3}H]-cAMP contained in 0.5 mL of 50% ethanol–water. After the column was rinsed with 10 mL of water, [^{3}H]cAMP was eluted with 10 mL of 2 N formic acid. The eluate was taken to dryness under a stream of nitrogen, and the [^{3}H]cAMP was redissolved in 50% ethanol–water (20 mL) for storage at −20 °C.

Preparation of Mono[^{125}I]iodoglucagon. Glucagon was iodinated by the chloroglycoluril method (Fraker & Speck, 1978; Markwell & Fox, 1978) with IODO-GEN as the iodinating reagent and reaction conditions adapted from von Schenck & Jeppsson (1977). Purified glucagon was dissolved at a concentration of 4 mg/mL in a 1:1 mixture of propylene glycol and 0.1 M glycine–NaOH buffer, pH 9.7. A 50-μL aliquot of this solution (~60 nmol of glucagon) was injected into a vial containing 2 mCi (~1 nmol) of carrier-free [^{125}I]iodine in 4 μL of 0.1 N NaOH. The mixture was transferred to a glass test tube (12 × 75 mm) plated with 20 μg of IODO-GEN, and the reaction was allowed to proceed for 20 min at room temperature.

Mono[^{125}I]iodoglucagon was chromatographically purified by anion-exchange procedures based upon the methodology of Desbuquois (1975). The reaction mixture was loaded directly on a Pasteur pipet column (1.8 mL) of DEAE-cellulose equilibrated with 1 mM ammonium bicarbonate buffer, pH 9.5. Unlabeled glucagon was eluted from the column by the passage of 20 mL of 10 mM ammonium bicarbonate buffer containing 6 M urea, pH 9.2. The column was then developed with a 70-mL linear gradient formed between the 10 mM buffer and a second buffer containing 100 mM ammonium bicarbonate in 6 M urea, pH 8.0. Fractions of about 0.5 mL were collected in tubes containing 50 μL of 10 mg/mL BSA in 20 mM Tris-HCl, pH 6.9. The fractions containing the mono[^{125}I]iodoglucagon which eluted at the beginning of the gradient were pooled and dialyzed for 24 h at 4 °C against 30 L of water and then for 12 h at 4 °C against four changes of 250 mL of 20 mM Tris-HCl, pH 6.9, and finally stored at 4 °C for use within 3 weeks.

Isolation of Liver Plasma Membranes. Partial purification of the membrane-bound adenylate cyclase system of rat liver was performed according to the method of Neville (1968) through step 11, as described by Pohl (1976). The final membrane pellet was resuspended in an equal volume of 1 mM sodium bicarbonate buffer and divided into 200-μL aliquots (~3 mg of protein) which were stored in liquid nitrogen and used within 2 months. Protein was determined by the Lowry method as modified for membrane proteins (Markwell et al., 1978).

Adenylate Cyclase Assay. The adenylate cyclase assay medium was essentially that of Wright & Rodbell (1979) and contained the following in a final volume of 0.1 mL: 2 mM [α-^{32}P]ATP (2 × 10^6 cpm); 10 mM $MgCl_2$; 0.2 mM cAMP (added to minimize the effects of phosphodiesterase activity); 1 mM dithiothreitol; 10 μM GTP; 30 mM Tris-HCl, pH 7.5; and an ATP regenerating system consisting of 5 mM creatine phosphate and 0.3 mg/mL (9.3 units/mL) creatine phosphokinase. Liver plasma membranes were thawed immediately before each assay and made into a suspension containing 3 mg/mL protein in 30 mM Tris-HCl–1 mM dithiothreitol, pH 7.5. Peptide samples were prepared by serial dilution from a fresh stock solution of the peptide made up in 25 mM Tris-HCl–1 mg/mL BSA, pH 7.5. Protein concentration was determined spectrophotometrically after Millipore filtration of the sample, and the 278-nm molar absorptivity was taken to be 8260 M^{-1} cm^{-1} (Gratzer & Beaven, 1969).

Assays were initiated by the addition of 20 μL of membrane suspension (60 μg of protein) to a mixture of 40 μL of assay medium and 40 μL of peptide solution held in an ice bath. Assay samples were incubated at 30 °C for 10 min, and the reaction was terminated by the addition of 100 μL of stopping solution (Salomon et al., 1974). A measured amount of [^{3}H]cAMP (about 20000 cpm) in 0.85 mL of water was added to each tube as an internal standard to monitor cAMP recovery.

Labeled cAMP was isolated by the method of combined Dowex 50 and alumina chromatography (Salomon et al., 1974; Franks & Malamud, 1976). Columns were prepared in Pasteur pipets plugged with glass wool and were not reused. The assay mixture (1.05 mL) was applied directly to a 1-mL column of Dowex AG 50W-X4 resin and followed by a wash with 1.6 mL of water. A second column containing 0.6 g of neutral alumina equilibrated with 10 mL of 0.1 M imidazole hydrochloride buffer, pH 7.5, was then placed beneath the Dowex column, and the Dowex column was eluted with an additional 3.8 mL of water. Labeled cAMP was eluted from the alumina column by a 5.0-mL wash with the 0.1 M imidazole hydrochloride buffer. The eluate was collected directly into a scintillation vial containing 10 mL of Aquasol and counted in a Beckman LS-230 scintillation counter.

Glucagon-stimulated adenylate cyclase activity was determined to be linear with respect to time for approximately 15 min and with respect to membrane protein up to about 100 μg. Recovery of the applied [^{3}H]cAMP was consistently on the order of 88–92%, and the assay blank (determined by adding stopping solution prior to membranes) was 5–10 cpm. Basal activity in the membranes averaged about 0.4 nmol of cAMP formed per mg of membrane protein in 10 min. This activity was increased 3–4-fold by maximal hormonal stimulation, in accord with the results of Wright & Rodbell (1979). Since different membrane preparations gave slightly different basal and maximal responses, results are expressed as the percentage of activation over basal level in order to facilitate comparison. The standard deviation for individual assay points was approximately 5%. Assays were normally carried out in pairs with either native glucagon or (*S*-methyl-Met^{27})glucagon serving as a control, although the two homoserine derivatives were also tested directly against each other.

Glucagon Binding Assay. The binding of glucagon derivatives was assayed essentially as described by Wright & Rodbell (1979). Peptide samples were initially dissolved in 1 mM HCl for concentration measurements and then diluted at least 20-fold with incubation buffer containing 1 mg/mL BSA, 1 mM dithiothreitol, and 20 mM Tris-HCl, pH 6.9. Protein concentration was determined by using the 278-nm and pH 3 molar absorptivity of 8310 M^{-1} cm^{-1} (Gratzer et al., 1967). The peptide samples (0.6 mL) were dispensed into 12 × 75 mm glass tubes held in an ice bath, and 0.2 mL of incubation buffer containing 0.13 pmol (240000 cpm) of mono[^{125}I]iodoglucagon was added to each tube. Binding was initiated by the addition of 0.2 mg of freshly thawed membrane protein suspended in 0.2 mL of incubation buffer. Reaction mixtures were incubated for 15 min at 30 °C and then rapidly (<5 s) vacuum filtered through oxoid membrane filters previously soaked at least 30 min in 100 mg/mL BSA. Each filter was immediately washed twice with 2 mL of incubation buffer

and then removed for counting in a Beckman Biogamma counting system.

