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Title PREPARATION AND PROPERTIES OF POLYGLYCYL INSULIN

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In order to understand more about insulin behavior in solution, Zn-free insulin was modified by adding polyglycyl chains to the molecule and then observing the effect of the peptide on the behavior of insulin under various conditions.

NCA-glycine (N-Carboxyglycine anhydride) of high purity was reacted with Zn-free insulin in aqueous solution to form polyglycyl insulin. It was found by dinitrophenylation, formaldehyde titration and amino acid analysis that two N-terminal amino acids and one lysine were acylated. The length of the added glycine peptide varied with the modification conditions.

Biological tests indicated that the polyglycyl insulin retained its biological activity, except for highly substituted derivatives. The derivatives were resistant to trypsin digestion.

Polyglycyl insulin was more soluble than native insulin. The solubility varied with pH in a manner similar to native insulin, but was about three times more soluble between pH 4 and pH 6.

Thin film dialysis experiments indicated that the polyglycyl insulin was heterogeneous with respect to the length of attached glycine peptides.

Insulin forms aggregates under various conditions. In the present study the gel filtration technique was used to analyze the aggregation state of modified insulins. The polyglycyl-insulin modified as its monomer behaved the same as the native insulin. However, when it was modified as the dimer, the product remained as dimer under conditions where native insulin could be converted to monomer.

PREPARATION AND PROPERTIES OF POLYGLYCYL INSULIN

by

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A THESIS

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Typed by Gwendolyn Hansen

To my parents and husband. . .

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PREPARATION AND PROPERTIES OF POLYGLYCYL INSULIN

INTRODUCTION

The polypeptide hormone insulin consists of two polypeptide chains; the A chain contains 21 amino acid residues and the B chain contains 30 amino acid residues. The chains are linked by two disulfide bridges. In addition, in the A chain there is an intrachain disulfide bridge between the cysteine residues occupying positions 6 and 11 (Sanger, 1959).

Insulin has been found to exist as aggregates of its molecular unit under various conditions. In aqueous solution the degree of association of insulin depends upon the concentration, the temperature, the pH, and particularly on the zinc content of the solution (Fredericq, 1956). By countercurrent distribution Harfenist and Craig (1952) established the fact that insulin could exist as monomer under certain conditions with a molecular of 6,000.

Below pH 2, the stable unit of Zn-insulin in aqueous solution is a dimer (Low and Einstein, 1960). Between pH 4-7 Zn-insulin is essentially insoluble, while in the pH range 7-9 it associates to form hexamers of 34,000 molecular weight. These associations are also affected by ionic strength (Oncley, 1952), with a greater tendency toward association seen at higher ionic strength. By comparison,

Zn-free insulin has a molecular weight of 12,000 at acidic pH but dissociates to monomer at pH 8-9. There is no association beyond dimer at pH 7.7 (Marcker, 1960). When Zn-insulin in solution is heated at a pH below 3.5, the molecules aggregate spontaneously to form fibers which are stable from pH 0 to pH 10. Small fibril fragments formed in this way and placed in an acid solution of insulin will then initiate fibril formation without heating. Four molecules are bound together to form a fibril, the length of which is limited only by available insulin.

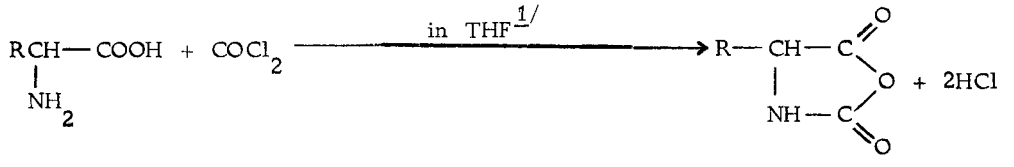
In view of this tendency to form aggregates, insulin can be used as a system for studying intermolecular forces between macromolecules. An approach to studying these forces is that of attaching selected amino acids (via the polymerization of the amino acid N-carboxy-anhydride) to insulin and studying the changes in the physical properties of the protein. An additional approach to studying these forces is that of chemical modification. Keresztes-Nagy (1962) successfully demonstrated a chemical modification method to dissociate a protein by reacting hemerythrin with succinic anhydride. The chemical modification did not abolish the biological activity of the protein. Fraenkel-Conrat (1953) prepared polyleucyl insulin which retained hormonal activity. Poly-DL-valyl-insulin prepared by Stracher, et al. (1956) was not hydrolyzed by trypsin and also showed a higher stability toward acid hydrolysis. Polyglycyl

ribonuclease, studied by Becker and Sawada (1963), was more heat stable than the native enzyme. Becker (1965) has reported that polyglycyl derivatives of chymotrypsin remain monomeric under conditions in which the native enzyme dimerizes. These are several examples of systems in which chemical modification prevents aggregation reactions. It was of interest to determine whether or not the addition of glycine peptides to insulin would result in the preparation of monomeric insulin.

The specificity of the reaction of N-carboxy amino acid anhydrides (NCAs) with proteins has been shown by Konigsberg and Becker (1959) to result in the attachment of chains to the free N-terminal amino groups and free epsilon amino groups of lysine. The degree of modification of the groups in proteins depends on the availability of the reactive groups as well as their reactivities with the NCAs.

The general scheme for the reaction involved in the preparation of polypeptidyl insulin can be visualized as follows:

