Insulin Methyl Ester. Specific Cleavage of a Peptide Chain Resulting from a Nitrogen to Oxygen Acyl Shift at a Threonine Residue*

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ABSTRACT: Zinc insulin reacted with anhydrous methanol–hydrogen chloride to yield two products (insulin methyl ester A and B in the ratio of 40:60) in which all six carboxyl groups were esterified. The two esters differed in net charge as well as in aggregation properties and conformation as shown by ion-exchange chromatography, Sephadex gel filtration, and optical rotatory dispersion measurements. The more positively charged ester (insulin methyl ester B) was irreversibly converted into insulin methyl ester A at pH values above 2.2; however, this reaction was inhibited in the presence of 7 M urea at pH 4.75. Deamination of each ester took place at the two α-amino groups of the N terminals and the ϵ-amino of lysine^{B29}. In addition, insulin ester B was also deaminated at threonine^{B27}, indicating a nitrogen to oxygen acyl shift had occurred at this residue during the esterification procedure. This hypothesis was further corroborated by the selective cleavage of the deaminated insulin ester B at the threonine position by mild base hydrolysis. The site of base cleavage was ascertained with the use of carboxypeptidase A to effect liberation of the newly exposed amino acid. At pH values above 2.2, insulin ester B underwent the reverse O to N shift, to afford a product with no intrachain ester bonds.

There have been many studies concerning the relationship of structure to the biological function of insulin. Attempts have been made to identify by chemical and enzymatic modification techniques the amino acid(s) and/or particular conformational state which might play an essential role in the manifestation of biological activity in the biosystem (Klostermeyer and Humble, 1966; Carpenter, 1966; Levy and Carpenter, 1967).

Several reagents have been used to effect the modification of the carboxyl groups in protein molecules; the reactions differ considerably in conditions and specificity of attack. Esterification has been effected with methanol and HCl (Fraenkel-Conrat and Olcott, 1945). Reactions with various diazo derivatives (Chibnall *et al.*, 1958; Delpierre and Fruton, 1965) as well as with several carbodiimide reagents (Riehm and Scheraga, 1965, 1966; Hoare and Koshland, 1966, 1967; Wilchek *et al.*, 1967) have been reported. Isoxazolium salts (Bodlaender *et al.*, 1968) and triethyloxonium tetrafluoroborate (Parsons *et al.*, 1969) have also been used in the modification of protein carboxyl groups.

The reaction of the carboxyl groups of insulin with various reagents has been studied (Mommaerts and Neurath, 1950; Hoare and Koshland, 1967). Mommaerts and Neurath (1950) reported that esterification in methanolic hydrogen chloride of approximately four of the six available carboxyl groups had little effect on the biological activity of the hormone; however, when total esterification was effected, complete inactivation of the hormone was observed. The activity was only partially regenerated upon mild saponification. This evidence indicated that some of the free carboxyl groups, or a particular conformational state, which may have been destroyed upon esterification, was required for the manifestation of biological activity. Also, Chibnall *et al.* (1958) reported suggestive evidence that esterification of insulin in methanolic hydrogen chloride produced some *N*- to *O*-acyl migration involving both serine and threonine residues.

The present report is concerned with the chemical modification of the carboxyl groups of insulin, effecting esterification with methanolic hydrogen chloride, and the subsequent characterization of products using physical, chemical, and enzymatic techniques. The reaction schemes are outlined in Figure 1. The esterification procedure was found to be accompanied in part by a nitrogen to *O*-acyl shift at threonine-27 of the B chain. After deamination of the rearranged product, the peptide chain was selectively cleaved by mild saponification (Figure 1).

Experimental Section

Materials

Bovine zinc insulin was the product of Eli Lilly and Co. (lot no. OLVOO). Carboxypeptidase A treated with diisopropyl fluorophosphate was obtained as a suspension of crystals in water from Worthington Biochemical Corp. (Lot No. 9DA). An enzyme stock solution was prepared by diluting one volume of enzyme suspension with ten volumes of 10% LiCl and stirring for several hours at 4°. The concentration of enzyme was obtained from optical density measurements at 278 mμ using the value of 1.94 for a 1-cm thick solution of 1 mg/ml (Vallee *et al.*, 1960). Methanol (Mallinckrodt) was prepared for use by refluxing with 1% calcium hydride for