All derivatives were assayed at least twice, with duplicate determinations generally agreeing to within 5%. As with the enzyme assays, results were converted to percentages to facilitate comparisons. Nonspecific binding measured in the presence of excess unlabeled peptide was 5–10% of total binding and was subtracted from the total to give specific binding. In the absence of unlabeled peptide, the specific binding of mono[^{125}I]iodoglucagon, taken as 100%, was about 1×10^5 cpm/mg of membrane protein. The two homoserine derivatives were assayed independently and also compared directly in a single assay.

Circular Dichroism. CD measurements were performed as previously described (Rothgeb et al., 1978). Spectra were recorded with protein concentrations of 0.09–0.10 mg/mL in a 0.1-cm path-length cuvette, and samples were repetitively scanned at 2 nm/min with a time constant of 4 s. Sample solutions were prepared by mixing fresh stock solutions of the peptides in 0.01 N HCl with varying proportions of 0.01 N HCl and 2-chloroethanol.

Isoelectric Focusing. Isoelectric focusing was carried out basically as described in the instructions provided by Bio-Rad Laboratories, using 7.5% polyacrylamide tube gels (5 × 110 mm) containing 6 M urea and 2% (w/v) carrier ampholytes (0.8% pH 3–10 and 1.2% pH 6–8). The pH profile of each gel was determined at room temperature with an Ingold microcombined electrode. Gels were subsequently stained with Commassie Brilliant Blue G250 to locate protein bands (Blakesley & Boezi, 1977).

Data Analysis. The CD spectra were analyzed by the method of Greenfield & Fasman (1969) by using the reference spectra of Chen et al. (1974) taken at 1-nm intervals over the range from 240 to 205 nm. The results of the binding and activation assays were analyzed by curve-fitting the data to a four-parameter equation describing a sigmoidal dose–response curve (De Lean et al., 1978). In both cases, nonlinear least-squares regression was performed by using a Gauss–Newton minimization routine adapted from the BMDX85 program (Dixon, 1970).

Results

Preparation of Glucagon Derivatives by Cyanogen Bromide Cleavage. The derivative generated by cyanogen bromide cleavage may exist in two different forms with either a free acid or a lactone ring at the carboxyl terminus (Figure 1, reaction II). Although the biological and physical properties of these two species might be expected to differ significantly at a pH where the terminal carboxyl group is ionized, there has been no attempt to differentiate between the properties of the two forms in previous studies of cyanogen bromide cleaved glucagon (Spiegel & Bitensky, 1969; Epand, 1972; Epand & Grey, 1973; Hruby et al., 1975; Lin et al., 1975; Tager et al., 1977; Hruby et al., 1981). Therefore, in this study we sought to isolate and independently test each species in order to compare their properties.

The lactone and open ring forms of homoserine are in chemical equilibrium and are readily interconverted under appropriate conditions (Armstrong, 1949; Offord, 1972). Nevertheless, our purification studies have shown that both forms of the homoserine derivative of glucagon are sufficiently stable in dilute aqueous acid to permit their chromatographic isolation in high purity by cation exchange (Jones & Gurd, 1981). Both forms are also sufficiently stable during isoelectric focusing analysis to allow chromatographically purified samples to be independently focused with only trace evidence of interconversion. The homoserine derivative was shown to cofocus with native glucagon at a p*I* of about 6.5, while the homoserine lactone derivative was found to band with (*S*-methyl-Met^{27})glucagon near p*I* 7.9. The latter result reflects the fact that both molecules have the same net change in charge relative to native glucagon and is consistent with the observation that both derivatives coelute on cation-exchange chromatography. Furthermore, both forms were determined by isoelectric focusing analysis to be relatively stable for several hours at 30 °C in the binding assay buffer of 20 mM Tris-HCl, pH 6.9.

Preparation of Glucagon Derivatives by Methionine Methylation. The isolation and analysis of the (*S*-methyl-Met^{27})glucagon derivative have been previously published (Rothgeb et al., 1977, 1978). In addition to providing the critical intermediate for the preparation of the enzymatically cleaved derivatives in this study, in preliminary experiments we have shown that methylation may be used as a protective mechanism to limit glucagon oxidation to tryptophan-25 so that subsequent demethylation yields a derivative selectively oxidized at the tryptophan residue (Coolican et al., 1979). Furthermore, the methylated [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})glucagon derivative prepared in this study is a suitable precursor for cleavage by carboxypeptidase B whose specificity is directed toward terminal residues bearing a positive charge (Ambler, 1972). We have demonstrated that the methionine residue can be removed from [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})glucagon by carboxypeptidase B to generate [des-Met^{27},Asn^{28},Thr^{29}]glucagon in better than 50% yield with a purity greater than 99.5% (Figure 1, reaction VIII) (Jones et al., 1980). Details for the preparation of this derivative will be described in a later report. Chemical preparation of [des-Met^{27},Asn^{28},Thr^{29}]glucagon in high purity should allow an extension of the studies conducted by Bromer (1976) on a "natural" form of this derivative isolated as a contaminant from native glucagon.

Preparation of Glucagon Derivatives by Cleavage with Carboxypeptidase Y. The extra methyl group and the associated positive charge in (*S*-methyl-Met^{27})glucagon restrict the action of carboxypeptidase Y to cleavage of the terminal threonine residue. Initial experiments demonstrated that quantitative release of threonine could be obtained with little or no release of asparagine though it was necessary to perform the digestion in 1 mM EDTA and 6 M urea in order to minimize contaminating endopeptidase activity which otherwise cleaved the desired product into a series of small peptides visible upon gel filtration. The time course for the digestion is shown in Figure 2A where it can be seen that quantitative release of the threonine residue was obtained after 24 h of digestion with less than 1% of the asparagine residue at position 28 being removed. However, residual endopeptidase activity resulted in a low yield (30%) after 24 h of digestion, so shorter reaction times were used, and the truncated product was isolated by HPLC, as shown in Figure 3A. By amino acid analysis, the first major peak to elute from the column was identified as [des-Thr^{29}](*S*-methyl-Met^{27})glucagon (Table I), and the second major peak was shown to be the uncleaved substrate. The minor peaks labeled a and b in the elution profile are presumably artifacts of the purification procedures and were not further characterized. The desired product, [des-Thr^{29}](*S*-methyl-Met^{27})glucagon, was obtained in 60% yield. Demethylation of this derivative produced [des-Thr^{29}]glucagon which was also purified by reverse-phase HPLC as shown in Figure 3B. [Des-Thr^{29}]glucagon was identified by amino acid analysis (Table II) as the single major