Step I. Preparation of N-Carboxy amino acid anhydride



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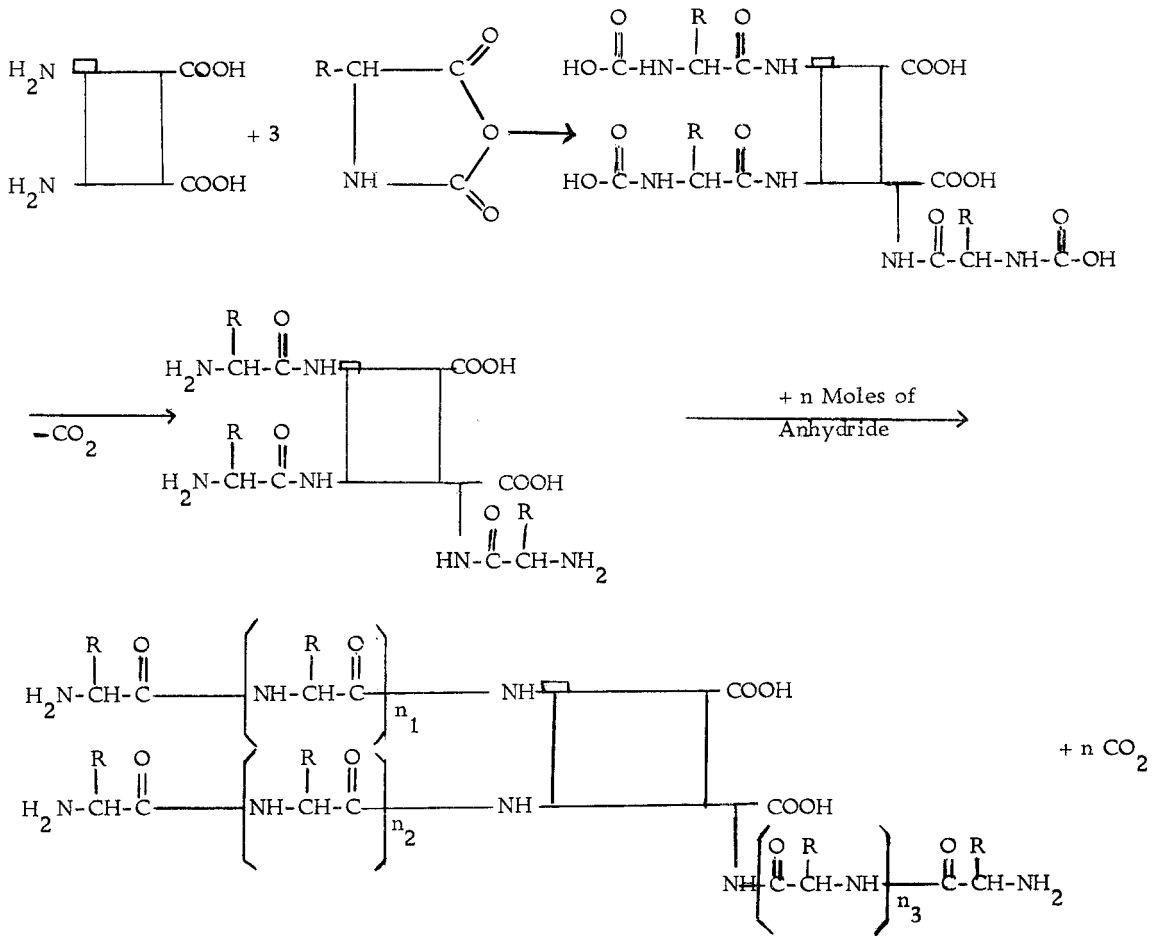
[1] Tetrahydrofuran

Step II. Modification of Insulin



To determine whether or not the treatment results in a product which is monomeric, the molecular weights must be established. Many attempts have been made to determine the molecular weight of proteins by measuring their ability to diffuse through materials of various porosity. Whitaker (1963) obtained a linear correlation between the logarithm of the molecular weight of a protein and the

ratio of its elution volume to the column void volume. The void volume (V_o) is the volume outside the gel grains, and is practically measured by determining the volume of liquid required to elute a substance through a column if the molecules are completely excluded from the gel particles. V_o varies with the size of the column. Another technique useful in molecular weight determination is that of Craig (1957). He studied a series of proteins and found that the time required for 50 percent of the protein to escape through cellophane membranes was roughly proportional to the molecular weight of the protein.

In this thesis, the preparation of polyglycyl insulin, and attempts to determine the molecular size of the derivatives by these two methods are described.

MATERIALS AND METHODS

Insulin: Crystalline pancreatic insulin, Lot No. 74B - activity 24 IU per mg., was purchased from Sigma Co. (St. Louis, Mo.)

Tetrahydrofuran (THF), reagent grade, was purchased from Matheson, Coleman, and Bell (East Rutherford, N. J.). It was refluxed with Lithium aluminum hydride overnight and distilled just prior to use (B.P. 65^{o}-66^{o}).

Ethyl ether, anhydrous and petroleum ether (B.P. 30^{o}-60^{o}), reagent grade, were obtained from J. T. Baker (Phillipsburg, N. J.).

Ethyl acetate, reagent grade, was obtained from Fisher Scientific Co. (Fair Lawn, N. J.). It was freshly distilled before use (B.P. 76^{o}-77^{o}).

Chromatographically homogeneous glycine, Lot No. 52589, A grade, was obtained from Calbiochem. (Los Angeles, California). Glycine was dried over P_2O_5 in vacuo at 100^{o} for three days before use.

Phosgene gas was purchased from Matheson Co. (East Rutherford, N. J.). It was passed through concentrated sulfuric acid before bubbling into the reaction flask.

Tris (hydroxymethyl) aminomethane was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Sodium methylate, Technical grade, was obtained from

J. T. Baker Chemical Co. (Phillipsburg, N. J.) F.W. 54.02, 25 percent in methanol solution. The normality as determined by titration with $KHC_8H_4O_4$ was 0.097.

P-Dioxane, reagent grade, was purchased from Matheson, Coleman, and Bell Co. (East Rutherford, N. J.). It was refluxed with potassium hydroxide and distilled before use (B.P. 100°-102°).

Sephadex G-150 and blue-dextran were purchased from Pharmacia Co. (Uppsala, Sweden).

Dialysis tubing, sizes 18/32 and 20/32, produced by Food Products Div., Union Carbide Corp., (Chicago, Ill.) were used.

Ultraviolet and visible spectra were determined using a Beckman DB spectrophotometer.

pH measurements were made with either a Corning pH meter Model 12, or with a Beckman Zeromatic pH meter.

A pH stat radiometer type TTTI (Copenhagen, Denmark) was used in the trypsin digestion experiment.

EXPERIMENTAL

Preparation of N-Carboxy-Glycine Anhydride

For synthesis of N-Carboxy-Glycine Anhydride (NCA-glycine) the Fuchs-Farthing (1950) method was used with minor modification. Five grams of dried glycine was suspended in 50 ml of freshly distilled THF. Phosgene gas was passed into the suspension which was stirred vigorously by a small magnetic bar. The reaction temperature was maintained at 45° C. The suspension became clear in three hours. Nitrogen gas was then bubbled through the reaction mixture for 30 minutes to remove excess phosgene. The solvent was removed via a rotatory evaporator at temperatures not over 40° C. The anhydrous NCA-glycine was recrystallized by dissolving it in a minimum amount of warm (50°) anhydrous ethyl acetate, filtering the solution through Celite (Johns-Mansville Corp.), and then cooling the solution until it became turbid. The solution was placed in a dry ice-acetone bath. Colorless crystals were obtained, which were dried at 0° C in vacuum and stored at 0° C over $CaCl_2$.

