Methylation of Glucagon, Characterization of the Sulfonium Derivative, and Regeneration of the Native Covalent Structure†

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ABSTRACT: The methylation of the single methionine residue of glucagon is accomplished at a pH of 3.5 in 8 M urea with methyl iodide. The reaction product is a soluble sulfonium derivative, *S*-methylglucagon, which can be isolated in a highly purified form. This derivative is characterized by amino acid analysis and its effect on the adenylyl cyclase system of rat liver plasma membranes. *S*-Methylglucagon does stimulate the adenylyl cyclase system; however, its activity is approximately 500 times less than that observed with the native hormone. The solubility of this derivative is great enough to allow for further modifications of the molecule which can be followed at a later stage by demethylation. Demethylation of *S*-methylglucagon regenerates the original covalent structure and is accomplished by treatment with Cleland's reagents at a pH of 10.5. The regenerated hormone is indistinguishable from native glucagon by its amino acid composition and its ability to stimulate the adenylyl cyclase system. The entire methylation-demethylation reaction sequence has been carried out with yields that approach 75%. The technique is suitable for the isotopic enrichment of native glucagon and may well be applicable to selected other methionine-containing peptides.

The 29-membered peptide hormone glucagon has a wide variety of biological effects, including those on intermediary metabolism (Unger and Orci, 1977), cardiac contractility (Glick et al., 1968; Parmley et al., 1969), and hepatic regeneration (Bucher and Swaffield, 1975; Farivar et al., 1976). Currently much interest attends the hyperglucagonemia characteristic of the diabetic state. The hepatic effects of glucagon upon carbohydrate metabolism are believed to be mediated through hormone specific adenylyl cyclase receptors situated on the plasma membrane of hepatocytes (Unger and Lefebvre, 1972).

Numerous studies of the structure of this peptide have been concerned with its crystal structure (Sasaki et al., 1975), its conformation in solution (Gratzer et al., 1967, 1968, 1972; Srere and Brooks, 1969; Edelhoch and Lippoldt, 1969; Panijpan and Gratzer, 1974), as well as the mechanism by which it binds to its specific receptor and subsequently activates the adenylyl cyclase system (Rodbell et al., 1971a-c; Epand, 1972; Birnbaumer and Pohl, 1973; Lin et al., 1975, 1976, 1977; Epand and Wheeler, 1975; Epand and Cote, 1976; Epand et al., 1977). As a result of these studies the structural features of the hormone which are responsible for its binding and its biological activity are currently being sought.

A striking feature of glucagon is its marked insolubility at physiological pH values (Bromer, 1972). This insolubility has hindered many studies and as a consequence numerous aspects of the structure-function relationships of the hormone remain unclear. The recent success in this laboratory with the methylation of the two methionines of sperm whale myoglobin followed by demethylation and renaturation (Jones et al., 1975, 1976) has prompted us to consider the usefulness of this strategy for the study of glucagon. The technique is capable of yielding a number of glucagon derivatives which can be isolated in highly purified forms. One particular derivative, the sulfonium intermediate, confers markedly increased solubility thereby making possible a number of previously inaccessible studies. The method is further suitable for isotopic enrichment of the native hormone.

The present report includes the methylation-demethylation procedure, purification procedures, chemical characterization of derivatives, and tests of biological activity. These results attest to the native structure of the regenerated hormone and provide evidence for the nearly full biological activity of the sulfonium intermediate at elevated concentrations.

Experimental Section

Materials. All chemicals used were reagent grade unless otherwise specifically designated. Distilled-deionized water was used throughout. In all preparations containing urea, the urea solution was deionized on the mixed bed ion-exchanger Rexyn I-300 immediately before use. Crystalline porcine glucagon was provided through the courtesy of Eli Lilly and Co. (Lot No. 358-V016-36).