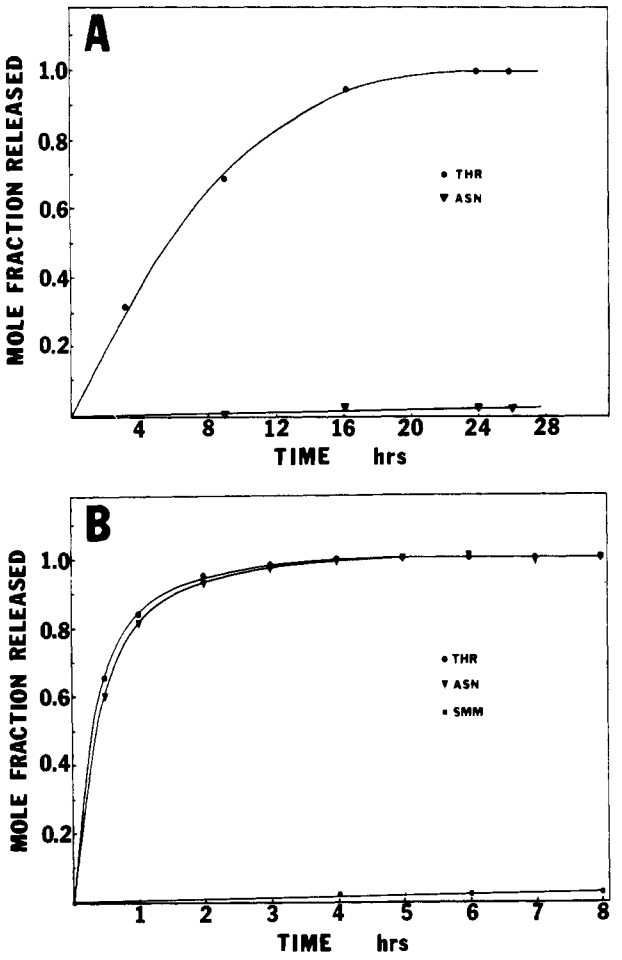

FIGURE 2: Time course release plots for the digestion of (*S*-methyl-Met[27])glucagon with carboxypeptidase. (A) Digestion with carboxypeptidase Y. (B) Digestion with carboxypeptidase A.

Table I: Amino Acid Composition of [Des-Thr[29]](*S*-methyl-Met[27])- and [Des-Asn[28],Thr[29]](*S*-methyl-Met[27])glucagon

amino acid	[des-Thr[29]]-(*S*-methyl-Met[27])-glucagon	[des-Asn[28],Thr[29]]-(*S*-methyl-Met[27])glucagon	
	acid hydrolysis	acid hydrolysis	enzymatic hydrolysis
Asp	3.84 (4)[a]	3.09 (3)	2.83 (3)
Asn	ND[b]	ND	<0.01 (0)
Thr	1.91 (2)	2.08 (2)	2.03 (2)
Ser	3.63 (4)	3.80 (4)	4.07 (4)
Glu	2.94 (3)	2.68 (3)	0.00 (0)
Gln	ND	ND	3.07 (3)
Gly	0.95 (1)	1.03 (1)	1.01 (1)
Ala	0.99 (1)	0.97 (1)	1.06 (1)
Val	0.92 (1)	0.71 (1)	1.05 (1)
Met	0.00 (0)	0.00 (0)	0.00 (0)
Leu	2.05 (2)	1.85 (2)	1.98 (2)
Tyr	2.15 (2)	1.98 (2)	1.96 (2)
Phe	2.19 (2)	1.79 (2)	1.93 (2)
Lys	1.07 (1)	1.02 (1)	1.07 (1)
His	1.02 (1)	0.95 (1)	1.04 (1)
Arg	2.03 (2)	2.03 (2)	2.07 (2)
Trp	0.88 (1)	0.96 (1)	1.08 (1)
SMM	0.93 (1)	0.99 (1)	0.75 (1)
Hse	0.00 (0)	0.02 (0)	0.21 (0)

[a] Expected values in parentheses are based on the glucagon sequence. [b] ND, not determined.

peak eluting at a 1-propanol concentration higher than that required for either [des-Thr[29]](*S*-methyl-Met[27])glucagon or (*S*-methyl-Met[27])glucagon.

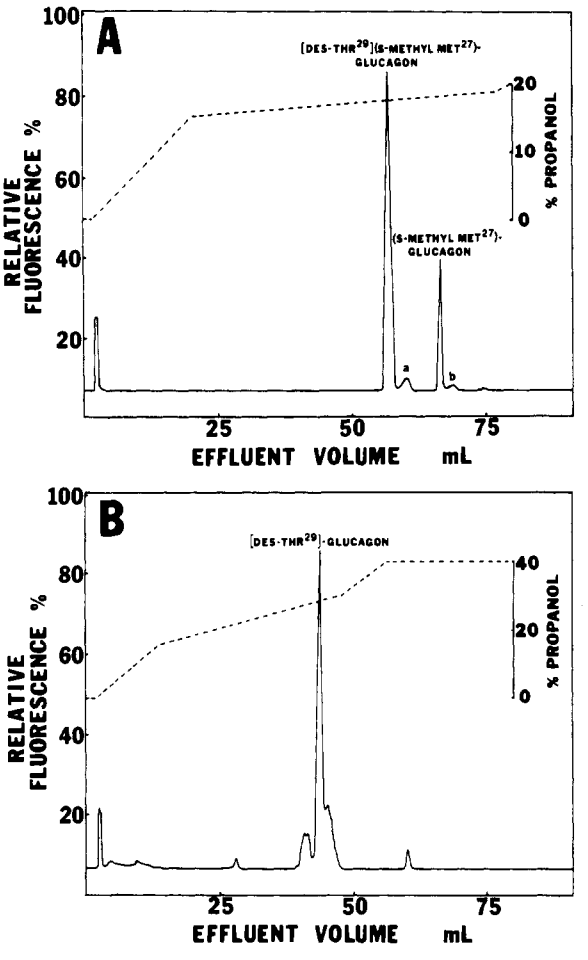

FIGURE 3: Reverse-phase HPLC of the glucagon derivatives generated by carboxypeptidase Y digestion. Experimental details are described in the text. (A) Purification of the desalted digestion mixture. (B) Purification of the demethylation reaction mixture.

Table II: Amino Acid Composition of [Des-Thr[29]]- and [Des-Asn[28],Thr[29]]glucagon

amino acid	[des-Thr[29]]-glucagon	[des-Asn[28],Thr[29]]-glucagon
Asx	3.96 (4)[a]	2.96 (3)
Thr	1.91 (2)	1.90 (2)
Ser	3.65 (4)	3.57 (4)
Glx	2.98 (3)	3.10 (3)
Gly	1.01 (1)	1.01 (1)
Ala	1.00 (1)	0.99 (1)
Val	0.99 (1)	1.03 (1)
Met	0.98 (1)	0.99 (1)
Leu	2.05 (2)	2.09 (2)
Tyr	2.01 (2)	1.93 (2)
Phe	1.98 (2)	2.00 (2)
Lys	1.01 (1)	1.03 (1)
His	0.92 (1)	0.97 (1)
Arg	2.09 (2)	2.06 (2)
Trp	0.89 (1)	0.85 (1)
SMM	0.00 (0)	0.00 (0)
Hse	0.00 (0)	0.00 (0)

[a] Expected values in parentheses are based on the glucagon sequence.