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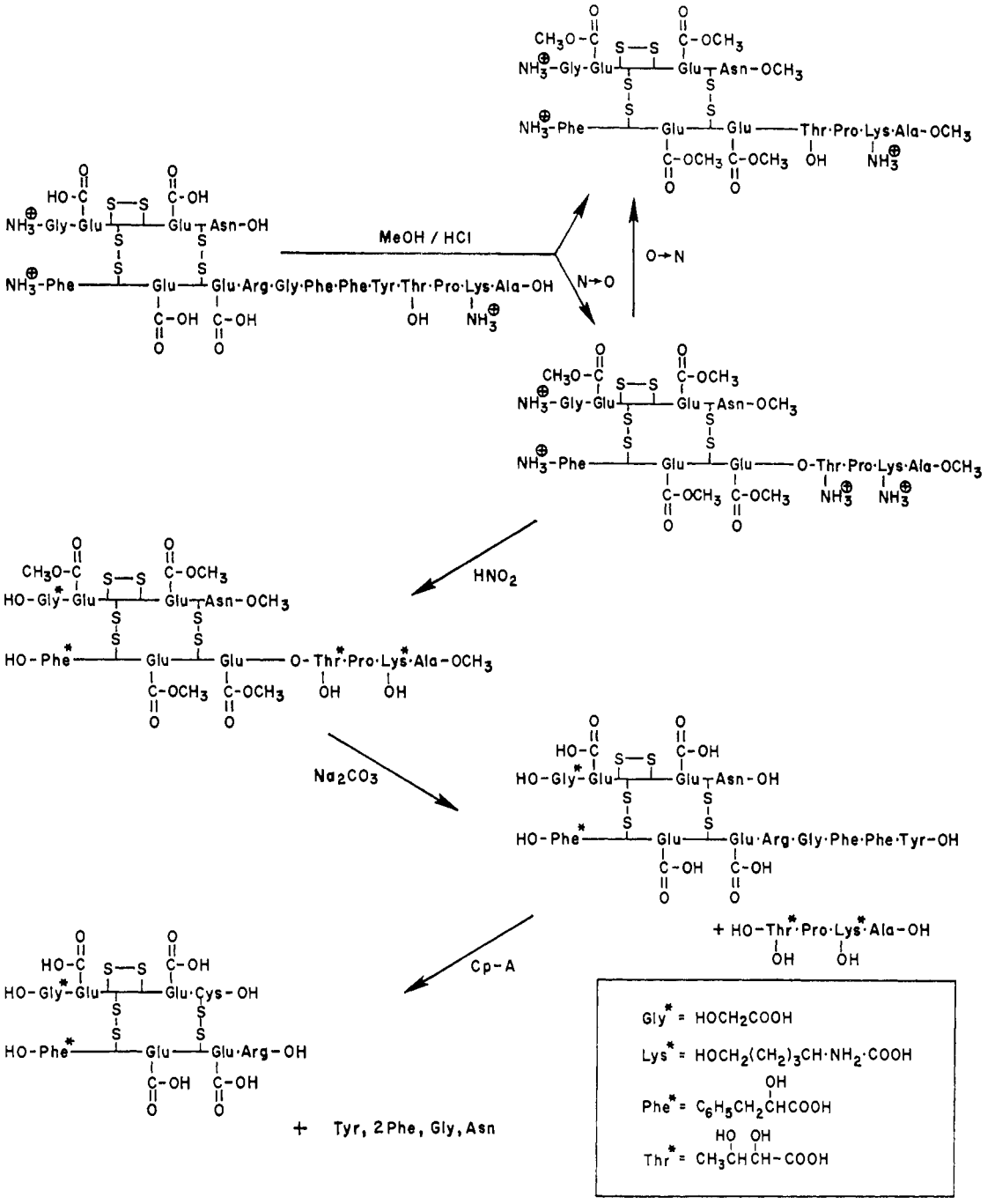

FIGURE 1: Scheme describing the synthesis of insulin methyl esters A and B and subsequent deamination, base hydrolysis, and carboxypeptidase treatment of insulin methyl ester B.