Methods. Routine acid hydrolysates were prepared in 5.7 N HCl at 110 °C for 22 h and were analyzed on a Beckman 121 amino acid analyzer equipped with DC-1A resin (Durrum). However, to avoid the partial destruction of *S*-methylmethionine during HCl hydrolysis (Toennies and Kolb, 1945) and its co-elution with lysine when the above analysis system is used, 24-h, 110 °C hydrolysates were prepared in 3 N *p*-toluenesulfonic acid containing 3-(2-aminoethyl)indole (Liu and Chang, 1971; Jones et al., 1976). These hydrolysates were analyzed on a Beckman 120 amino acid analyzer fitted with a column (0.9 × 19 cm) of PA-35 resin (Beckman). The column was eluted at 65 °C with pH 5.28 citrate buffer, 0.3 N in sodium ion. The elution position of *S*-methylmethionine under these conditions was 14.4 min after histidine (Jones et al., 1976). Enzymatic digestion was carried out with "insoluble" protease from *Streptomyces griseus* (Sigma) followed by aminopeptidase M (Sigma). To the enzymatic digestion media,

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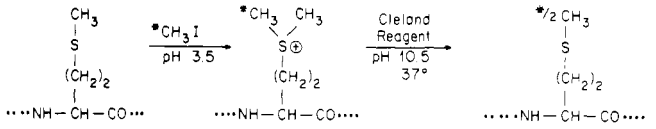

FIGURE 1: The sequence of steps employed to effect the methyl exchange reaction involving the methionine methyl group in a peptide.

0.05 mg/mL chloramphenicol (Parke Davis and Co.) was added to inhibit bacterial growth. Amino acid analysis of the enzymatic hydrolysates made use of the Beckman Model 120 or 121 systems above as well as the lithium citrate buffer system to achieve separation of asparagine and glutamine (Garner et al., 1973). Performic acid oxidation followed by HCl hydrolysis was performed according to the method of Hirs (1967).

Glucagon and its derivatives (25-mg lots) were purified on a 1.6 × 20 cm column of Whatman CM 52 cation exchange resin. The column was equilibrated with 10 mM ammonium acetate at pH 4.5 in 6 M urea as a first buffer. The column was then eluted with a linear gradient of the first buffer and a second buffer of 20 mM ammonium acetate at pH 5.4 in 6 M urea. The column effluent was continuously monitored for both conductivity and absorbance at 280 nm. Each fraction from the column was pooled, dialyzed against 3% acetic acid (using previously boiled Spectrapor 3 membrane tubing), lyophilized, and stored at −20 °C until characterized.

Methylation of purified glucagon was achieved by suspending 20 mg of purified glucagon in 2 mL of 8 M urea. The pH of the solution was adjusted to 3.5 with 1 N HCl at which time the glucagon completely dissolved. To this solution was added a 100-fold molar excess of methyl iodide (Mallinkrodt, copper free). The biphasic reaction mixture was placed in the dark and allowed to stir at room temperature for 18 h. At the end of this time the slightly turbid reaction mixture was diluted with an equal volume of 2% acetic acid and applied to a 1.6 × 35 cm Bio-Gel P-2 column equilibrated with 2% acetic acid. Again the column was monitored at 280 nm. The peptide-containing fractions were pooled and lyophilized. The methylated glucagon was then repurified on the CM52 column described above.

Demethylation of the sulfonium derivative was achieved as previously described (Jones et al., 1976). Purified methylated glucagon was dissolved to a final concentration of 2.5 mg/mL in 0.5 M dithiothreitol or dithioerythritol (Calbiochem) at a final pH of 10.5. The reaction vial was sealed and placed at 37 °C and the reaction allowed to proceed without stirring for 24 h. At the end of the 24-h reaction period the reaction mixture was dialyzed against 3% acetic acid, lyophilized, and purified as described above.

Glucagon Activity Assay. Crude rat liver membranes were prepared following the method of Cohen and Bitensky (1969). All procedures were carried out at 0 °C. The livers were removed from adult Sprague-Dawley rats immediately following decapitation and placed in a medium consisting of 0.02 M potassium acetate (pH 7.4). The livers were trimmed free of connective tissue and minced in the medium. The minced tissue was then homogenized in 5 vol of medium with 6–10 strokes of a loosely fitting Teflon pestle in a Potter-Elvehjem homogenizer, filtered through three layers of cheesecloth, and centrifuged at 12 000*g* for 5 min. The supernatant was discarded, the pellet resuspended in 10 vol of medium, and the centrifugation repeated. This step was repeated until the supernatant was clear. The final pellet was suspended in 2 vol of medium. Aliquots of the suspension were frozen in liquid nitrogen and stored at −20 °C until needed.