Preparation of Glucagon Derivatives by Cleavage with Carboxypeptidase A. The presence of methionine in the sulfonium form further limits the activity of carboxypeptidase A whose specificity is generally for neutral amino acids (Ambler, 1972). Figure 2B illustrates the time course of release of threonine, asparagine, and *S*-methylmethionine. Cleavage is essentially complete at 3–4 h with minimum cleavage of *S*-methylmethionine. The use of a large enzyme

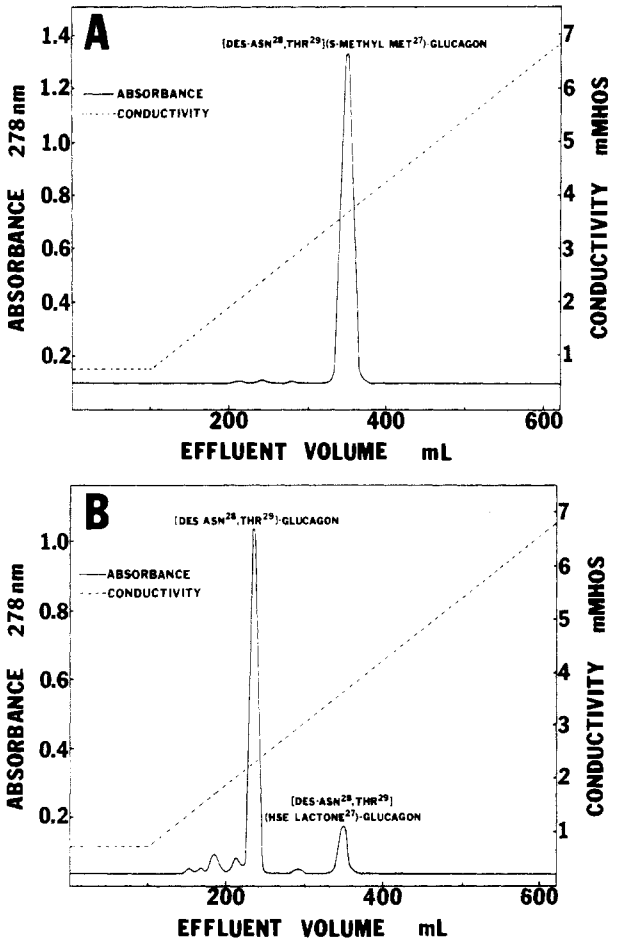

FIGURE 4: CM-cellulose cation-exchange chromatography of the glucagon derivatives generated by carboxypeptidase A digestion. Details are given in the text. (A) Purification of the desalted digestion mixture. (B) Purification of the demethylation reaction mixture following trifluoroacetic acid treatment.

to substrate ratio (1:1 w/w) resulted in the quantitative release of both the threonine and asparagine residues. Any peptides cleaved beyond the *S*-methylmethionine residue at position 27 would have been removed during purification by gel filtration and ion-exchange chromatography since such peptides would differ in size and charge from the desired product, [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})glucagon.

The elution profile for the purification of this derivative by cation-exchange chromatography is presented in Figure 4A. In this figure, a single peak is seen whose amino acid composition (Table I) corresponds to that of [des-Asn^{28},Thr^{29}]-(*S*-methyl-Met^{27})glucagon. The purity of this derivative was greater than 99% based on its asparagine content. However, as can be seen in Table I, trace amounts of homoserine were detected in the acid hydrolysate; even larger amounts of the amino acid with a concomitant decrease of *S*-methylmethionine were found in the total enzymatic hydrolysate. The homoserine content of this derivative increased with time and correlated with a decrease in *S*-methylmethionine even when solutions of the derivative were stored frozen. A possible explanation for this phenomenon is presented in Figure 5. In this figure, an analogy is drawn between the mechanism for cyanogen bromide cleavage of methionine residues and a proposed mechanism for the spontaneous decomposition of the carboxyl-terminal *S*-methylmethionine residue of [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})glucagon. While we have no experimental evidence proving this mechanism, it is consistent with our experimental observations; i.e., decomposition of *S*-methylmethionine is only observed when it occurs as the

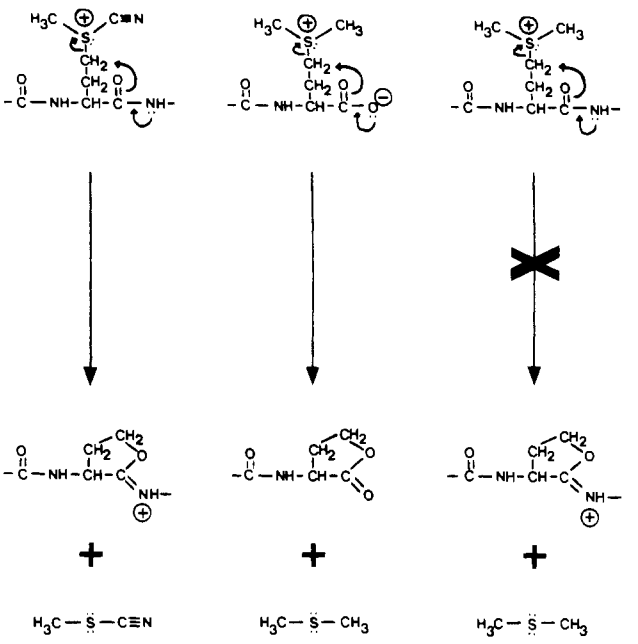

FIGURE 5: Possible reaction mechanisms for the spontaneous conversion of a carboxyl-terminal *S*-methylmethionine residue to homoserine lactone. The reaction mechanism shown on the left illustrates the mechanism for cyanogen bromide cleavage of the methionyl peptide bonds (Gross, 1967). The center reaction scheme illustrates a similar mechanism for the decomposition of a carboxyl-terminal *S*-methylmethionine residue. The reaction mechanism shown on the right involving a *S*-methylmethionine residue at an internal position has not been observed to occur.

carboxyl-terminal residue of a peptide. This difference in stability may be the result of the increased nucleophilicity of the carboxylate anion with respect to the nucleophilicity of the carbonyl group from the amide linkage (see Figure 5). These results indicate that particular care should be taken in assessing the properties of any truncated and methylated derivative and suggest that the true activity of [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})glucagon may in fact be even less than our data indicate.