1 hr followed by distillation. Ether (Mallinckrodt) was dried over "Linde" Type 4A Molecular Sieves (Matheson, Coleman & Bell). Hydrogen chloride gas was purchased from Matheson. Anhydrous hydrogen chloride in methanol was prepared by bubbling the gas, which had been previously passed through a sulfuric acid drying trap, into dried methanol (50 ml) under anhydrous conditions at room temperature. The normality of the solution was ascertained by titration of a 50% aqueous solution with 1.0 N sodium hydroxide. Glacial acetic acid, sodium nitrite, sodium carbonate, sodium acetate, sodium chloride, sulfuric acid, and urea were purchased from Mallinckrodt and used without further purification. The chromatography resins (CM-Sephadex C-25; Sephadex G-50, fine; and Sephadex G-25, medium) were obtained from Pharmacia Fine Chemicals, Inc.

Methods

Amino acid analyses were performed on a Technicon automatic amino acid analyzer according to the procedure of Spackman *et al.* (1958). All hydrolyses were carried out in 6 N HCl in sealed, evacuted tubes for 24 hr at 110°. *Ultraviolet* absorption measurements were obtained from a

Zeiss spectrophotometer (PM Q II). Enzyme reactions were performed on a pH-Stat (Radiometer). *Methoxyl analyses* were performed by the Chemistry Department, University of California, Berkeley, Calif. *Optical rotatory dispersion* measurements were performed on a Cary-60 spectropolarimeter. *Gel electrophoresis* experiments were run on a Büchler instrument. *Removal of salts* and urea from solutions of insulin derivatives was performed in part using an Amicon ultrafiltration cell with a UM-2 diaflow membrane.

Chromatographic Procedures. A CM-Sephadex C25 column (2.5 × 40 cm) was used to assess the homogeneity of insulin and insulin methyl ester. The chromatographic buffer contained 0.01 M sodium acetate in 7 M urea with the pH adjusted to 4.75 with glacial acetic acid. Elution was performed by a linear gradient obtained by running 0.35 M NaCl (1 l.) into the stirred reservoir containing 0.10 M NaCl (1 l.) (all in 7 M urea–0.01 M sodium acetate at pH 4.75). Flow rates were 50 ml/hr and fractions of 10 ml were collected. Protein was determined by absorbance at 278 mμ. The samples (30 mg/ml) were dissolved in the buffer containing 0.01 M sodium acetate–0.10 M NaCl in 7 M urea and applied to the column.

A Sephadex G-25, medium, column (5.0 × 50 cm) was used to remove urea and salts from protein solutions obtained from the CM-Sephadex column. Samples were eluted with 2.0 M acetic acid with a flow rate of 70 ml/hr. A Sephadex G-50, fine, column (2.5 × 95 cm) was used to assess the homogeneity as to size of insulin and insulin methyl ester. Samples (5 mg/ml) were eluted at a flow rate of 20 ml/hr using either 2.0 M acetic acid or 0.01 M sodium acetate–0.1 M NaCl in 7 M urea at pH 4.75 as eluents.

Preparation and Isolation of Insulin Methyl Ester. Zinc insulin crystals (1.0 g) were suspended in anhydrous methanol (1600 ml) to which was added anhydrous 4 N hydrochloric acid–methanol (4.0 ml) resulting in a 0.01 N hydrochloric acid solution. The protein dissolved immediately. After 1 hr at room temperature under anhydrous conditions, a further addition of hydrochloric acid–methanol was made to bring the final concentration of hydrochloric acid to 0.10 N. After 23 hr, a small amount of insoluble material was filtered off, anhydrous ether (6 l.) was added to the clear solution, and the resulting precipitate was permitted to sit at 4° for 20 hr. The precipitate was isolated by centrifugation, washed three times with fresh anhydrous ether, and then dried at room temperature under vacuum over phosphorus pentoxide to yield the mixed esters as a colorless amorphous solid (850 mg). The methoxyl analysis value for the insulin derivative was 3.10% as compared with a calculated value of 3.03% for insulin hexamethyl ester.

Esters A and B were separated using large chromatographic runs (200 mg) on CM-Sephadex followed by removal of urea and salts from the respective protein solutions utilizing a 400-ml Amicon ultrafiltration cell with a UM-2 diaflow membrane at 85 psi. Solutions were first concentrated (300–30 ml) and then desalted using 0.01 N hydrochloric acid. Insulin methyl ester A or B was isolated by precipitation with anhydrous acetone. Rechromatography of fractions A and B showed them to be homogeneous and stable to the conditions of chromatography and isolation. Methoxyl analysis of insulin methyl ester A and B was 3.09 and 3.15%, respectively.

