Reaction of Insulin with Ethyl Glycinate and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide*

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ABSTRACT: The coupling reaction between insulin and ethyl glycinate using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide has been investigated. A preliminary study, using this carbodiimide, of the reaction between *m*-nitrobenzoic acid and ethyl glycinate as a function of pH showed an increase in reaction velocity with decreasing pH over the range pH 6.75–3.75. Similar pH dependency was observed in the reaction with insulin. The stability of the carbodiimide employed, investigated over a wide range of pH, was minimal in 0.1 N HCl solution. When the coupling reaction of ethyl glycinate with insulin was carried out overnight at pH 4.5, between 5.0 and 5.3 moles of ester was bound per mole of insulin. All six insulin carboxyl groups are thus accessible to the added reagents. It was not possible to find reaction conditions which would restrict the reaction to one specific carboxyl group. The main fraction, however, of the product after reaction at pH 7.0 for 1 hr had only one carboxyl group modified. This preparation was purified and degradation studies were carried out. The results showed that 80% of the reaction had occurred in the B chain at either one or both of the glutamic acid residues and 20% in the A chain, probably at the C-terminal asparagine.

In order to determine the three-dimensional structure of insulin by multiple-isomorphous, single-isomorphous, and/or heavy-atom anomalous dispersion techniques, heavy atoms must be bound to specific sites on the insulin molecule. Problems associated with a loss of isomorphism and disorder effects are frequent in the preparation of orthorhombic heavy-atom insulin derivatives by use of immersion techniques (B. W. Low, private communication). Previous efforts in this insulin study to introduce heavy atoms by chemical modification have largely involved reactions of the amino groups. Hunter and Nozaki (private communication) studied the carbamoylation reactions using *p*-iodophenyl isocyanate and Birnbaum (private communication) investigated the reaction with *p*-iodophenyl isothiocyanate. Zeidenberg and Erlanger (Zeidenberg, 1964) have prepared tri-*p*-iodobenzamido-insulin. These reactions were all carried out at alkaline pH and the reaction products in most instances subsequently fractionated. Orthorhombic insulin crystals (Low and Berger, 1961) studied in this laboratory are grown and are stable in the pH range pH ~2.2–4.5; none of these modification reactions, therefore, could be carried out in the crystal. Unfortunately, the solubilities of most of these derivatives were greatly reduced by the loss of ionizable amino groups and recrystallization procedures were unsuccessful.

Several investigators have reported chemical modification studies in solution designed to provide heavy atom derivatives (Fenselau *et al.*, 1967; Wilcox and Cobain, 1962) and other investigators have subsequently crystallized modified material (*e.g.*, Green *et al.*, 1954; Sigler *et al.*, 1964; and Venkatappa and Steinrauf, 1967). Drenth *et al.* (1968) have reported the *in situ* modification of amino groups in papain crystals.

The reactivity of the carboxyl groups in proteins has not previously been exploited for the introduction of heavy atoms, either by reaction in solution followed by crystallization or by direct reaction in the crystal. A potentially useful reaction, the coupling between carboxyl groups of proteins and some amines, using water-soluble carbodiimides as coupling agent, was first reported by Hoare and Koshland (Hoare and Koshland, 1966). The reaction was studied at pH 4.75 (Hoare and Koshland, 1966, 1967) and at pH 4.80 (Wilchek *et al.*, 1967) with 1-benzyl- and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, respectively. This reaction which proceeds in acid media is of special interest for the study of insulin, a protein which is much more stable under acid than under alkaline conditions (Freudenberg and Münch, 1940; Beychok, 1965). It has the further advantage of usefulness for modification studies within crystals grown and stable at acid pH. Under such *in situ* reaction conditions isomorphous crystalline heavy-atom derivatives might be expected. We were encouraged, therefore, to investigate this reaction in insulin which has six carboxyl groups—Glu (A4), Glu (A17), Asn (A21, C terminal), Glu (B13), Glu (B21), and Ala (B30, C terminal).