Demethylation of the [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})-glucagon yielded [des-Asn^{28},Thr^{29}]glucagon which was purified by cation-exchange chromatography. In the initial preparation, amino acid analysis of this derivative indicated the presence of homoserine (0.2 residue) and low methionine content (0.8 residue). Based on the above discussion concerning the instability of the carboxyl-terminal *S*-methylmethionine residue of the starting material, it was believed that the desired product, [des-Asn^{28},Thr^{29}]glucagon, contained [des-Asn^{28},Thr^{29}](homoserine lactone^{27})glucagon (or the open form of this derivative) as a 20% contaminant. Furthermore, since the desired product and the contaminant coeluted on cation-exchange chromatography, it seemed feasible that the lactone ring must have been opened by the basic conditions used in the demethylation procedure. In order to remove the contaminating peptide, the peptide mixture was treated with anhydrous trifluoroacetic acid to close the lactone ring and create a net charge difference between the two peptides allowing their separation by cation-exchange chromatography as seen in Figure 4B. The amino acid composition of the [des-Asn^{28},Thr^{29}]glucagon is presented in Table II. No homoserine could be detected in this peptide. Amino acid analysis of the contaminant peptide confirmed the composition as suggested above.

Preparation of Mono[^{125}I]iodoglucagon. The IODO-GEN method of iodination worked very effectively for the iodination of glucagon at pH 10 in the presence of propylene glycol.

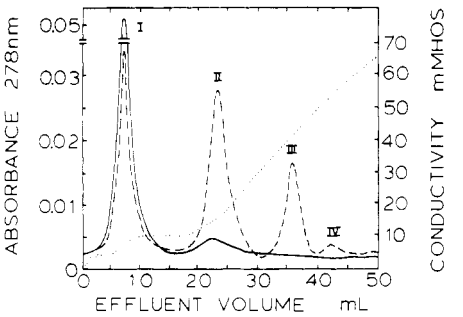

FIGURE 6: DEAE-cellulose anion-exchange chromatography of the iodoglucagon reaction mixture. Conditions for the IODO-GEN-mediated iodination and column elution are described in the text. Shown are elution profiles for reactions conducted with a glucagon to iodine molar ratio of 15 to 1 (—) and 1 to 2 (---) and the conductivity of the eluate (···).

Under these conditions, lactoperoxidase-mediated iodination is known to favor the Tyr-13 residue of glucagon 4:1 over Tyr-10 (von Schenck & Jeppsson, 1977), and perhaps this specificity is retained for IODO-GEN-mediated iodination. The IODO-GEN reaction was more efficient than enzymatic iodination using a solid-phase lactoperoxidase–glucose oxidase system (unpublished results). Timing was found to be far less critical for the IODO-GEN reaction than for the standard chloramine-T procedure (Rodbell et al., 1971). Chromatographic and isoelectric focusing analyses of iodination reaction mixtures revealed very little evidence of structural damage induced by IODO-GEN even after reaction times as long as an hour. Although the oxidation of methionine is known to be a potential hazard of peptide hormone radioiodination (Stagg et al., 1970), methionine oxidation did not appear to be a significant side reaction during IODO-GEN-mediated iodination, with about 5% oxidation being observed in control studies conducted with the free amino acid.

The elution profile of the crude iodoglucagon reaction mixture from a pipet column of DEAE-cellulose is shown in Figure 6. The elution pattern follows closely that reported by Desbuquois (1975) for the DEAE chromatographic fractionation of iodoglucagon prepared by the iodine monochloride method, with peak I being native glucagon and peaks II, III, and IV being mono-, di-, and multiiodinated species, respectively. In this study, peak II was identified as monoiodoglucagon by its elution position at the beginning of the gradient and by the fact that monoiodoglucagon prepared and purified exactly according to Desbuquois (1975) eluted at the same position. The material in peak II was shown to be homogeneous by isoelectric focusing analysis and to cofocus with native glucagon at p*I* 6.5. Figure 6 also shows that increasing the glucagon to iodine molar ratio reduces the potential for multiple iodination but does not affect the elution pattern. At the greater than 50 to 1 molar ratio of glucagon to iodine used for radioiodination, the potential for multiple iodination is minimal, and virtually all of the [^{125}I]iodine is incorporated into mono[^{125}I]iodoglucagon. Only a trace peak of di[^{125}I]-iodoglucagon eluting at a volume corresponding to peak III was observed in the radioactivity profile of crude ^{125}I-labeled glucagon. Exclusive monoiodination would be expected to enhance the stability of labeled glucagon by minimizing radiolysis (von Schenck et al., 1976), and our preparation was found to be usable for at least 3 weeks without significant change in specific or nonspecific binding. Additionally, displacement of a more homogeneous labeled hormone by derivatives should yield more reproducible binding constants. The specific activity of our sample was not determined experimentally, but based upon the method of iodination and the chromatographic data, it was assumed to be the same as that of the initial [^{125}I]iodine preparation.

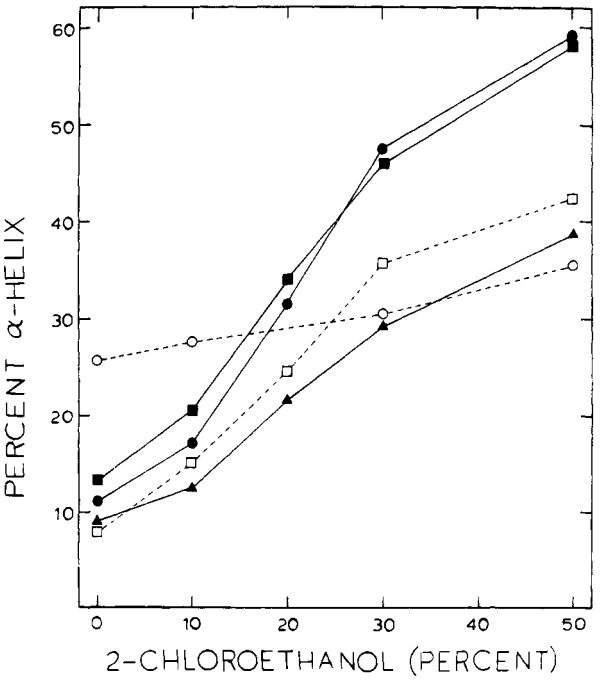

FIGURE 7: Effect of 2-chloroethanol on peptide α-helix content determined by CD analysis. (●) Native glucagon; (■) (*S*-methyl-Met^{27})glucagon; (▲) [des-Asn^{28},Thr^{29}](homoserine lactone^{27})glucagon; (○) [des-Asn^{28},Thr^{29}]glucagon; (□) [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})glucagon.

Circular Dichroism Analysis of Glucagon Derivatives. The CD spectra of the carboxyl-terminal derivatives obtained at low peptide concentration in 0.01 N HCl were very similar to that of native glucagon with the exception of [des-Asn^{28},Thr^{29}]glucagon. ([Des-Thr^{29}]glucagon and its sulfonium precursor were not studied.) Curve fitting of the CD data for [des-Asn^{28},Thr^{29}]glucagon was consistent with a secondary structure containing about 25% α helix, as compared to an estimated 10% α helix for native glucagon and the other derivatives. The latter results are in close agreement with values reported by other laboratories (Srere & Brooks, 1969; Epand, 1972). The altered conformation of [des-Asn^{28},Thr^{29}]glucagon was also evident in CD spectra obtained in increasing concentrations of the helix-favoring solvent 2-chloroethanol. As shown in Figure 7, while native glucagon and the other derivatives exhibited the expected large increase in α helicity in this solvent (Gratzer & Beaven, 1969; Srere & Brooks, 1969; Epand, 1972), [des-Asn^{28},Thr^{29}]glucagon showed only a minimal increase in helicity. Although this phenomenon needs to be further investigated, it may be an indication that [des-Asn^{28},Thr^{29}]glucagon tends to self-associate into structures of higher helical content at low pH and at low concentration. The fact that [des-Asn^{28},Thr^{29}]glucagon differs in helicity from [des-Asn^{28},Thr^{29}](homoserine lactone^{27})glucagon in the absence of chloroethanol may be an indication of the important role played by the hydrophobic helix-favoring methionine residue (Bromer, 1976; Ross et al., 1977; Wu & Yang, 1980).