Determination of the Purity of NCA-glycine

Owing to the fact that NCA-glycine decomposes before melting, its purity cannot be checked by melting point determination as in the

case of other NCAs. Hence, Katchalski's non-aqueous titration method (1953) was used for estimating the purity of NCA-glycine.

0.349 mM of NCA-glycine was dissolved in 5 ml of dioxane and 1 ml of thymol blue (0.5 gm per 100 ml methanol) as an indicator was added, and the solution was titrated to a blue end point with 0.097 N standardized sodium methylate in methanol solution. 5 ml dioxane without NCA-glycine was used as a titration blank. The results are as shown below:

NCA-glycine (mM)	CH_3ONa used - blank (mM)	purity of NCA-glycine (%)
0.349	0.37	105.6
0.349	0.34	97.3
0.349	0.35	100.0

Within experimental error the data show that the NCA-glycine prepared was almost 100 percent pure.

Preparation of Polyglycyl Insulin

Because of the low solubility of Zn-insulin, the soluble chloride salt of insulin was prepared according to the method of Carpenter (1958). Crystalline Zn-Insulin was dissolved in 0.25 N HCl (15 mg/ml) and cooled to 0° C. To this solution was added 18 volumes of cold acetone; the resulting precipitate was collected after standing

overnight at 0°, washed with acetone and then three times with ether. After the insulin salt had been dried in air, it was stored at -10°.

A. Modification of insulin as its dimer.--Zn-free insulin salt was dissolved in phosphate buffer, pH 8.0, ionic strength 0.1, to make a one percent protein solution. Under these conditions, the insulin molecules were dimers as tested by Sephadex technique. NCA-glycine was then added to the solution with stirring at 4°. The molar ratio of NCA to protein was 60 to 1. After reaction for 24 hours the pH of the solution had dropped to 7.6. The solution was poured into cellophane tubing No. 18/32 and dialyzed against distilled water for two days. It was then centrifuged at 13,000 x g for one hour and the supernatant was retained for further experiments.

B. Modification of monomeric insulin.--In this procedure a 0.5 percent protein solution was made by dissolving the Zn-free insulin salt in 0.1 M bicarbonate buffer, pH 9.2. The insulin existed as monomers under these conditions. NCA-glycine was added to the solution with stirring to a final molar ratio of NCA to protein of 60 to 1. Immediately after the addition of NCA-glycine, the pH of the solution dropped from 9.2 but was adjusted back to 9.2 with N NaOH. The mixture was then stirred at 4° for 24 hours in order to complete the modification reaction. Further dialysis and centrifugation were carried out as in procedure A.

Dinitrophenylation and Amino Acid Analysis of Polyglycyl-Insulin

To about 1 ml protein (5 mg/ml) was added 100 mg of sodium bicarbonate, followed by the addition of 3.0 ml five percent dinitrofluorobenzene in absolute ethanol. The flask, wrapped with aluminum foil to exclude light, was shaken mechanically at room temperature for six hours, at which time a yellow precipitate was obtained. A few drops of 6 N HCl were added to acidify the yellow suspension, which was then centrifuged in a clinical centrifuge. The yellow precipitate was washed with 95 percent ethanol and then ether to remove unreacted dinitrofluorobenzene and dinitrophenol.

For hydrolysis, 1 ml of 5.7 N HCl was added to 5-6 mg of protein in an ampule which was then alternately evacuated and purged with pure nitrogen several times. The tube was sealed under vacuum and placed in a refluxing toluene bath (110.6^{o}) for 24 hours. After hydrolysis the mixture was evaporated to dryness, and the amino acid content was analyzed by the Spinco Automated Amino Acid Analyzer (Spackman, Stein and Moore, 1958), with accelerated columns. For epsilon-DNP-lysine analysis the improved method of Nishikawa (1966) was used.

Formaldehyde Determination of ε-amino Groups

Insulin and polyglycyl$_{29}$ insulin[2] were dissolved in 6 ml 0.1 N KCl and titrated to pH 9.2 with 0.05 N NaOH. Protein concentration was one percent. After the addition of 4 ml of 35 percent formaldehyde, the protein solution was then titrated back to pH 9.2. In a control experiment, 6 ml of 0.1 N KCl was brought to pH 9.2, 4 ml of formaldehyde added and the solution titrated back to pH 9.2.

Trypsin Digestion of Polyglycyl Insulin

For trypsin digestion a pH stat was used. The pH-stat was set to maintain pH 9.4 at 25°. Polyglycyl$_{29}$ insulin (20 mg in 4 ml of 0.004 M HCl) was placed in the digestion cell of the instrument, the solution brought to pH 9.4, and equilibrated at pH 9.4 for 30 minutes. Trypsin solution (10 mg in 4 ml of 20 mM $CaCl_2$ 0.004 N HCl), adjusted to pH 9.4, was added and the reaction was allowed to proceed for about two hours. After readjusting the pH to 4.8, the digestion mixture was allowed to stand overnight at 4°. The precipitate of desocta-peptide insulin was collected by centrifugation, washed three times with water, two times with acetone and three times with ether. The precipitate was dried in vacuo and hydrolyzed

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[2] Polyglycyl$_{29}$ insulin indicates insulin with 29 glycine residues added.

with 5.7 N HCl at 110° for 24 hours. The amino acid content was analyzed by Beckman Spinco 120 B amino acid analyzer.

Solubility Studies

The solubility of Zn-free insulin and poly-glycyl$_{29}$ insulin in the range pH 2.0-9.0 was determined in the following manner: 0.5 ml of 0.2 mM protein solution was added to 1.0 ml of buffer and KCl was used to adjust the ionic strength to 0.1. The solutions were sealed and equilibrated with constant shaking at 25° for 36 hours. Aliquots of the supernatant solution were removed with fritted glass immersion tubes by allowing the supernatant to diffuse into the tube. 0.25 ml of the supernatant was diluted with 1 ml of 0.01 N HCl, and the optical density was measured at 282 mμ .

Dialysis of Polyglycyl Insulin

The dialysis cell used in these studies was constructed according to the designs of Craig (1957, 1962).