Conversion of Insulin Methyl Ester B into A. The stability of insulin methyl ester B was found to be extremely sensitive to pH and to the presence or absence of urea. Insulin methyl ester B (20 mg) was dissolved in aqueous solutions with the following pH values: 2.0, 2.8, 4.5, and 6.0. After 1 hr urea was added to give a final concentration of 7 M, the pH was adjusted to 4.75, and the material was chromatographed on CM-Sephadex. Insulin methyl ester A or B was exposed to pH 4.75 in 7 M urea for periods up to 35 hr and then chromatographed on CM-Sephadex.

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate was adapted from the procedure of Shapiro *et al.* (1967) with the omission of mercaptoethanol. Samples of the various proteins were dissolved in electrophoresis buffer (0.025 M phosphate–0.5% sodium dodecyl sulfate, pH 7.1) to make a concentration of 2 mg/ml, and the solution was kept at 37° for 3 hr. Each sample was then mixed with an equal volume of a 2 M sucrose solution containing 0.5% sodium dodecyl sulfate and 0.025 M phosphate (pH 7.1). To 0.4 ml of this sucrose solution was added 10–15 μl of a concentrated bromophenol blue solution and 25–40 μl of this sample sucrose–bromophenol blue solution was applied to the top of each polymerized gel (gels contained 20% acrylamide–0.5% sodium dodecyl sulfate–0.025 M phosphate, pH 7.1; *N,N'*-methylenebisacrylamide:acrylamide ratio was 1:50; gel length was 50 mm) and run at constant current (5 mA/tube) until the band of tracking dye approached the lower end of the gel (about 2 hr). The electrophoresis buffer contained 0.5% sodium dodecyl sulfate and 0.025 M phosphate (pH 7.1). After electrophoresis the dye fronts were marked with fine copper wire. The gels were fixed overnight in a 50% methanol–10% acetic acid solution, stained overnight in a solution of 1% Amido-Schwartz in 20% methanol and 20% acetic acid, and destained electrophoretically using 7% acetic acid. The relative migrations of Zn insulin, ribonuclease A (Sigma), α-chymotrypsinogen (C. F. Boehringer und Söhne), and insulin mixed esters A and B were determined.

Deamination of Insulin Methyl Esters. Insulin methyl ester A (10 mg) was dissolved in 50% acetic acid (10 ml). Sodium nitrite (500 mg in 2 ml of water) was added to this solution over a period of 25 min at room temperature with vigorous stirring. After 30 min the mixture was diluted with water (200 ml) and then lyophilized. The resulting residue was thoroughly washed with distilled water (four 30-ml positions) with vigorous mixing followed by centrifugation, and then relyophilized. The deaminated product was characterized by amino acid composition and was used for the alkaline hydrolysis experiments described below. A duplicate reaction was run on insulin methyl ester B and on unmodified insulin; however, in the latter case the product, after lyophilization, was purified on Sephadex G-25 with 2 N acetic acid as an eluent.

Sodium Carbonate Hydrolysis of Deaminated Insulin Methyl Esters. Deaminated methyl ester A (5 mg) was dissolved in 0.1 N sodium carbonate (14 ml) and stirred at room temperature for 23 hr. The pH of the solution was adjusted to 2.0 with concentrated HCl and the resulting precipitate was isolated by centrifugation. The precipitate was washed twice with 0.01 N HCl and then lyophilized. The precipitation and washing of the large fragment removed the deaminated material arising from the Thr-Pro-Lys-Ala portion of the molecule. The large, acid-insoluble fragment was used for the carboxypeptidase A reaction. Duplicate experiments were performed on deaminated methyl ester B, native insulin, and deaminated insulin.

TABLE I: Amino Acid Analyses of Insulin and Insulin Derivatives.