An alternative explanation for the anomalous CD behavior of [des-Asn^{28},Thr^{29}]glucagon might be that secondary structural changes were caused by the anhydrous trifluoroacetic acid treatment which was unique to the preparation of this derivative. Anhydrous acid has been reported to induce $\alpha \rightarrow \beta$ shifts at the three aspartic acid residues in glucagon (Bromer, 1976). However, native glucagon identically treated with anhydrous trifluoroacetic acid exhibited no change in its ability to activate adenylate cyclase (data not shown), making it

Table III: Biological Potency and Binding Affinity of the Glucagon Carboxyl-Terminal Derivatives

peptide	half-maximal activation concn[a] (nM)	rel biological potency[b] (%)	half-maximal displacement concn[c] (nM)	rel binding affinity[d] (%)
native glucagon	4.0[e]	100.0	1.7	100.0
[des-Thr^{29}]glucagon	5.0	80.0	ND[f]	ND
[des-Asn^{28},Thr^{29}]glucagon	104.3	3.8	61.4	2.8
[des-Asn^{28},Thr^{29}](homoserine^{27})glucagon	55.9	7.2	79.7	2.1
[des-Asn^{28},Thr^{29}](homoserine lactone^{27})glucagon	104.1	3.8	384.3	0.4
(*S*-methyl-Met^{27})glucagon	95.1[g]	4.2	81.2	2.1
[des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})glucagon	516.0	0.8	316.7	0.5

[a] Determined by curve fitting the data of Figure 8. [b] Relative biological potency equals (glucagon concentration required for half-maximal activation)/(derivative concentration required for half-maximal activation) × 100. [c] Determined by curve fitting the data of Figure 9. [d] Relative binding affinity equals (glucagon concentration required for half-maximal displacement of the ^{125}I-labeled glucagon)/(derivative concentration required for half-maximal displacement of the ^{125}I-labeled glucagon) × 100. [e] Average of five separate assays. This same value was obtained whether the data were collectively analyzed or the assays were independently analyzed and the results averaged. The standard deviation for the independent analyses was approximately 1 nM. [f] ND, not determined. [g] Average of three separate assays.

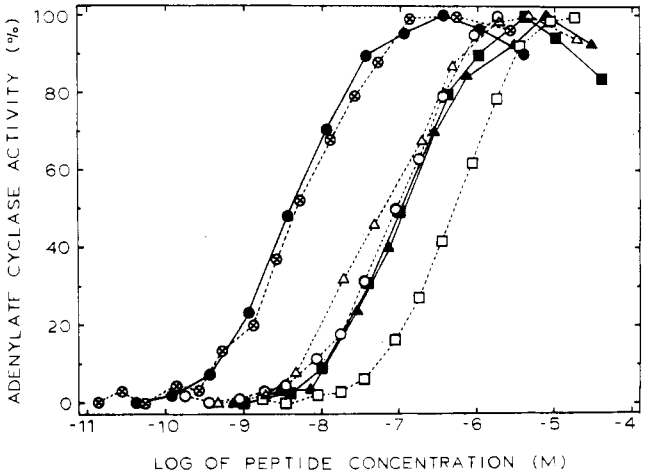

FIGURE 8: Dose–response curves for the activation of rat liver adenylate cyclase activity under conditions described in the text. (●) Native glucagon; (⊗) [des-Thr^{29}]glucagon; (○) (*S*-methyl-Met^{27})glucagon; (■) [des-Asn^{28},Thr^{29}]glucagon; (□) [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})glucagon; (▲) [des-Asn^{28},Thr^{29}](homoserine lactone^{27})glucagon; (△) [des-Asn^{28},Thr^{29}](homoserine^{27})glucagon. The curves for native glucagon and (*S*-methyl-Met^{27})glucagon represent the averaged results of five and three separate assays, respectively; other curves represent single point determinations.

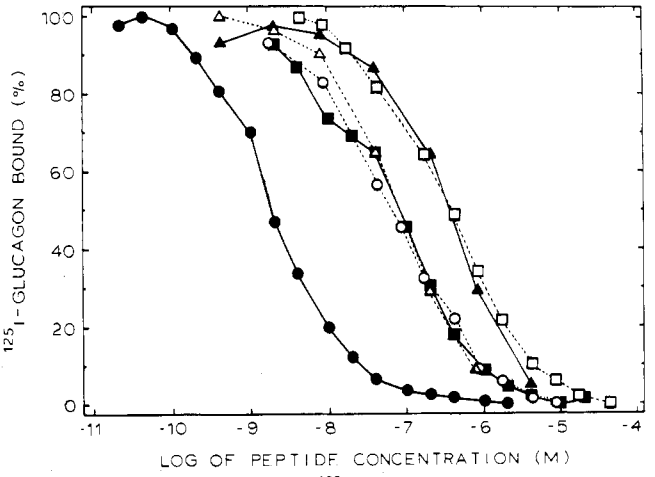

FIGURE 9: Displacement of mono[^{125}I]iodoglucagon bound to rat liver plasma membranes. Experimental details are given in the text. (●) Native glucagon; (○) (*S*-methyl-Met^{27})glucagon; (■) [des-Asn^{28},Thr^{29}]glucagon; (□) [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})-glucagon; (▲) [des-Asn^{28},Thr^{29}](homoserine lactone^{27})glucagon; (△) [des-Asn^{28},Thr^{29}](homoserine lactone^{27})glucagon.

rather unlikely that gross structural changes could have occurred.

Biological Characterization of Glucagon Derivatives. Dose–response curves for the activation of rat liver adenylate cyclase by glucagon and the carboxyl-terminal derivatives are shown in Figure 8. The derivative concentrations required for the half-maximal activation of adenylate cyclase, as determined by computer curve fitting, are given in Table III. At sufficiently high peptide concentrations, all the derivatives were full agonists capable of eliciting a maximal adenylate cyclase activity equivalent to that produced by native glucagon. The composite curve for native glucagon is nearly identical with that obtained by Rodbell and co-workers (Rodbell et al., 1971), and the half-maximal concentration, 4×10^{-9} M, agrees with their published value.