Stimulation of the adenylyl cyclase in liver membranes was patterned after the procedure of Epand and his colleagues (Epand et al., 1974; Epand and Epand, 1973). To a 150-μL volume containing final concentrations of 25 mM Tris[1]-HCl at pH 7.5, 5 mM theophylline, 20 mM phosphocreatine (Calbiochem), 50 units of creatine phosphokinase (Calbiochem), 1 mg/mL bovine serum albumin, 5 mM KCl, 0.25 mM EDTA,[1] 6 mM $MgSO_4$, 1 mM ATP[1] (Calbiochem), 0.5 mg/mL bacitracin, and the appropriate hormone concentration when applicable was added the liver membrane preparation in a 50-μL aliquot containing 1000–1500 μg of membrane protein in order to initiate the reaction. Membrane protein concentrations were determined by a modified Lowry technique (Schacterle and Pollack, 1973). Hormone solutions were made by carrying out serial dilutions of a stock hormone solution using 4 mg/mL albumin as the diluting medium and substituting a 50-μL aliquot of a particular hormone-albumin solution for the albumin mentioned above. The concentration of the hormone stock was determined by amino acid analysis using HCl hydrolysis as described above.

The stimulation reaction was allowed to proceed for 10 min at 37 °C. The reaction was terminated by the addition of 50 μL of 0.3 N HCl (final pH of 1.0–1.5) and heating at 85 °C for precisely 20 min. The resulting mixtures were then lyophilized, diluted to an appropriate volume (final cAMP[1] concentration between 1 and 10 pmol per assay aliquot), frozen, and stored at −20 °C until assayed for cAMP content.

The extent to which the adenylyl cyclase system was activated was determined by assaying for cAMP content in each of the samples above. The assay procedure made use of a specific cAMP binding protein, isolated from rodent liver (Calbiochem). The procedure was a slightly modified version of the technique of Maguire and Gilman (1974) detailed in the Calbiochem "Guide for the Assay of Cyclic AMP in Biological Samples" (Document No. 3082A). The amount of cAMP generated in each stimulation sample was compared to that contained in standard solutions by measuring the degree of displacement of tritiated cAMP from the cAMP binding protein. Each hormone concentration was assayed using triplicate determinations on at least two samples.

A dose-response curve for glucagon and its derivatives was constructed as follows. The basal adenylyl cyclase activity (without addition of hormone) and the activity of the fully stimulated system (glucagon concentration $\sim 10^{-5}$ M) were determined. The percent stimulation, ± its standard error (SE), was calculated by subtracting the basal activity from the observed activity (± SE) and dividing this by the difference between the fully stimulated and basal activities.

Results and Discussion

Reaction Scheme. The reaction scheme utilized for this study is summarized in Figure 1. The scheme shows the sequence of steps which effect a methyl exchange reaction involving the methionine methyl group in a peptide linkage. The treatment with methyl iodide was carried out at pH 3.5 to minimize the reactivity of other nucleophiles in the peptide (Jones et al., 1975, 1976) as well as to take advantage of the enhanced solubility of native glucagon at this pH (Bromer, 1972). Examples of selective alkylation of methionine residues in proteins and peptides (Gurd, 1967; Link and Stark, 1968; Naider and Bohak, 1972; Jones et al., 1975) under conditions

[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; ATP, adenosine triphosphate; cAMP, cyclic 3′,5′-adenosine monophosphate.