Cellophane tubing (number 18/32) was used, and the solvents were 0.1 N acetic acid and 0.01 N acetic acid. The protein was dissolved in the same solvent that was used for the diffusate. The membrane was then placed on the glass collar and 1 ml of one percent protein solution was then inserted into the dialysis membrane. The stirring motor was started, and the dialysis cell was placed into

the cuvette, which contained 10 ml of solvent. The dialysis was allowed to proceed for 30 minutes, then the diffusate was replaced with the same amount of fresh solvent. After each change of solvent a syringe was used to withdraw and reinject the retentate into the dialysis bag several times to thoroughly mix the solution. Several 10 ml volumes of the diffusates were collected over a period of time and set aside for analysis. At the end of the experiment the retentate was removed from the dialysis membrane and diluted to 10 ml. The diffusate and the retentate were then analyzed for protein content by measuring optical density at 280 mμ.

Sephadex Gel Filtration

Dry Sephadex G-150 (bead form) was suspended in 0.5 percent aqueous NaCl solution and allowed to swell for five days, during which time the fine particles were decanted. A glass column of 18 mm diameter was filled with gel to a height of 40 cm (at equilibrium). Following the initial packing, a disk of Whatman No. 1 filter paper was applied to the top of the gel and the column material was allowed to settle for two days at 4° under flow of buffer.

The G-75 column was prepared according to the description given in the Pharmacia booklet, No. 2 "Theory and Experimental Technique." The void volume was determined by using a 0.1 percent solution of blue-dextran (molecular weight 1×10^{6}).

The protein sample was dissolved in 1 ml of the appropriate buffer and applied to the column. The sample was allowed to pass into the gel, and the gel was washed by addition of 3 ml of buffer. After the latter volume had passed into the gel an overlaying buffer was added, a reservoir attached, and elution allowed to proceed under hydrostatic pressure. The flow rate was 6 ml per hour and fractions of 2.0 ml were collected by means of an automatic fraction collector. The optical density of the fractions collected was measured at 280 mμ using a Beckman DB spectrophotometer.

RESULTS AND DISCUSSION

Preparation and Analysis of Polyglycyl-Insulin

The number of glycine residues added to the insulin molecule after the NCA-glycine modification can be obtained by first completely hydrolyzing the modified insulin and then determining the quantities of amino acids with the amino acid analyzer. Subtraction of four glycine residues, the number present in native insulin, from the total number found after hydrolysis of the glycine modified insulin gives the number of glycine residues added to the native insulin during modification.

Three groups in insulin can react with NCA-glycine. Two of them are the N-terminal amino groups (glycine and phenylalanine), and the third is the epsilon-amino group of lysine in the B-chain. When insulin is treated with FDNB all these groups, in addition to others, would form DNP-derivatives. On complete hydrolysis these three DNP-derivative amino acids would not appear as free amino acids and thus three less amino acids would be found on total amino acid analysis. On the other hand, when insulin was modified with NCA-glycine first, then treated with FDNB reagent, these three would be recovered as free amino acids. In the latter successive reactions three glycines of the total modified insulin formed

DNP-derivatives, which could be interpreted as the result of the reaction of three glycines at the N-terminal of added glycine peptide chains with the FDNB reagent. Therefore, simply by analyzing the amino acids which formed DNP-derivatives on reaction of native insulin with FDNB and similarly analyzing modified insulin after FDNB treatment the degree as well as the number of sites of the NCA-acylation can be determined. By dividing the total added glycines in the modified insulin by the number of reaction sites just mentioned, the average length of the added glycine peptide chains can be obtained.

The results shown in Table 1 indicate that one glycine, one phenyalanine and from 0.4 to 0.9 of the lysine of the native insulin were reacted with FDNB reagent. After hydrolysis, one fourth of the total glycine and two thirds of the total phenylalanine could still be found after hydrolysis, but almost no free lysine existed after the FDNB treatment. After NCA-glycine modification the total number of glycine residues in the insulin molecule were increased. After reaction of the derivative with FDNB and hydrolysis of the DNP-modified insulin, the number of glycine residues was decreased from 3 to 6 moles/moles. The lysine analysis were considered more reliable, and these data were used in the calculation of the average chain lengths. Phenylalanine was completely recovered within the experimental error when the modified insulin was treated with FDNB.

Table 1. Amino Acid Analysis of Insulin and Insulin Derivatives

	No.	Sample	Glycine	Phenyl-alainine	Lysine	DNP-N-terminal res.	Glycine residues added	Average chain length
Native Control Insulin		Zn-insulin	4. 1	3. 1	1. 0			
		DNP-insulin	3. 2	2. 0	0. 1	DNP-gly. DNP-phe	---	---
Modified insulin (polyglycyl-insulin)	I	PG-insulin	32. 0	2. 8	0. 9	---	28	11. 7
		DNP-PG-insulin	27. 4	3. 0	0. 4	DNP-gly	---	---
	II	PG-insulin	33. 5	2. 9	1. 0	---	29	10. 6
		DNP-PG-insulin	29. 7	2. 9	0. 7	DNP-gly	---	---
	III	PG-insulin	8. 3	2. 8	0. 9	---	8.	2. 8
		DNP-PG-insulin	5. 4	2. 6	0. 8	DNP-gly	---	---
	IV	PG-insulin	23. 9	2. 8	1. 0	---	20	6. 9
		DNP-PG-insulin	18. 1	2. 6	0. 9	DNP-gly	---	---

This is clearly indicated that phenylalanine was completely reacted with NCA-glycine.

Since some DNP-lysine is destroyed during hydrolysis, the epsilon-amino groups were determined independently by formaldehyde titration. As is shown in Table II, about 20 percent of the epsilon amino groups were available for titration, that is, 80 percent of the lysine residues in insulin were acylated. By amino acid analysis, 70 percent were found to be acylated in this derivative.

Table 2. Formaldehyde titration of lysine epsilon-amino group in insulin and polyglycyl-insulin

	NaOH - NaOH control (mole)	Protein conc. (mole)	ϵ-amino acid group titrated %
Insulin	0.460×10^{-2}	0.465×10^{-2}	98.9
Polyglycyl$_{29}$ insulin	0.159×10^{-2}	0.812×10^{-2}	19.0

It appears from the results shown in Table 3 that at either higher protein concentration or higher molar ratios of NCA to protein more highly modified derivatives are obtained. At pH 9.2 the light modification of the protein must be due to hydrolysis of NCA-glycine at this relatively high pH.

From a time course study of the hydrolysis of DNP-polyglycyl-insulin, it was revealed that hydrolysis was complete in 24 hours.

The result is shown in Table 4. It differed from polyvalyl-insulin which was resistant to acid hydrolysis as reported by Stracher, et al. (1956).

Table 3. Effect of insulin concentration and the molar ratio of NCA-glycine to insulin on degree of modification.