The results of the receptor binding assays are shown in Figure 9, and Table III also gives the derivative concentrations required for the half-maximal displacement of mono[^{125}I]-iodoglucagon bound to the membranes. For native glucagon, half-maximal displacement occurred at about 2×10^{-9} M, in good agreement with the results of the activation assays and with other laboratories (Rodbell et al., 1971). The glucagon carboxyl-terminal derivatives appear to comprise three general groups in terms of both receptor binding and biological activity (Figures 8 and 9), and in every case, it may be seen that reduced biological potency is associated with an equal or greater reduction in the apparent binding affinity. Thus, it would appear that the carboxyl-terminal modifications affected only the process of receptor recognition and binding and did not interfere with subsequent events leading to the activation of adenylate cyclase. This observation is consistent with the fact that glucagon$_{1-21}$ contains all the information required for maximal activity at elevated concentration (Wright et al., 1978).

Discussion

The studies described herein provide a stepwise series of well-characterized glucagon derivatives modified in a region known to be critically involved in the receptor binding process which leads ultimately to the activation of adenylate cyclase. A key point in the preparative protocol is the use of the purified sulfonium derivative as a substrate for restricted enzymatic cleavage reactions so that contamination with the more active native hormone is limited not only by the initial purification but also by the susceptibility of any contaminant native hormone to subsequent cleavage. Possible mechanisms leading to undesirable side reactions have been considered, and strategies for their detection and minimization have been developed so that yields have been brought within a satisfactory range.

The procedures used for the preparation and purification of mono[^{125}I]iodoglucagon provide a highly desirable alternative to lactoperoxidase methods which are less effective or

result in lower specific activity (von Schenck & Jeppsson, 1977) and to chloramine-T procedures with their potential for oxidative damage (Shima et al., 1975), short half-life of the labeled product (Lin et al., 1975), and heterogeneous binding characteristics of the different iodinated species (Bromer et al., 1973).

The binding and activation assays show that all the derivatives are full agonists, confirming the essential role of the glucagon carboxyl region for binding to hormone-specific receptors rather than for the activation of adenylate cyclase (Wright et al., 1978).

Since [des-Thr^{29}]glucagon is virtually indistinguishable from native glucagon in the activation assay, it appears that neither the polar side chain of Thr-29 nor the terminal carboxylate charge is necessary for full activity. However, the additional removal of the penultimate asparagine residue as in [des-Asn^{28},Thr^{29}]glucagon leads to considerably reduced binding and activity, indicating a critical role for this residue, either directly through its role in binding or indirectly through its role in preserving the hormone structure appropriate for binding.

The biological equipotency of [des-Asn^{28},Thr^{29}]glucagon, the similarly truncated homoserine derivative, and the homoserine lactone derivative precludes differentiating between the role of the hydrophobic side chain of methionine and the role of the dislocated carboxylate charge. An equivalent disruption of the stabilizing forces necessary for binding is seen for the intact sulfonium derivative, (*S*-methyl-Met^{27})glucagon, with its additional positive charge and extra methyl group. The loss of functional effectiveness associated with methylation is likewise demonstrated in the truncated derivatives [des-Asn^{28},Thr^{29}]glucagon and [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})glucagon. Hence, it is apparent that receptor binding involves multiple interactions that are ionic, polar, and nonpolar in nature (Epand et al., 1981) and that similar perturbations to binding may result from a disruption of either electrostatic or hydrophobic forces which contribute to the receptor interaction.

The derivatives generally show a comparable loss of relative biological potency and relative binding affinity; thus, it is interesting that the free acid and lactone forms of the homoserine derivative activate similarly but appear to differ significantly in binding affinity. These results would seem to indicate that the derivatives differ in the rate at which they dissociate from the receptor but that both derivatives remain bound for sufficient time to bring about whatever change is necessary to activate the adenylate cyclase system (Cuatrecasas, 1975).

The lack of correlation between the biological activity of the derivatives and their secondary structure at low pH or in the helix-favoring solvent and the flexible nature of glucagon under physiological conditions of pH and concentration (Panijpan & Gratzer, 1974) suggest that the initial nucleation structure for receptor binding may not involve the α-helical conformation proposed for the receptor-bound state (Sasaki et al., 1975). Binding may be associated with a process of mutual conformation selection by the hormone and the receptor occurring subsequent to an initial nucleation by the flexible ligand (Burgen et al., 1975; Laiken & Némethy, 1971).

The availability of these derivatives in high purity and yield should facilitate additional structural studies by CD (Wu & Yang, 1980) or other techniques such as NMR (Rothgeb et al., 1978; Bösch et al., 1978; Brown et al., 1981), optically detected magnetic resonance (Ross et al., 1977, 1979), or fluorescence analysis (Epand & Wheeler, 1975; Johnson et al., 1978). Furthermore, since these derivatives are modified within such a restricted range of the hormone sequence, they provide a suitable series with which to probe the binding interaction between the hormone and antibodies directed against the carboxyl-terminal region (Shima et al., 1975; Berzofsky et al., 1980). Finally, their pattern of binding affinities could be extremely useful for assessing the identity of receptor proteins isolated from a variety of glucagon target cells and freed of the constraints imposed by associated regulatory and catalytic units.

Acknowledgments

We are grateful to Professor Frank R. N. Gurd for helpful discussions and the provision of laboratory space and equipment and to Dr. Ward W. Moore of the Medical Sciences Program for his constant interest and support. We are also deeply indebted to Robert Boeglin for help in preparing the glucagon derivatives and to Kevin Thomas for performing the CD analyses. The excellent technical assistance of Kenney Green and the typing assistance of Laurie Steiger McCafferty are gratefully acknowledged, and gratitude is expressed to Doreen H. Mar for much encouragement and many helpful suggestions.

References

Ambler, R. P. (1972) *Methods Enzymol. 25*, 143–154.
Armstrong, M. D. (1949) *J. Am. Chem. Soc. 71*, 3399–3402.
Berzofsky, J. A., Hicks, G., Fedorko, J., & Minna, J. (1980) *J. Biol. Chem. 255*, 11188–11191.
Blakesley, R. W., & Boezi, J. A. (1977) *Anal. Biochem. 82*, 580–582.
Böhlen, P., Stein, S., Stone, J., & Udenfriend, S. (1975) *Anal. Biochem. 67*, 438–445.
Bösch, C., Bundi, A., Oppliger, M., & Wüthrich, K. (1978) *Eur. J. Biochem. 91*, 209–214.
Bregman, M. D., Trivedi, D., & Hruby, V. J. (1980) *J. Biol. Chem. 255*, 11725–11731.
Bromer, W. W. (1976) *Metab. Clin. Exp. Suppl. 1 25*, 1315–1316.
Bromer, W. W., Boucher, M. E., & Patterson, J. M. (1973) *Biochem. Biophys. Res. Commun. 53*, 134–139.
Brown, L. R., Bösch, C., & Wüthrich, K. (1981) *Biochim. Biophys. Acta 642*, 296–312.
Burgen, A. S. V., Roberts, G. C. K., & Feeney, J. (1975) *Nature (London) 253*, 753–755.
Chen, Y.-H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350–3359.
Coolican, S. A., Jones, B. N., & Gurd, R. S. (1979) 15th Midwest Regional Meeting of the American Chemical Society; Abstr. 215.
Counis, R., & Mongongu, S. (1978) *Anal. Biochem. 84*, 179–185.
Cuatrecasas, P. (1975) *Adv. Cyclic Nucleotide Res. 5*, 79–104.
De Lean, A., Munson, P. J., & Rodbard, D. (1978) *Am. J. Physiol. 235*, E97–E102.
Deranleau, D. A., Ross, J. B. A., Rousslang, K. W., & Kwiram, A. L. (1978) *J. Am. Chem. Soc. 100*, 1913–1917.
Desbuquois, B. (1975) *Eur. J. Biochem. 53*, 569–580.
Dixon, W. J., Ed. (1970) *BMD Biomedical Computer Programs X-series Supplement*, pp 177–186, University of California Press, Berkeley, CA.
Epand, R. M. (1972) *J. Biol. Chem. 247*, 2132–2138.
Epand, R. M., & Grey, V. (1973) *Can. J. Physiol. Pharmacol. 51*, 243–246.
Epand, R. M., & Wheeler, G. E. (1975) *Biochim. Biophys. Acta 393*, 236–246.