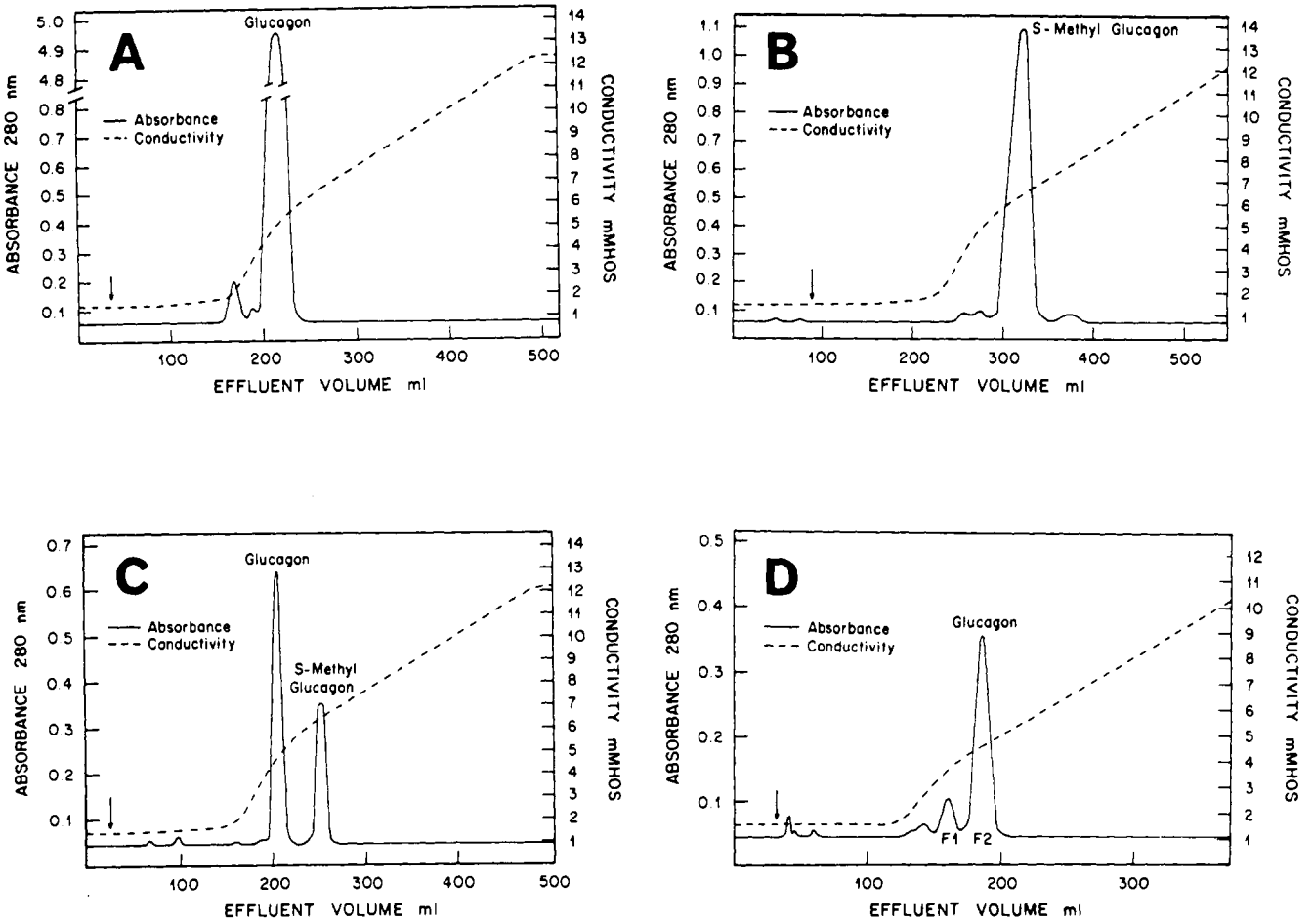

FIGURE 2: The elution profiles for the purification of glucagon and its derivatives. In all cases the sample was loaded at zero effluent volume; the arrow indicates the time at which the gradient was started. Other details are in the text: (A) the purification of native glucagon; (B) the purification of *S*-methylglucagon; (C) the elution profile demonstrating the separation between a mixture of native and *S*-methylglucagon; (D) the purification of demethylated glucagon.

similar to those reported here have been reported previously.

The regeneration of the methionine-containing hormone from the methylated sulfonium intermediate was best effected by exposure to mercaptans (Schejter and Aviram, 1972; Naider and Bohak, 1972; Jones et al., 1975, 1976). The optimum conditions found to date are those stated in the Experimental Section. These appear adequate for our purposes, although milder conditions for dealkylation are currently being investigated.