Some fundamental information concerning optimal conditions for this reaction was unavailable although Horinishi *et al.* (1968) have reported a reduction in the reaction rate below pH 5.0 using *N*-ethyl-*N'*-morpholinylpropylcarbodiimide. This carbodiimide is unstable at pH 3.0. A study was, therefore, undertaken of the stability of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and of the reaction of insulin with ethyl glycinate using this carbodiimide. Ethyl glycinate was chosen as the model amine both because Hoare and Koshland (1966, 1967) and Carraway and Koshland (1968) used this amine in their studies at pH 4.75 and because the analytical procedure for the determination of ethyl glycinate is

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simple and accurate. Furthermore, this amine is a relatively small molecule, appropriate in size as a model compound for studies of the reactions in the crystal.

Experimental Procedures

Materials and Analytical Methods. Insulin was purchased from Boots Pure Drug Co., Ltd. (batch 26918, 23.7 IU/mg, Zn content 0.439%). Our analysis of this material following carboxypeptidase digestion showed that this preparation contains less than 5% desamido-insulin.

The DEAE-cellulose–8 M urea system has been shown to yield good separation of desamido- from hexamido-insulins (Thompson and O'Connell, 1960). There is evidence that 8 M urea causes some irreversible conformational changes in insulin (Beychok, 1965). The insulin employed in these reactions was, therefore, initially fractionated using a CM-cellulose chromatographic system similar to that of Smith (1964), but using microgranular CM-32 and pH 3.5 citrate buffer as eluent. Unfortunately, with this method the desamido-rich fractions were eluted only a little faster than the native (hexamido) insulin, which led to a low recovery of the latter. Insulin may exist in the aggregated state even at this pH (3.5); a small amount of desamido-insulin might then aggregate with large amounts of the hexamido fraction. Owing to this difficulty, unfractionated commercial insulin preparations were used in this study.

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was obtained from Ott Chemical Co. Carboxypeptidase A (DFP treated) was obtained from Worthington Biochemical Corp. CM-cellulose (microgranular CM-32) was obtained from Whatman. Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) was purchased from Eastman Organic Chemicals. The urea solutions used were passed through a mixed bed resin to free them from cyanate ion.

All spectrophotometric measurements were made using the Beckman DB recording spectrophotometer. A value of $E_{1\text{ cm}}^{0.1\%}$ 1.04 at 276 mμ was used to determine insulin concentration.

Acrylamide gel electrophoresis was carried out at pH 4.50 with a gel prepared by mixing the following solutions (2:1:1:4 volume ratios): acrylamide (40% acrylamide, 0.3% *N,N'*-methylenebisacrylamide); 0.28% *N,N,N',N'*-tetramethylethylenediamine; 1.7 M sodium acetate buffer at pH 4.5; and 0.28% ammonium persulfate. A sample of 10–20 μg was usually applied to the gel.

Amino acid analysis was carried out using the Beckman Spinco amino acid analyzer, Model 120C. The biological activity of an insulin sample was measured by the mouse convulsion test at the Eli Lilly Research Laboratories.

pH Dependence of the Reaction. The coupling reaction between *m*-nitrobenzoic acid and ethyl glycinate using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (hereafter referred to as carbodiimide) was studied. Equal volumes of 3 M ethyl glycinate, 0.3 M carbodiimide, and 5×10^{-3} M *m*-nitrobenzoic acid were mixed and the pH was adjusted to the appropriate value (within the range 3.75–6.75) using either 0.01 M hydrochloric acid or 0.01 M sodium hydroxide. At suitable time intervals, 2.0-ml portions of the reaction mixture were withdrawn and added to 5 ml of 0.5 M sodium carbonate. The ethyl *m*-nitrohippurate which had formed was then extracted immediately with 5 ml of chloroform. The chloroform layer was washed once with 0.5 M sodium carbonate (5 ml) and brought to 20-fold dilution with ethanol. The optical density of the solution was then measured at 255 mμ where the molar extinction coefficient of ethyl *m*-nitrohippurate is 8.26×10^3.

pH-Dependent Stability of Carbodiimide. The decomposition of carbodiimide was followed spectrophotometrically at 212 mμ, its absorption maximum; at this wavelength, the absorption of its urea derivative is very small. Carbodiimide was dissolved in solutions of hydrochloric acid (to give a final pH in the range pH 6.75 to that of 6 N HCl and a final carbodiimide concentration of 10^{-4} M). The pH drifts upward in the course of the reaction; it was readjusted after 1 hr for the solution with an initial pH of 3.75, after 6 hr for the solution with the initial pH of 4.75, and twice daily for solutions of higher pH. The upward drifts observed were about 0.2 to 0.3 pH unit.