Solvent	pH	protein conc. (%)	NCA/insulin molar ratio	glycine residues added
0.1 M bicarbonate solution	8.0	1	60	31
"	8.0	1	30	21
"	9.2	0.5	60	4
40% dioxane in 0.067 M phosphate buffer	7.0	1	60	15
"	7.0	1	30	8

Table 4. Effect of time on the hydrolysis of insulin derivatives.

Sample	time of hydrolysis (hours)	glycine residues per Mole	phenylalanine residues per Mole
Polyglycyl insulin	24	32.0	2.8
DNP-polyglycyl-insulin	12	25.7	2.2
"	24	27.4	3.0
"	36	27.6	2.7

Trypsin Digestion of Polyglycyl Insulin

According to the Sanger structure of insulin, trypsin should cleave the molecule at two points to give alanine, a heptapeptide (gly-phe-phe-try-thr-pro-lys) and the desoctapeptide-insulin. However, Hunter and Ludwig (1962) found that by amidination of the lysine residue next to the C-terminal of the B-chain, release of alanine with trypsin digestion was no longer possible. In the present study the same lysine residue on the B-chain was linked to a long glycine peptide chain which resulted from the modification. It was found that after such modification, the polyglycyl$_{29}$ insulin was not digested by trypsin under the same condition that 80 percent of the native insulin could be digested. It is possible, therefore, that steric hindrance produced by the glycine peptide chain attached to lysine blocked the approach of trypsin at the arginine site and cleavage at either point (lysine and arginine) was prevented. Since 20 percent of the lysine in this preparation was modified, one might expect 20 percent cleavage. However, under these conditions, the molecule is dimeric, and hydrolysis may be prevented for this reason.

Solubility of Zn-free Insulin and Polyglycyl-Insulin

The solubility of glycine modified insulin was higher than that of non-modified insulin in the pH range of 2.0 to 8.0 in 0.1 ionic

strength buffer (Figure 1). This result may be due to the increased solvation of the proteins resulting from the addition of polyglycyl chains. The validity of this reasoning gains support from the following observations. If one looks at the soluble fraction obtained when polyglycyl insulin is prepared in 40 percent dioxane, it is found that a maximum of 13 glycine residues have been added. But if polyglycyl insulin is prepared in aqueous buffered solutions, the soluble fraction contains a derivative with about 35 glycine residues added.

Polyglycine itself is one of the least soluble polypeptides dissolving only in strongly interacting solvents such as trifluoroacetic and dichloroacetics acids or in concentrated aqueous lithium and calcium halide solutions. Becker (1953) reported that only 15 percent of the products obtained from the polymerization of NCA-glycine in aqueous buffered solution were soluble. In addition, polyglycyl peptides with chain lengths greater than ten were insoluble. As mentioned above, polyglycyl insulin with more than about 35 glycine residues added is insoluble in the aqueous buffer solutions used in this work. A possible explanation to account for this phenomenon is the following: When an average glycine chain length of greater than 12 is present, the polyglycyl peptide chains are long enough to allow formation of hydrogen bonding of one chain to the other either intra- or inter-molecularly. Thus, an insoluble

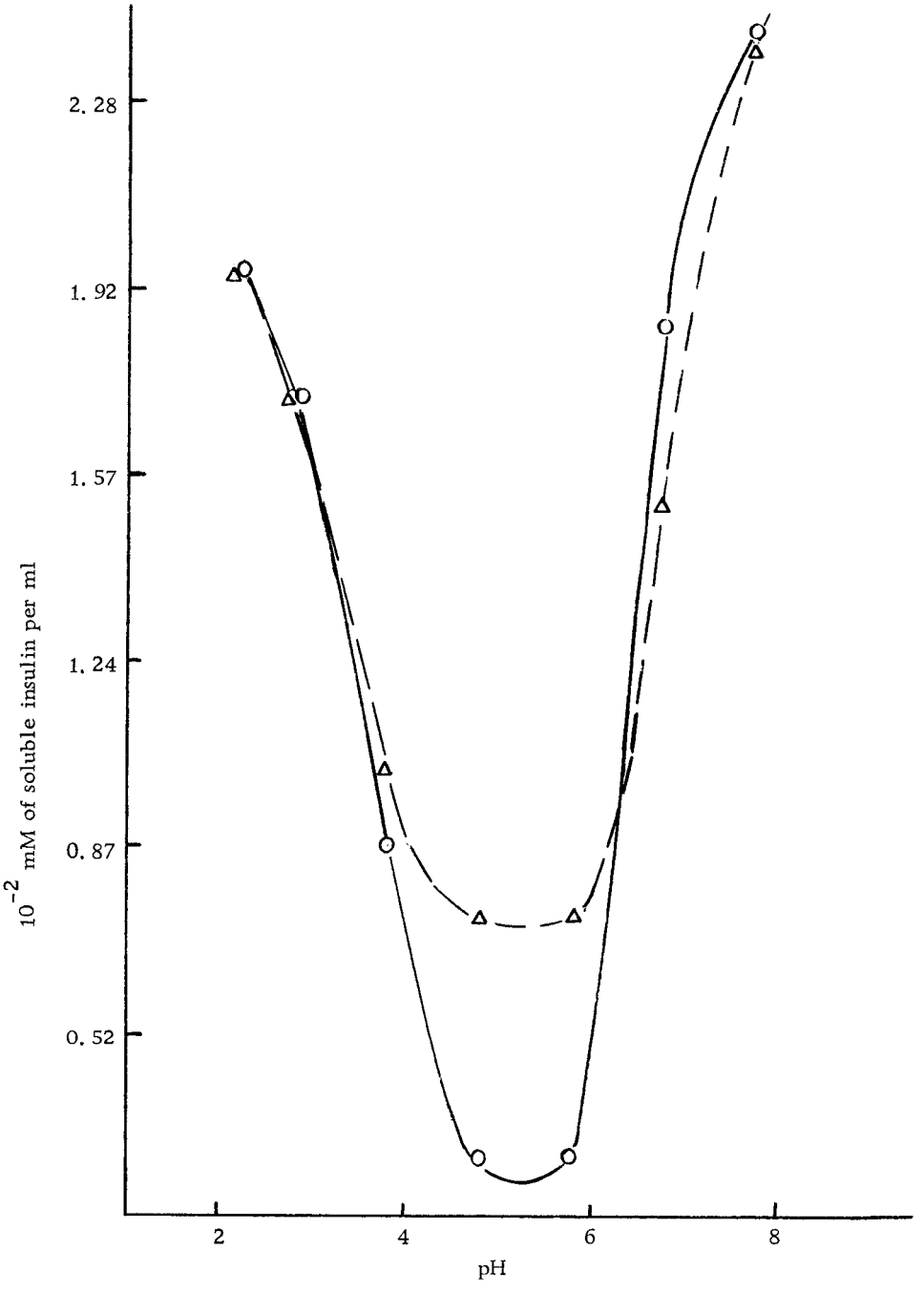


Figure 1. Effect of pH on the solubility of Zn-free insulin and polyglycyl-insulin. μ = 0.1, temperature 24-25°
Δ polyglycyl insulin, O Zn-free insulin

derivative is formed as evidenced by the formation of a white precipitate.