Purification and Characterization of Native Glucagon. The purification of native glucagon, as shown in Figure 2A, revealed some minor impurities (less than 5%) which were not recognizable by amino acid composition as either glucagon or insulin derivatives (Tager and Sterner, 1973; Bromer et al., 1972; Nolan et al., 1971). Their activities have not yet been assayed. The major peak in Figure 2A was shown by amino acid analysis to agree with the predicted values for native glucagon as shown in Table I (Bromer et al., 1957). Little or no deaminated product could be identified in any of the preparations. The ability of this fraction to stimulate the adenylyl cyclase system of liver membranes (see below) also indicated that this fraction was glucagon (Pohl et al., 1971).

Purification and Characterization of S-Methylglucagon. The elution profile for the purification of a typical glucagon methylation reaction mixture is shown in Figure 2B. As can be seen from this figure, the major fraction of the profile is well separated from several impurities in the reaction mixture. Figure 2C shows the separation of a mixture of *S*-methylglucagon, the major fraction in Figure 2B, and native purified glucagon. The clear separation seen in Figure 2C indicates that there should be little or no native glucagon in the methylation reaction mixture of Figure 2B. The amino acid composition of the major fraction in Figure 2B is given in Table I. As can be seen from Figure 2B and Table I, the methylation reaction appears to have proceeded extensively and specifically, modifying only the single methionine residue of native glucagon. The yield of the reaction from native glucagon to purified methylated glucagon is greater than 90%.

Although the purification of *S*-methylglucagon was accomplished on a column containing 6 M urea, it is conceivable that an association between native and *S*-methylglucagon could carry native glucagon along with the sulfonium derivative. To rule out this possibility, the absolute purity of *S*-methylglucagon was checked as follows.

A sample of purified *S*-methylglucagon was subjected to *p*-toluenesulfonic acid hydrolysis while a second sample was subjected to performic acid oxidation followed by HCl hydrolysis. The amino acid, *S*-methylmethionine, is resistant to both *p*-toluenesulfonic acid hydrolysis and performic acid oxidation and can be recovered quantitatively with both procedures. Therefore, the presence of methionine in the *p*-toluenesulfonic acid hydrolysate or methionine sulfone in performic acid hydrolysate would indicate contamination of the *S*-methylglucagon by native glucagon. Using this strategy and overloading the amino acid analyzer (>300 nmol of hydroly-

TABLE I: Amino Acid Compositions of Purified Native, *S*-Methyl-, and Methylated-Demethylated Glucagons.

Amino acid	Native glucagon		*S*-Methylglucagon		Methylated-demethylated glucagon	
	Acid hydrolysis	Enzymatic hydrolysis	Acid hydrolysis	Enzymatic hydrolysis	Acid hydrolysis	Enzymatic hydrolysis
Asp [3][a]	4.0[c]	2.9	4.0	2.9	4.1	2.9
Thr [3]	2.9	3.0	2.9	2.9	3.0	3.0
Ser [4]	3.9	4.2	3.9	4.0	3.9	3.4
Asn [1]	N[d]	0.9	N	1.0	N	1.0
Glu [0]	3.0	0	3.2	0	3.0	0
Gln [3]	N	3.0	N	2.9	N	3.0
Gly [1]	1.0	0.9	1.0	1.0	1.0	1.0
Ala [1]	1.0	1.3	1.0	1.0	0.9	1.2
Val [1]	0.9	1.2	0.9	1.0	1.0	1.1
Met [1]	1.0	1.0	0	0	1.0	1.0
Leu [2]	2.0	1.9	2.0	2.0	2.0	2.1
Tyr [2]	2.1	2.0	2.1	2.1	2.0	1.8
Phe [2]	2.0	2.0	2.0	2.0	2.0	2.0
Lys [1]	1.1 (1.0)[e]	1.0	1.8[f] (1.0)	1.9 (1.1)	0.9	1.0 (1.0)
His [1]	1.2 (1.0)	0.9	0.9 (1.0)	0.9 (0.9)	1.0	1.0 (0.8)
Arg [2]	2.1 (2.0)	1.9	1.9 (2.1)	1.8 (2.0)	1.9	1.8 (2.1)
Trp [1]	N (1.0)	1.1	N (1.0)	N (1.0)	N	N (1.1)
SMM[b]	N	N	N (1.0)	N (1.1)	N	N (0)