Reaction of Insulin with Ethyl Glycinate and Carbodiimide. Insulin and ethyl glycinate were dissolved in 0.01 N hydrochloric acid to final concentrations of 0.6 mg/ml and 0.5 M, respectively. The solution was brought either to pH 4.5 or to pH 7.0 by the addition of 1 N sodium hydroxide. Solid carbodiimide was then added to a final concentration of 0.1 M. The solution was maintained at room temperature for the desired period and excess carbodiimide was decomposed by the addition of 0.5 M acetic acid. The solution was then dialyzed, concentrated under reduced pressure at 40° to about 40 ml,[1] passed through Sephadex G-25 (4 × 50 cm column, with 0.5 M acetic acid as eluent), and finally lyophilized.

Absorbance measurements at 276 mμ of the reaction mixture showed no detectable changes, suggesting that tyrosine groups had reacted little if at all. The procedure (Carraway and Koshland, 1968) for regenerating these groups, using hydroxylamine, was followed nonetheless, to remove any possibility of error arising from that source.

Chromatographic Separation of Ethyl N-Insulinglycinate on CM-Cellulose with 8 M Urea. The initial separation on a CM-cellulose column was carried out with a 0.0–0.2 M sodium chloride gradient. The CM-cellulose was equilibrated with 0.01 M citrate buffer, pH 4.50, containing 8 M urea, packed in a 3 × 52 cm column, and washed again with the same buffer. The modified insulin preparation (300 mg) was dissolved in 5 ml of buffer and placed on the column. A linear gradient elution was carried out, with 800 ml of the initial buffer and 800 ml of buffer containing 0.10 M sodium chloride. No protein came off the column with this gradient; therefore it was followed by a gradient produced by 800 ml of buffer containing 0.10 M sodium chloride and 800 ml of buffer containing 0.20 M sodium chloride. This procedure does not provide a good separation. A different gradient, employing 1 l. of buffer containing 0.05 M sodium chloride and 1 l. of buffer containing 0.15 M sodium chloride, was used for the refractionation of some material from this column and for the fractionation of subsequent samples.

The flow rate was about 18 ml/hr, with 13-ml fractions. Progress of the elution was followed by absorption measurements at 276 mμ. The appropriate fractions were combined, dialyzed, concentrated, and passed through Sephadex G-25.

[1] The insulin concentration was not allowed to rise above about 7.5 mg/ml, so larger final volumes were sometimes necessary.

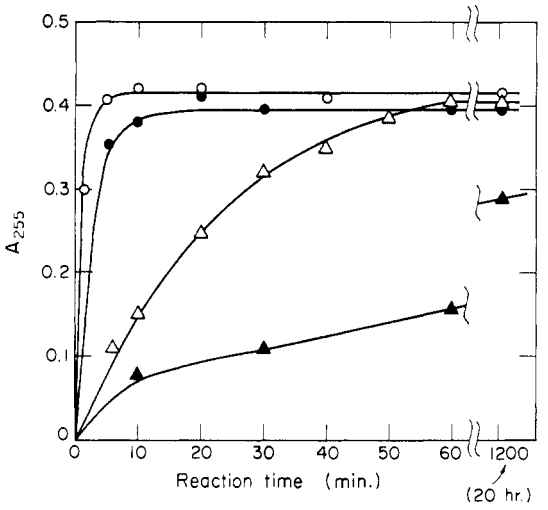


FIGURE 1: The production of ethyl *m*-nitrohippurate (increasing optical density at 255 mμ), plotted as a function of time for the reaction between 5×10^{-3} M *m*-nitrobenzoic acid and ethyl glycinate (1.0 M) using 0.10 M carbodiimide: (a) ▲——▲, reaction at pH 6.75; (b) △——△, reaction at pH 5.75; (c) ●——●, reaction at pH 4.75; (d) ○——○, reaction at pH 3.75.