Dialysis Studies of Polyglycyl Insulin

The quantitative dialysis technique with cellophane membranes offers a very useful and simple means for detecting variations in protein shape and size. Dialysis is a diffusion process, where the diffusion rate depends on the concentration gradient across the membrane. In order to separate molecules of similar size, it is important to select membranes of the proper pore size. When the dialysis membrane has pore sizes larger than all the molecules of the solutes, the escape rates of all the molecules would only relate to their individual rate of free diffusion. On the other hand, when the pore sizes are reduced to the molecular dimensions of the small molecule, then only the small molecules can pass through and the rest would be greatly retarded. The highest selectivity as far as the porosity of the membrane is concerned will be obtained when the pores are of such size that they will barely permit the smallest solute to pass. However such high selectivity will result in a very slow process. One of the best ways to speed up such a process is to increase the maximum membrane area for a given retentate volume. That is the technique of thin film dialysis which is used in the present experiment (Craig, 1962).

It was pointed out by Craig and King (1962) that escape patterns can be classified as a straight line, a continuously curving line, a line with a more or less sharp break, etc. A single solute behaving ideally will give a straight line. In the case of continuous curving line, it may indicate that the solute contains a distribution of molecular sizes; if the curvature is positive, it could be the result of association or other deviation from ideality that is concentration dependent; if the curvature is negative, then diffusion becomes more rapid with dilution, which is to be expected with the dissociation of dimer, trimer, etc. A mixture of two molecular sizes in solution would dialyze at different rates giving two straight lines of different slope with a break between them. The 50 percent escape time, that is the time necessary for half of the solutes to pass through the membrane, is used by Craig as a measure of size and shape.

Zn-insulin shows a straight line over a considerable concentration range. A break in the curve appeared only when the percentage remaining approached 20 percent (Figure 2, Curve B). According to Craig and King (1962), these results arise from dissociation of the dimer at the lower concentration. The polyglycyl insulin curve shows a distribution of molecular size owing to the various length of polyglycyl peptide chains attached. Compared to native insulin, the 50 percent escape time (5.8 hours) of the modified insulin was too high to be related only to the increase of the molecular size by the addition

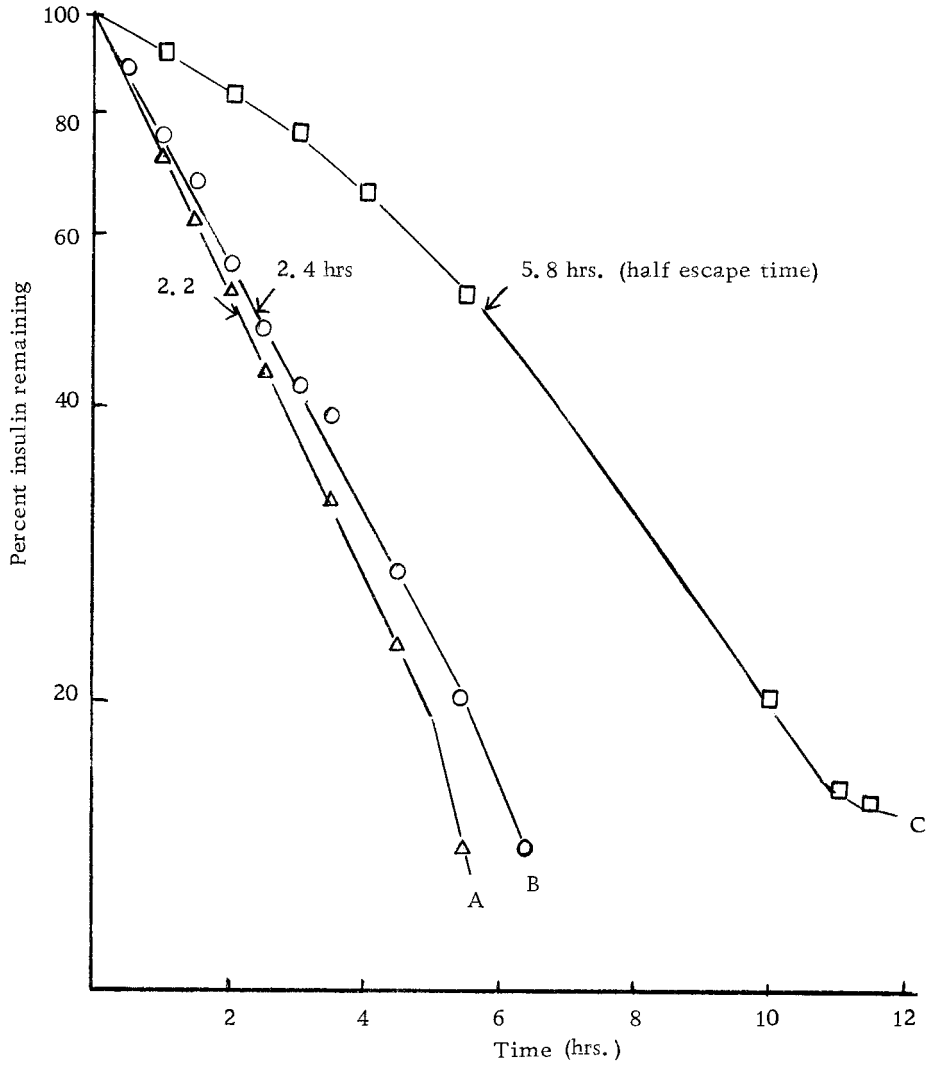


Figure 2. Comparative escape rates of insulin and polyglycyl insulin through dialysis membrane.

Curve A. Zn-Insulin, solvent 0.2 N HoAc
Curve B. Zn-Insulin, solvent 0.1 N HoAc
Curve C. Polyglycyl$_{29}$ insulin, solvent 0.2 N HoAc, pH 2.77

of the glycine peptide chains. Since shape of the molecules also affect the escape rate through the membrane, the result of the high 50 percent escape time probably was due to the deviation in shape of the modified insulin. The added glycine peptide might protrude from the insulin molecule causing the escape rate to be much slower than the native one.