Epand, R. M., Rosselin, G., Hoa, D. H. B., Cote, T. E., & Laburthe, M. (1981) *J. Biol. Chem. 256*, 1128–1132.
Fraker, P. J., & Speck, J. C., Jr. (1978) *Biochem. Biophys. Res. Commun. 80*, 849–857.
Franks, D. J., & Malamud, D. (1976) *Anal. Biochem. 73*, 486–492.
Gratzer, W. B., & Beaven, G. H. (1969) *J. Biol. Chem. 244*, 6675–6679.
Gratzer, W. B., Bailey, E., & Beaven, G. H. (1967) *Biochem. Biophys. Res. Commun. 6*, 914–919.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.
Gross, E. (1967) *Methods Enzymol. 11*, 238–255.
Hiyashi, R. (1977) *Methods Enzymol. 47*, 84–93.
Hruby, V. J., Wright, D. E., Lin, M. C., & Rodbell, M. (1975) *Metab. Clin. Exp., Suppl. 1 25*, 1323–1325.
Hruby, V. J., Agarwal, N. S., Griffen, A., Bregman, M. D., Nugent, C. A., & Brendel, K. (1981) *Biochim. Biophys. Acta 674*, 383–390.
Johnson, M. L., Formisano, S., & Edelhoch, H. (1978) *J. Biol. Chem. 253*, 1353–1356.
Jones, B. N., & Gurd, R. S. (1981) *Arch. Biochem. Biophys. 206*, 227–231.
Jones, B. N., Boeglin, R. J., & Gurd, R. S. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1930.
Jones, B. N., & Pääbo, S., & Stein, S. (1981) *J. Liq. Chromatogr. 4*, 565–586.
Jones, W. C., Jr., Rothgeb, T. M., & Gurd, F. R. N. (1976) *J. Biol. Chem. 251*, 7452–7460.
Laiken, N., & Némethy, G. (1971) *Biochemistry 10*, 2101–2106.
Lewis, R. V., Stein, S., & Udenfriend, S. (1979) *Int. J. Pept. Protein Res. 13*, 493–497.
Lin, M. C., Wright, D. E., Hruby, V. J., & Rodbell, M. (1975) *Biochemistry 14*, 1559–1563.
Liu, T. V., & Chang, Y. H. (1971) *J. Biol. Chem. 246*, 2842–2848.
Markwell, M. A. K., & Fox, C. F. (1978) *Biochemistry 17*, 4807–4817.
Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206–210.
Neville, D. M., Jr. (1968) *Biochim. Biophys. Acta 154*, 540–552.
Offord, R. E. (1972) *Biochem. J. 129*, 499–501.
Panijpan, B., & Gratzer, W. B. (1974) *Eur. J. Biochem. 45*, 547–553.
Pohl, S. L. (1976) in *Methods in Receptor Research* (Blecher, M., Ed.) pp 159–174, Marcel Dekker, New York.
Rodbell, M. (1980) *Nature (London) 284*, 17–22.
Rodbell, M., Krans, H. M. J., Pohl, S., & Birnbaumer, L. (1971) *J. Biol. Chem. 246*, 1861–1871.
Ross, J. B. A., Rousslang, K. W., Deranleau, D. A., & Kwiram, A. L. (1977) *Biochemistry 16*, 5398–5402.
Ross, J. B. A., Rousslang, K. W., de Haën, C., Lavis, V. R., & Deranleau, D. A. (1979) *Biochim. Biophys. Acta 576*, 372–384.
Rothgeb, T. M., Jones, B. N., Hayes, D. F., & Gurd, R. S. (1977) *Biochemistry 16*, 5813–5818.
Rothgeb, T. M., England, R. D., Jones, B. N., & Gurd, R. S. (1978) *Biochemistry 17*, 4564–4571.
Salomon, Y., Londos, C., & Rodbell, M. (1974) *Anal. Biochem. 58*, 541–548.
Sasaki, K., Dockerill, S., Adamiak, D. A., Tickle, I. J., & Blundell, T. L. (1975) *Nature (London) 257*, 751–757.
Schwartzel, E. H., Jr., Bachman, S., & Levine, R. A. (1977) *Anal. Biochem. 78*, 395–405.
Shima, K., Sawazaki, N., Tanaka, R., Tarui, S., & Nishikawa, M. (1975) *Endocrinology (Philadelphia) 96*, 1254–1260.
Simpson, R. J., Neuberger, M. R., & Liu, T.-Y. (1976) *J. Biol. Chem. 251*, 1936–1940.
Spiegel, A. M., & Bitensky, M. W. (1969) *Endocrinology (Philadelphia) 85*, 638–643.
Srere, P. A., & Brooks, G. C. (1969) *Arch. Biochem. Biophys. 129*, 708–710.
Stagg, B. H., Temperley, J. M., Rochman, H., & Morley, S. (1970) *Nature (London) 228*, 58–59.
Tager, H. S., Hohenboken, M., & Markese, J. (1977) *Endocrinology (Philadelphia) 100*, 367–372.
Unger, R. H., & Orci, L. (1981) *N. Engl. J. Med. 304*, 1518–1524.
von Schenck, H., & Jeppsson, J.-O. (1977) *Biochim. Biophys. Acta 491*, 503–508.
von Schenck, H., Larsson, I., & Thorell, J. I. (1976) *Clin. Chim. Acta 69*, 225–232.
White, A. A., & Karr, D. B. (1978) *Anal. Biochem. 85*, 451–460.
Wright, D. E., & Rodbell, M. (1979) *J. Biol. Chem. 254*, 268–269.
Wright, D. E., Hruby, V. J., & Rodbell, M. (1978) *J. Biol. Chem. 253*, 6338–6340.
Wu, C.-S. C., & Yang, J. T. (1980) *Biochemistry 19*, 2117–2122.