Carboxypeptidase A Digestion. The CPase A[2] digestion of insulin and of insulin derivatives was carried out at pH 7.4 using the reaction conditions of Slobin and Carpenter (1963a). These investigators showed that the hydrolysis proceeds more rapidly with zinc-free than with zinc-insulin. Insulin was, therefore, converted into zinc-free insulin acetate by passage through Sephadex G-25 before digestion using the procedure of Katsoyannis (P. Katsoyannis, 1966, private communication). CPase A solution was prepared according to the procedures of Ambler (1967) and added to the insulin solution. The ratio of insulin to CPase A used initially was 20 to 1 by weight. For complete digestion, a ratio of 10 to 1 was employed. After incubation at 37° the reaction was stopped by adding an equal volume of 10% trichloroacetic acid. The material was then centrifuged and the supernatant was decanted. The precipitate was washed once with 5% trichloroacetic acid and the wash solution was combined with the supernatant. This solution was used for amino acid analysis.

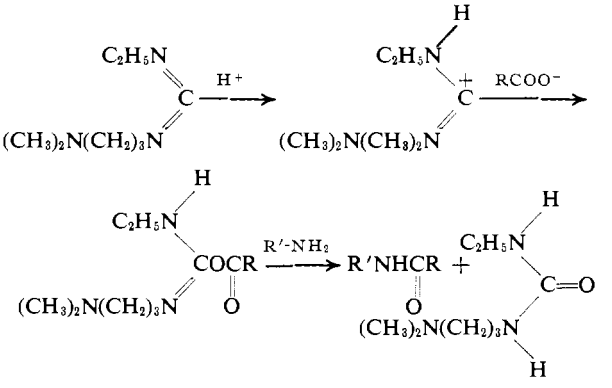
Reduction, Carboxymethylation, and Separation of A and B Chains. The reduction of the disulfide bonds of insulin or modified insulin, and subsequent carboxymethylation, was carried out according to the procedures reported by Crestfield *et al.* (1963). To a 7-ml solution of insulin (2 mg/ml), 4.8 g of urea, 15 mg of EDTA, and 1.7 g of Tris were added. The pH was adjusted to 8.6 by the addition of 6 N hydrochloric acid, and water was added to fill up the 10-ml screw-top test tube. To this solution, 0.1 ml of β-mercaptoethanol was added, and the solution was kept for 4 hr at room temperature.

At the end of the reduction reaction, 0.268 g (1 mmole) of iodoacetic acid in 1.0 ml of 1 N sodium hydroxide was added. The solution was kept for another 30 min and then passed through a column of Sephadex G-25, using 50% acetic acid as solvent. The reduced, carboxymethylated insulin or insulin derivative was lyophilized and stored in the cold.

The separation of the A and B chains of RCM-insulin or RCM-insulin derivatives was carried out by the method of Humbel and Crestfield (1965), using a Dowex 50-X2 column (1.5 × 17 cm, NH_4 cycle). RCM-insulin (about 7 mg) was dissolved in 1.0 ml of 0.2 M ammonium acetate buffer containing 8 M urea and placed on the column. The column was washed with 20 ml of the buffer solution, which eluted the RCM-A chain. The chain was then washed with 10 ml of 0.2 M ammonium acetate buffer (without urea), and finally with 1 N ammonia, which eluted the RCM-B chain. The RCM-A and RCM-B chains were isolated by passage through a column of Sephadex G-25, using 50% acetic acid as the eluent, and lyophilized.

Results

Characteristics of the Coupling Reaction. The pH dependence of the coupling reaction between *m*-nitrobenzoic acid and ethyl glycinate using carbodiimide as the coupling reagent is shown in Figure 1. The reaction is faster when the pH is lower. The first-order reaction constant was about 2×10^{-2} sec^{-1} at pH 3.75 and 1×10^{-4} sec^{-1} at pH 6.75. The reaction is almost complete within 5 min when the pH is lower than 4.75. The reaction velocity was not affected when the ethyl glycinate concentration was reduced from 1.0 M to 0.5 M. The high pH dependence of the reaction indicated that the protonation step of the carbodiimide (Khorana, 1952) is essential for initiation of the reaction.



The decomposition of carbodiimide as a function of pH is shown in Figure 2. Carbodiimide is fairly stable above pH 5.75, but becomes less stable in the lower pH range; thus at pH 3.75, 80% is decomposed within 1 hr. The decomposition was most rapid in 0.1 M hydrochloric acid but as the pH decreases further the rate of the decomposition is reduced again. The initial absorbance at 212 mμ increased as the acidity of the medium in which the carbodiimide was dissolved was increased (Figure 2). At the same time, the shape of the spectrum changed, so that in 6 N hydrochloric acid no peak was observed near 212 mμ (Figure 3, spectrum 3). The latter curve, which may represent the protonated intermediate, did not change significantly during the course of an hour; this suggests that the intermediate is quite stable,

[2] Abbreviations used are: CPase A, carboxypeptidase A; RCM, reduced, carboxymethylated.