Sephadex Gel Filtration of Polyglycyl-Insulin

The determination of the molecular weight of proteins by means of filtration on cross-linked dextran gels has been very successful. Sephadex gel filtration is a fractionation method which utilizes the sieving properties of a bed of porous gel granules. During passage through the bed, solutes which are small enough to penetrate the porous gel are slowed down relative to large molecules which are unable to enter the pores. Thus large molecules, which can only move in the liquid outside the gel granules are eluted first. Smaller molecules, which, penetrate into the gel particles to a varying extent depending on their size are eluted later. Whitaker (1963) obtained an excellent linear correlation between the logarithm of the molecular weight of a protein and the ratio of its elution volume, V, to the column void volume, V_o.

For making a standard curve, five proteins covering a molecular weight range of 13,600 to 68,000, gave a linear plot of V/V_o

against the logarithm molecular weight, as shown in Figure 3. Lysozyme was eluted much later than expected. The explanation is that it forms a weak complex with the dextran at the center of the enzyme, that is the dextran serves as substrate or inhibitor for the enzyme (Whitaker, 1963).

In order to find out the behavior of various modified insulins the zinc-free insulin (native insulin) was modified under the conditions where it existed as monomers or as dimers. The modified insulins were then passed through the Sephadex column. After the determination of V_o and V_e, the molecular weights of the eluted poly-glycyl insulins were calculated from the standard curve. The results provide information as to the association or dissociation of the modified insulin under different controlled elution conditions. The results are shown in Table 5.

Zn-free insulin indeed exists as monomer both in pH 8-9 buffer and in 40 percent dioxane (Fredericq, 1956). When the Zn-free insulin was dissolved in pH 7.7 buffer, it associated to its dimer form; if it was dissolved in pH 3.5-7.0 buffers, higher aggregates of the insulin molecules resulted. Modification of the insulin in its monomeric form was found not to change the association or dissociation behavior under the various conditions just described. However, when the insulin was modified while in its dimer form, the modified insulin remained as a dimer even when dissolved in pH 8-9 buffers

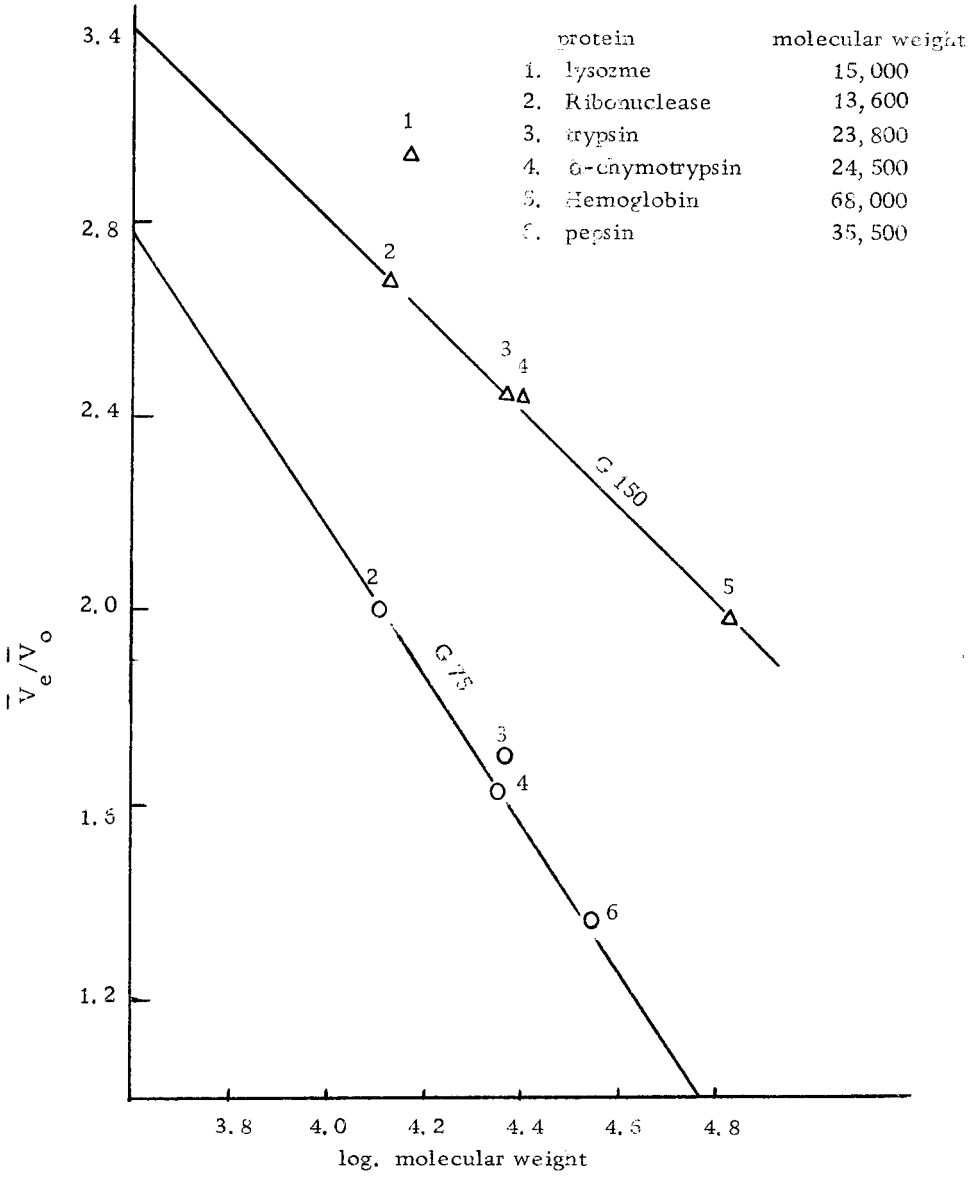


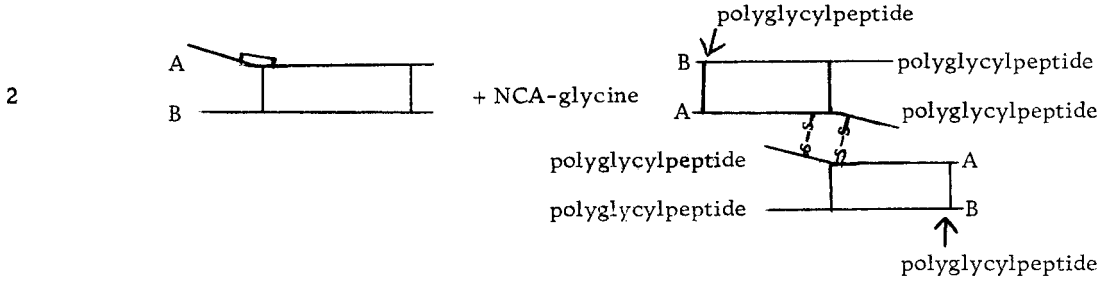
Figure 3. Relationship between elution volume and molecular weight of proteins on G-150 Sephadex. At 4° C column 1.8 x 40 cm, flow rate 6 ml per hour. 10 mg of each protein were used. G-75 standard curve from Whitaker (1963).