Amino Acid	Insulin		Insulin Methyl Ester A	Insulin Methyl Ester B	Deaminated Insulin	Deaminated Insulin Methyl Ester A	Deaminated Insulin Methyl Ester B
	Calcd	Obsd					
Asp	3	3.10	2.88	2.85	3.05	3.02	3.10
Thr	1	1.05	0.91	0.97	0.95	0.92	**0.07**
Ser	3	2.98	2.80	2.75	2.81	2.85	2.80
Glu	7	6.95	6.82	7.02	6.94	7.04	6.92
Pro	1	0.89	0.95	0.88	0.80	0.82	0.78
Gly	4	4.06	3.85	3.81	**3.06**	**3.05**	**3.08**
Ala	3	3.00	3.00	3.00	3.00	3.00	3.00
Half-Cys	6	5.80	4.74	5.70	5.65	5.75	5.71
Val	5	4.68	4.65	4.73	4.69	4.70	4.72
Ile	1	0.78	0.77	0.77	0.72	0.75	0.76
Leu	6	5.90	5.86	5.75	5.91	5.93	5.95
Tyr	4	3.84	3.69	3.72	3.10	3.15	3.05
Phe	3	2.94	2.98	2.91	**2.04**	**2.06**	**2.03**
Lys	1	1.02	1.08	1.10	**0.08**	**0.10**	**0.05**
His	2	1.91	2.03	2.06	2.03	1.89	1.92
Arg	1	1.05	1.02	0.98	0.96	0.95	0.06

Hydrolysis with Carboxypeptidase A. Each of the base-hydrolyzed esters (3 mg) was dissolved in water (7 ml) and the pH was adjusted to 7.4 with dilute sodium hydroxide. The pH was maintained in a pH-Stat. To the stirred solution was added carboxypeptidase A stock solution (50 μl). After 4 hr, an additional portion of carboxypeptidase A solution (25 μl) was added and the reaction was continued for an additional 4 hr. At this point a 2-ml aliquot was removed, the pH was adjusted to pH 2.88, and the resultant precipitate was removed by centrifugation. The clear supernatant of the aliquot was analyzed for free amino acids. To the remaining reaction mixture was added an additional portion of carboxypeptidase A (25 μl) and the reaction permitted to continue for 16 hr. After a total of 24 hr the pH was lowered to 2.88, the precipitate was removed by centrifugation, and the supernatant was analyzed for free amino acids.

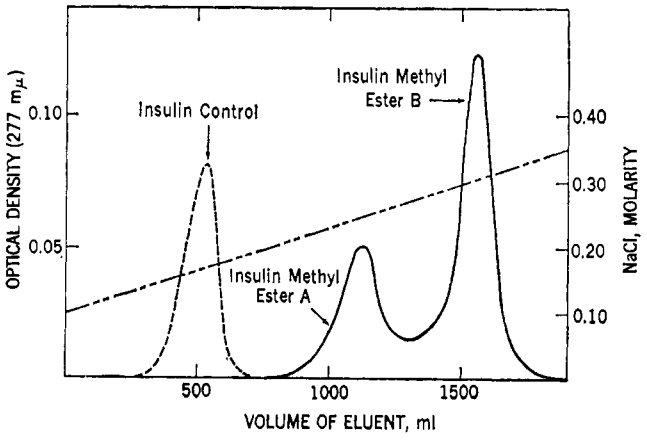

FIGURE 2: Chromatography on CM-Sephadex of insulin (---) and insulin methyl esters A and B (—) with 0.01 M sodium acetate buffer in 7 M urea with 0.10–0.35 M NaCl gradient, pH 4.75; sodium chloride gradient (—·—·—·—).

Results

Synthesis of Insulin Methyl Ester. The esterification of insulin was carried out under rigorously maintained anhydrous conditions in 0.1 N HCl in methanol at 25°. The isolated crude reaction products were then dissolved in 7 M urea–0.01 N sodium acetate buffer and chromatographed on CM-Sephadex, with the results shown in Figure 2. The materials present in peaks A and B were pooled separately and each fraction was desalted using an Amicon ultrafiltration cell. The isolated products were then rerun on the same column yielding homogeneous products.

The stability of the product in peak B was observed to be very dependent on the pH of the solution to which it was exposed. Exposure of pure insulin methyl ester B to solutions of various pH values for 1 hr effected different degrees of conversion as shown in Figure 3.

The stability of insulin methyl ester B was, however, appreciably altered in the presence of 7 M urea. This ester, when exposed to a solution of pH 4.75 for 1 hr, underwent approximately a 70% conversion into a product with an elution volume characteristic of insulin methyl ester A. However, in the presence of 7 M urea at this pH there was no observed conversion, even after 36 hr.