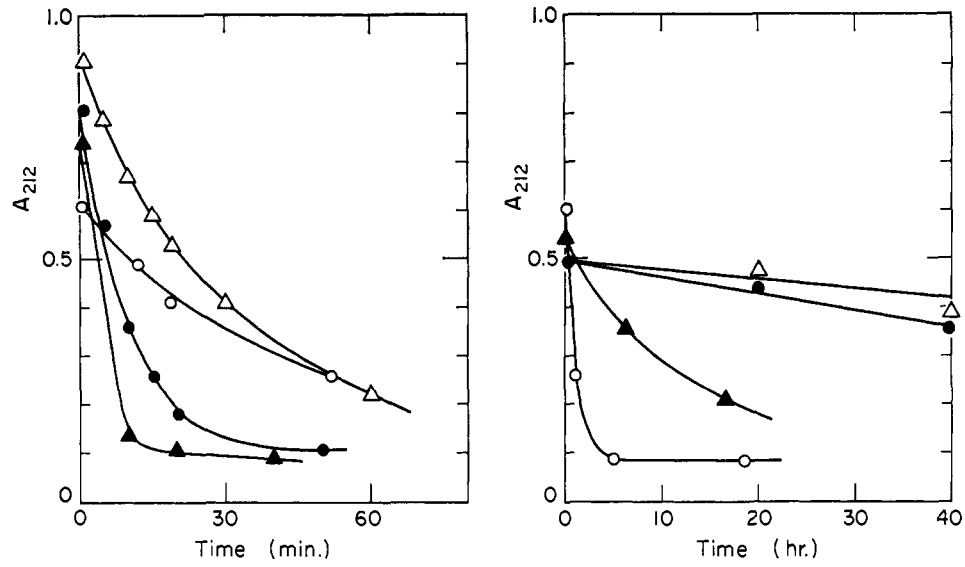


FIGURE 2: Stability of carbodiimide under acidic conditions. Carbodiimide was dissolved (10^{-4} M) in various concentrations of hydrochloric acid, and the decreases in absorption at 212 mμ were plotted as a function of time: (a–d) the short-range (min) stability in highly acidic solutions is shown; (e–h) the long-range (hr) stability in weakly acidic solutions is shown; (a) ○—○, dissolved at pH 3.75; (b) ▲—▲, dissolved in 0.1 N HCl; (c) ●—●, dissolved in 0.5 N HCl; (d) Δ—Δ, dissolved in 3 N HCl; (e) ○—○, dissolved at pH 3.75; (f) ▲—▲, dissolved at pH 4.70; (g) ●—●, dissolved at pH 5.75; (h) Δ—Δ, dissolved at pH 6.80.

due to the virtual absence of any hydroxide ion or carboxylate anion at very low pH.

From the results shown in Figures 1 and 2, the earlier choice of pH 4.75–4.80 for this reaction is seen to be quite reasonable, but it is also possible to use pH 3.5, at which pH carbodiimide is not so stable but the coupling reaction itself is faster. For the purpose of modifying selected carboxyl groups, the use of higher pH (near neutrality) might also be promising.

Reaction of Insulin with Ethyl Glycinate and Carbodiimide. In order to minimize aggregation and thus to reduce the possibility for insulin intermolecular cross-linking, the reaction of insulin with ethyl glycinate and carbodiimide was carried out at low insulin concentrations (600 μg/ml). The extent of the reaction could be determined semiquantitatively by acrylamide gel electrophoresis.

The reaction can be controlled by pH; it proceeds faster at lower pH. After an overnight reaction at pH 4.5, the main electrophoretic band was the fastest moving one. The increase in glycine content of this band was 5.0–5.3 moles per mole of insulin. The biological activity of this preparation was less than 0.1 IU/mg.

The electrophoretic pattern after reaction at pH 7.0 for 1 hr showed a main band corresponding to one carboxyl group reacted. The preparation as a whole contained 1.7 moles of additional glycine per mole of insulin on the average (1.42 moles, 1.75 moles, and 2.0 moles for separate experiments, respectively).