[a] Values in brackets refer to the theoretical number of residues per molecule based on sequence analysis (Bromer et al., 1957). [b] SMM indicates the amino acid *S*-methylmethionine. [c] Values were rounded off to the nearest tenth of a residue. [d] N, not determined. [e] Values in parentheses were obtained from either *p*-toluenesulfonic acid hydrolysis or enzymatic hydrolysis followed by analysis on the PA-35 column as described in the text. [f] Lysine and *S*-methylmethionine co-elute under these conditions of analysis.

sate) no methionine or methionine sulfone could be detected in the respective hydrolysates. Given the detection limit for an amino acid of less than 0.5 nmol, this procedure puts an upper limit on the levels of contamination of less than 0.17%.

The above results clearly demonstrate the feasibility of modifying the single methionine in native glucagon and isolating highly purified *S*-methylglucagon from unreacted material and other impurities.

One potentially important aspect of the physical properties of this derivative is its increased solubility. In contrast to native glucagon (Bromer, 1972) the solubility of this derivative at physiological pH values is greater than 25 mg/mL. This greater than 500-fold increase in solubility could result from the additional positive charge on the molecule or a disruption of the hormone–hormone interactions which have been postulated to account for the limited solubility of native glucagon (Blanchard and King, 1966; Srere and Brooks, 1969; Gratzer et al., 1972; Panijpan and Gratzer, 1974). Further studies concerning the structural basis for this solubility enhancement are in progress.

Purification and Characterization of Methylated-Demethylated Glucagon. The elution profile for the purification of a typical demethylation reaction mixture is shown in Figure 2D. The second peak of this elution profile (F2) elutes at the same position and conductivity as does native glucagon. The amino acid analyses of this fraction are shown in Table I. As can be seen from Figure 2 as well as the results summarized in Table I, this fraction appears to be chemically identical with native glucagon. The results of the dose-response curve given below also indicate that the native structure has been regenerated.

The location of the first peak (F1) in Figure 2D indicates that this fraction contains more net negative charge than does F2 or native glucagon. Amino acid analysis of F1 (not shown) suggests that this fraction represents a product of nonspecific deamidation, a monodesamidoglucagon. It is estimated from the comparison of the areas of F1 and F2 that the yield of fraction F2 is greater than 75%. This yield is consistent with those observed for other preparations.

Adenylyl Cyclase Activity of Native Glucagon and Its Derivatives. Shown in Figure 3 are the dose-response characteristics of native glucagon, methylated-demethylated glucagon, and the intermediate *S*-methylglucagon. The response for each peptide or derivative was calculated as described above using native glucagon as the standard. The activity of the fully stimulated adenylyl cyclase system (concentration of native glucagon $\sim 10^{-5}$ M) was approximately 12 times greater than the basal activity (~5 pmol of cAMP per 10 min per mg of membrane protein). The characteristics of the curve for native glucagon are very similar to the curves obtained by other investigators using the same crude liver membrane preparation (Epand, 1972; Epand et al., 1973; Epand and Cote, 1976). This curve has been used as a basis for judging the activity of the other glucagon derivatives.

While the physical and chemical characterization of the methylated-demethylated compound indicate it is identical with the native hormone, the most rigorous proof of identity includes its ability to activate adenylyl cyclase to a comparable extent as the native peptide. As can be seen from Figure 3, the curve for the regenerated hormone is indistinguishable from that of the native. It would appear then that the procedures described herein were successful in that a product with physical, chemical, and biological properties identical with native glucagon can be obtained following methylation and demethylation.