The products of the esterification reaction were also analyzed using gel chromatographic techniques (Sephadex G-50) with 2 N acetic acid as the eluent. The results shown in Figure 4a indicate that the two esters behave differently in the 2 N acetic acid; ester A has the same elution volume as insulin, whereas ester B has a smaller elution volume indicative of a higher molecular radius. When the chromatography was performed in the presence of 7 M urea, insulin and insulin methyl esters A and B chromatographed with the results

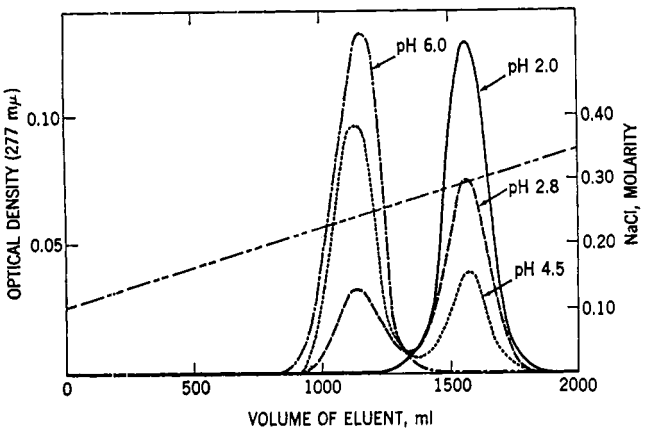

FIGURE 3: Chromatography on CM-Sephadex of insulin methyl ester B, showing the effect of pH on the rate of conversion into insulin methyl ester A. pH 2.0 (—), pH 2.8 (— — —), pH 4.5 (·····), pH 6.0 (·—·—·—); sodium chloride gradient (— — — —). Conditions are the same as for Figure 2.

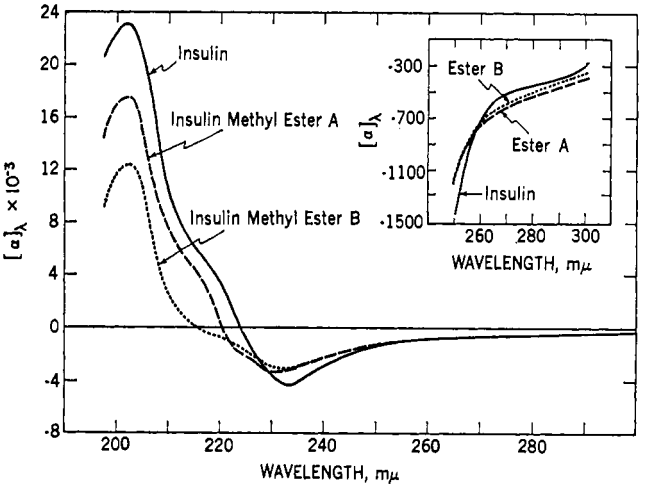

FIGURE 5: Optical rotatory dispersion spectra at 1.0 mg/ml at pH 1.90 of insulin (—), insulin methyl ester A (— — —), and insulin methyl ester B (·····).

shown in Figure 4b. The identical elution volumes of the three different compounds indicate that their molecular weights in urea are identical. The fact that in 2 N acetic acid, insulin methyl ester B exhibited a higher apparent molecular weight than ester A may be attributed to a difference in their abilities to aggregate.

Methoxyl analysis of esters A and B gave approximately the same value which was equivalent to a reaction at each of the six carboxyl groups. The amino acid analyses of both products were also essentially identical as shown in Table I. Gel electrophoresis was carried out in sodium dodecyl sulfate with the results shown in Table II. These data, as in the gel chromatography work, indicated that, in dissociating solvents, insulin and a mixture of the two esterified products had the same molecular weight.

Optical rotatory dispersion spectra were obtained of insulin and the two esterified products in 0.01 N HCl solutions in which all products are stable. These spectra are shown in Figure 5.