Chromatography of Ethyl Glycinate Derivatives of Insulin. The products of the reaction at pH 7.0 were fractionated by chromatography on CM-cellulose as described earlier. The elution pattern for a separation using only the improved procedure is shown in Figure 4. The total recovery of protein from the column was 97% in that case, and the peaks contained 12% (peak 1), 28% (peak 2), 25% (peak 3), 21% (peak 4), and 11% (after peak 4) of the total, respectively.

All the analytical studies reported here were carried ou with fractions from material containing 1.42 moles of additional glycine, which was initially chromatographed using a 0.00–0.20 M sodium chloride gradient. Acrylamide electrophoresis of the four main fractions before rechromatography is represented in Figure 5. Most of peak 1 has the same mobility as native insulin, whereas peaks 2, 3, and 4 have higher mobilities. All the fractions of peaks 1 and 2 were combined, dialyzed, concentrated, and finally desalted by passage through Sephadex G-25. This material was rechromatographed, using a 0.5–0.15 M sodium chloride gradient, and gave three peaks. The first was presumably insulin,

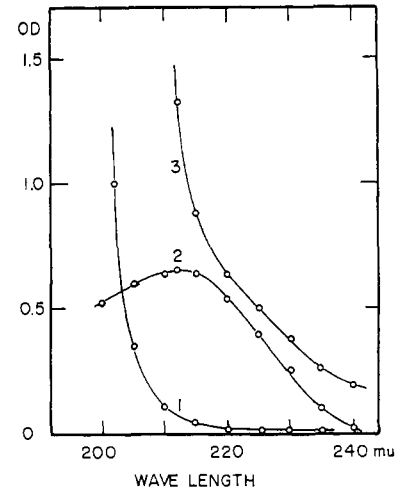


FIGURE 3: Absorption spectra of carbodiimide and its derivatives. Carbodiimide at 10^{-4} M was dissolved in water (curve 2) and in 6 N hydrochloric acid (curve 3). *N*-Ethyl-*N'*-dimethylaminopropylurea (curve 1) was dissolved in water at 10^{-4} M.

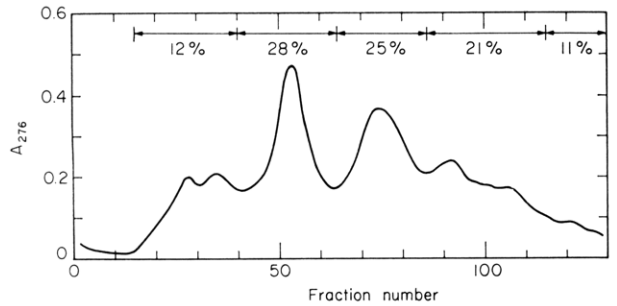


FIGURE 4: Chromatogram of ethyl glycinate modified insulin. Ethyl glycinate insulin (300 mg) was placed on a column of CM-cellulose (3.0 × 52 cm), and eluted with 0.01 M citrate buffer at pH 4.50 containing 8 M urea. Sodium chloride concentration was increased linearly from 0.05 M to 0.15 M during 2 l. of elution. Elution curve was drawn by measuring optical density at 276 mμ.

the second was small and poorly separated from the first, and the third was larger and well separated from the second.

Amino acid analyses of the second (2A) and third (2B) peaks revealed enhanced glycine contents of 1.10 mole/mole of insulin and 0.92 mole/mole of insulin, respectively, indicating that only one carboxyl group had been modified in each case. The biological activity of peak 2B was 17.7 IU/mg.

Carboxypeptidase A Digestion of Peak 2B and Insulin. To confirm whether or to what extent C-terminal asparagine and alanine were modified by reaction with ethyl glycinate and carbodiimide CPase A digestion was carried out for both peak 2B and insulin acetate. Slobin and Carpenter (1963a) have reported the precise reaction conditions under which both asparagine and alanine can be quantitatively released from insulin acetate. The results of CPase A digestion, using their reaction conditions, are summarized in Table I.