In contrast to the methylated-demethylated species, the methylated sulfonium derivative is clearly distinguishable from the native hormone. The dose-response curve shown in Figure 3 reveals that *S*-methylglucagon can stimulate the adenylyl cyclase system. At $\sim 10^{-5}$ M its activity, however, is considerably less than that observed with native glucagon. The concentration of *S*-methylglucagon required to obtain 50% activity of the native hormone is ~500 times greater for this sulfonium derivative. However, the increased solubility of this derivative

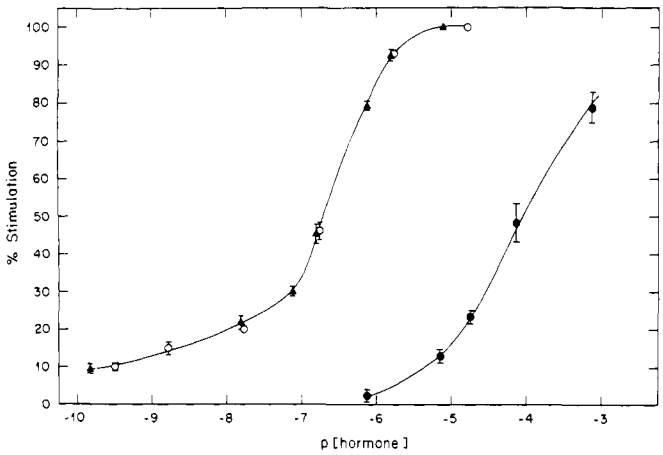

FIGURE 3: The dose-response curve for the stimulation of the adenylyl cyclase system with native, methylated-demethylated, and *S*-methylglucagon. The symbol ▲ represents native glucagon, O represents methylated-demethylated glucagon, and ● represents *S*-methylglucagon. The scale for the abscissa of this figure (p[hormone]) is the log of the molar hormone concentration. The bars on each point indicate the standard error of the determination. Details for the assay and the calculation of the response are given in the text.

permits the use of concentrations which give activities up to 80% of that observed with the native hormone. At the highest concentration of *S*-methylglucagon used, the dose-response curve has not yet begun to taper off and hence it is conceivable that with even higher concentrations of the sulfonium derivative, nearly 100% activity could be observed.

A decreased adenylyl cyclase activity for the sulfonium derivative could result from several different factors: (a) there might be an increase in the nonspecific binding of the sulfonium derivative to the membrane preparation; (b) the sulfonium derivative might have a decreased affinity for the glucagon specific receptors; (c) the derivative might have a decreased activation potentiality for the adenylyl cyclase system once it is bound to the receptor; (d) the sulfonium derivative may be totally unable to bind and the observed activity may be the result of undetected native glucagon contamination.

This assay technique cannot distinguish between the first three of the above possibilities; however, significant contamination of the sulfonium derivative fraction has been ruled out by chemical analysis. The possibilities leading to the decreased activity of the sulfonium derivative can be evaluated only after detailed analyses are made of the binding and activation characteristics with a more highly purified membrane preparation.

The methylation-demethylation procedure described above offers the opportunity to further characterize the physical, chemical, and biological properties of the hormone glucagon (Morrow et al., 1974a,b, 1976). These studies are made possible to a large degree by the enhanced solubility of the sulfonium derivative. Chemical or enzymatic modifications of this derivative are possible and can be followed at a later stage by demethylation. Studies are currently in progress in which the sulfonium derivative is subjected to digestion by carboxypeptidase A to obtain a series of des-Asn_{28}-Thr_{29} glucagon derivatives. The properties of these derivatives should prove to be interesting when compared to those of other such derivatives (Spiegel and Bitensky, 1969; Epand, 1972; Lin et al., 1975; Bromer, 1976).

The fact that the demethylation reaction on the sulfonium derivative yields a product indistinguishable from native glucagon is significant in several respects. First, this demonstrates that *S*-methylglucagon has probably been modified at only the single methionine residue. Second, any differences observed between the native hormone and a derivative whose synthesis makes use of the sulfonium intermediate will be the result of the derivatization reactions and not the methylation-demethylation reactions. Third, the technique makes available a method for covalently labeling this methionine-containing peptide hormone without iodination. There are a number of reports which suggest that iodination may itself distort interpretation of the properties which are of importance for the study undertaken (Bromer et al., 1973; Desbuquois, 1975; Lin et al., 1976, 1977). This covalent labeling of the native molecule without de novo synthesis will be useful for a number of potentially important studies (Lin et al., 1977), while the technique itself is general enough such that it may be applied to a number of other biologically important peptides as well.

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