Table 5. Gel filtration of insulin and polyglycyl-insulin on Sephadex

Sephadex	Sample	modification condition native insulin exists as	Elution Condition		V/V_o	Molecular weight
			Solvent	pH		
G-75	PG_{29}-insulin & insulin	dimer	0.01 N HCl	2	2.07	1.6×10^4
	PG_{29}-insulin & insulin	dimer	40% dioxane in 0.01 N HCl		2.07	1.6×10^4
	PG_8-insulin & insulin	monomer	0.01 N HCl	2	2.14	1.1×10^4
	PG_8-insulin & insulin	monomer	40% dioxane in 0.01 N HCl		2.28	6.3×10^3
	PG_{29}-insulin	dimer	0.2 N NH_4COOH-0.2 N HoAc	4.2	1.26	3.0×10^4
	Zn-free insulin		0.2 N NH_4COOH-0.2 N HoAc	4.2	1.29	2.4×10^4
G-150	Zn-free insulin		0.1 M bicarbonte	9.2	3.02	6.0×10^3
	Zn-free insulin		phosphate buffer, $\mu = 0.1$	7.5	2.71	1.2×10^4
	PG_4-insulin	monomer	0.1 M bicarbonte	9.2	2.93	7.4×10^3
	PG_4-insulin	monomer	phosphate buffer, $\mu = 0.1$	7.5	2.68	1.3×10^4
	PG_{28}-insulin	dimer	tris-buffer, $\mu = 0.1$	8.4	2.72	1.2×10^4
	PG_{28}-insulin	dimer	0.1 M bicarbonate	9.2	2.75	1.1×10^4
	PG_{32}-insulin	monomer	0.1 M bicarbonate	9.2	2.92	7.6×10^3
	PG_{32}-insulin	monomer	phosphate buffer, $\mu = 0.1$	7.5	2.70	1.3×10^4

or in 40 percent dioxane (Table 5). In pH 3.5 to 7.0 buffers, the modified insulin solution showed a great deal of turbidity, indicating that higher aggregates were produced.

It has been well known that the polymerization of insulin in aqueous solution depends on the presence of zinc ion. The association of zinc-free insulin, however, was not well understood. From a study of the reaction of silver ions or cyanide with insulin, Marcker (1960) suggested that dimerization of zinc-free insulin proceed by a disulfide interchange mechanism



as shown in Figure 3. The position of most of this glycine peptides in monomeric insulin does not interfere with the interchange reaction between the two disulfide linkages of the different monomers. Therefore, the association of the modified monomer to its dimer form is not surprising. When the native dimeric insulin was modified, it may be suggested that the added glycine peptides would most probably reinforce the intermolecular disulfide linkage by formation of further hydrogen bonding between the two monomeric insulin

molecules and hold them strongly together. The dissociation of the modified dimeric insulin molecule thus becomes more difficult.

Biological Activity of Polyglycyl Insulin

The hormone activity determination was carried out by Charles R. Park, M.D. of Vanderbilt University using the glucose uptake test in perfused heart. As shown in Table 6, insulin with only 26 glycyl residue added to its N-terminals retain 100 percent of its original activity, but with 39 residues attached, 90 percent activity was lost. Therefore steric hindrance from the added groups may affect the activity of insulin.

Table 6. Hormone activity of polyglycyl insulin

Sample	Activity
Native Insulin	100%
Polyglycyl Insulin (39 res./mol)	10%
Polyglycyl Insulin (26 res./mol)	100%

Fraenkel-Conrat and Fraenkel-Conrat (1950) studied the effect of modification of the various functional groups of insulin on its biological activity. They found that changes in the insulin molecule which affect only the side groups on the structure have little effect on its biological activity. Evans and Saroff (1957) have reported

studies of the modification of insulin with O-methylisourea. After approximately half of the α-amino group of glycine and about 10 percent of the α-amino group of phenylalanine of its monomeric form have been converted to the guanidine group the modified insulin possessed its full hypoglycemic activity. However, when these amino groups were covered with large molecules such as fluorescein isothiocyanate, 68-80 percent of the biological activity was lost (Tietre, 1962). This loss of activity was presumably by steric hindrance.

SUMMARY AND CONCLUSIONS

Polyglycyl insulin with various added glycine peptide chain lengths were produced by reaction of NCA-glycine with insulin. Glycine peptides were attached at three sites in the insulin molecule. For both glycine and phenylalanine at the two N-terminal ends, acylation was hundred percent completed, whereas only 80 percent acylation was measured for the epsilon-lysine group.

By varying the insulin concentration or the molar ratio of NCA-glycine to insulin during the acylation reaction, different chain lengths of the added glycine peptide chain could be obtained. Usually a heavier modification would result either from higher insulin concentration or higher molar ratio of NCA-glycine to insulin during the reaction.

Polyglycyl insulins were more soluble in aqueous solution but were less soluble in organic solvents than native insulin. Modified insulin with more than 40 added glycine residues exhibited a much lower solubility in aqueous solution as compare to insulin containing 20 to 35 glycine residues.

Differing from native insulin, the heavily modified insulin was found resistant to trypsin digestion, but it still retained biological activity.

When the modified insulin was dialyzed against buffer through

dialysis tubing of selected pore size, it appeared that polyglycyl-insulin was heterogeneous based upon the escape rate curves.

The results of gel filtration experiments showed that when insulin was reacted with NCA-glycine under conditions where the insulin molecule was in its monomeric form, the resulting polyglycyl-insulin behaved the same as the native insulin, it followed the same kind of pH dependent aggregation and dissociation reactions as does the native insulin molecule. However when the insulin was modified as its dimer, the modified dimeric insulin molecules became much more stable; it no longer dissociated into its monomeric form as did the native insulin when the environment was changed.

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