Dicyclohexylcarbodiimide is known to cause racemization of the C-terminal group of proteins (Weygand *et al.*, 1963; Goodman and McGahren, 1967). The finding that approximately 20% of the asparagine was not released by carboxypeptidase A after the modification of insulin by ethyl glycinate and carbodiimide might reflect such an occurrence in the presence of carbodiimide. However, the short reaction time (1 hr) and the low carbodiimide concentration (relative to that of ethyl glycinate) make racemization rather unlikely

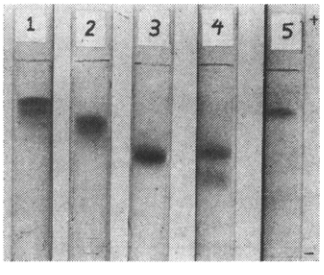


FIGURE 5: Photograph of polyacrylamide gel electrophoresis of ethyl glycinate modified insulin after separation on CM-cellulose. Samples (10 μg) were placed on top of the gel (pH 4.5) and electrophoresis was carried out for 90 min at 3 mA/tube (top side as anode). The gel was stained with Amino Black 10-B: (1) fraction 135; (2) fraction 139; (3) fraction 147; (4) fraction 152; (5) insulin.

TABLE I[a]

	CPase A, w/w, %	Hydrolysis Time (hr)	Ala (μmole)	Asp-NH_2 (μmole)	Asp (μmole)
Insulin	5	4	0.0590	0.0407	
	5	8	0.0580	0.0476	
	10	10	0.0585	0.0541	0.0017
Peak 2B	5	4	0.0657	0.0257	
	5	8	0.0595	0.0324	
	10	10	0.0596	0.0435	0.0035

[a] These results show that the expected amount of alanine residue was released from both insulin and peak 2 after 4-hr hydrolysis, indicating that the C-terminal alanine residue of the B chain was not modified at all during the reaction. The release of C-terminal asparagine from the A chain of peak 2B was much slower than from insulin, and under conditions where the release is complete for insulin (95.4%), only 81% of the asparagine plus aspartic acid was released.

in this case. The 20% reaction in the A chain probably, therefore, occurred at the C-terminal asparagine.

Studies on Separated A and B Chains of Peak 2. The reduction of the disulfide bonds and the subsequent carboxymethylation and separation of the A and B chains of the peak 2 fraction proceeded nicely, as described in the experimental procedures.

The results of amino acid analysis were as expected for the RCM-A and RCM-B chain, except for glycine. The RCM-A chain of peak 2 contained 1.18 moles of glycine (an excess of 0.18 mole of glycine/mole of A chain), indicating that about 20% of the reaction with ethyl glycinate occurred in the A chain. Previous experiments with CPase A digestion showed that 19% of the asparagine could not be released. These two results could suggest that the most reactive carboxyl group in the A chain is that of the C-terminal asparagine, the two glutamic acid residues (A4 and A17) being less reactive.

The RCM-B chain of peak 2 contained 3.78 moles of glycine, that is, 0.78 mole of extra glycine/mole of B chain. The CPase A digestion showed that the C-terminal alanine is completely free in the peak 2 fraction, so almost 80% of the reaction with ethyl glycinate must have occurred at either one or both of the two glutamic acid residues (B13 and B21) of the B chain.

Discussion

In conclusion, this study has shown that all of the carboxyl groups of insulin can be made to react with ethyl glycinate and a water-soluble carbodiimide, under appropriate conditions. The reactivities of all the groups are not the same, however, and some specificity can be introduced at higher pH and shorter reaction times. The most reactive groups are one or both of the glutamic acid residues in the B chain.

It is interesting to note that the peak 2B fraction had

a biological activity of 17.7 IU/mg, 75% of the activity of native insulin. It has been reported that removal of the C-terminal (A21) asparagine residue by CPase A digestion causes nearly complete inactivation of insulin (Slobin and Carpenter, 1963a), but that removal of the C-terminal (B30) alanine does not (Slobin and Carpenter, 1963b; Nicol, 1960; Harris and Li, 1952). If the carboxyl group of asparagine has been modified this suggests that it is the carboxyl group of the C-terminal asparagine, and not the whole asparagine residue which is essential for insulin activity. The importance of some of the carboxyl groups for insulin activity has also been reported by Carr *et al.* (1929) and Landauer and Lang (1946), who observed that insulin loses its activity by esterification with ethanol and hydrochloric acid, and regains it by subsequent saponification.

Acknowledgments

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