Deamination Studies. In a further attempt to characterize the difference between insulin methyl esters A and B a reaction effecting deamination was carried out on insulin as a control and then on the two ester products. As judged by loss of amino acids (Table I), deamination of insulin using sodium nitrite in acetic acid was effected at the N-terminal amino acids, phenylalanine and glycine, and at the ε-amino group of lysine-29 of the B chain. The same reaction was carried out on insulin ester A or B. Amino acid analysis of the products (Table I) showed that in both cases there was complete loss of the two N-terminal amino acids and the ε-amino group of lysine, indicating that the amino groups of these amino acids were free to react with nitrous acid and were not involved in any inter- or intrachain peptide linkages. However, with ester B there was a further loss of threonine owing to deamination of this residue which only occurs at position 27 of the B chain. These latter results indicate that a *N*- to *O*-acyl shift occurred during esterification to yield ester B. The conversion of a peptide to an ester bond at threonine introduced a new amino group and increased by one the number of positive charges on ester B. As a result ester B was more strongly adsorbed to the CM-Sephadex than was ester A (Figure 2).

Specific Cleavage. Since insulin contains only one threonine residue, a nitrogen to *O*-acyl shift would introduce one ester

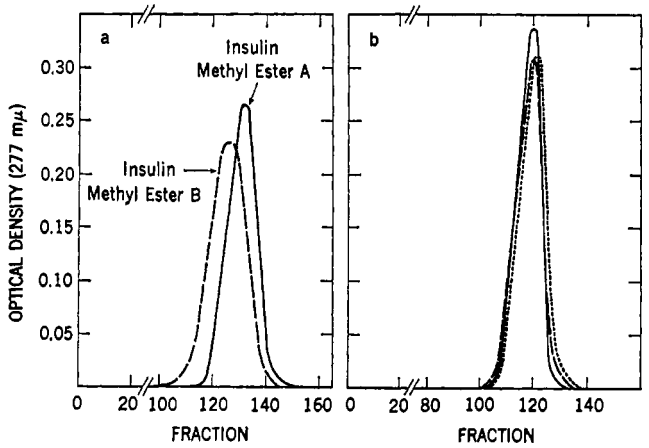

FIGURE 4: Chromatography on Sephadex G-50. (a) With 2 M acetic acid of purified insulin methyl ester A (—) and insulin methyl ester B (— — —). (b) With 0.01 M sodium acetate buffer in 7 M urea at pH 4.75 of insulin methyl ester A (— — —), insulin methyl ester B (·····), and insulin (——).

TABLE II: Gel Electrophoresis in Sodium Dodecyl Sulfate Buffer.

Sample	R_F	Mol Wt
Insulin	0.62	5,733
Ribonuclease	0.51	13,700
Chymotrypsinogen	0.35	23,200
Insulin methyl esters (A and B)	0.60	

TABLE III: Amino Acid Analysis of Free Amino Acids Released by the Action of Carboxypeptidase A on Insulin and Insulin Derivatives.

Amino Acid	Insulin[a]	Insulin[b]	Insulin[c] Methyl Ester	Insulin[d] Methyl Ester	DAIE-A[e] Hydrolyzed	DAIE-B[f] Hydrolyzed 8 hr	DAIE-B[f] Hydrolyzed 24 hr
1	2	3	4	5	6	7	8
Asn, Asp	0.90	0.88	0.00	0.85	0.90	0.89	0.91
Gly						0.92	0.85
Ala	0.94	0.92	0.00	0.88	0.88	0.05	0.06
Tyr						0.75	0.79
Phe						2.00	2.00

[a] Insulin control. [b] Insulin treated with sodium carbonate. [c] Insulin ester A and insulin ester B. [d] Insulin ester treated with sodium carbonate. [e] Deaminated insulin ester A treated with sodium carbonate. [f] Deaminated insulin ester B treated with sodium carbonate.

bond which should be susceptible to a selective hydrolysis under mild basic conditions where no peptide bonds would be broken. However, in order to avoid a return migration under alkaline conditions, the neighboring amino group must be removed or protected (Iwai and Ando, 1967). In this instance all of the amino groups were removed by the nitrous acid reaction (Ramachandran and McConnell, 1955). Deaminated esters A or B, deaminated insulin, as well as insulin and mixed esters A and B, were treated with 0.1 N sodium carbonate at room temperature for 20 hr to effect saponification of the ester groups. If ester B contained an ester bond rather than a peptide bond between residues 26 and 27, saponification of the deaminated ester B should cleave the chain at this point, releasing a modified tetrapeptide from the C-terminal end and exposing a new C-terminal amino acid, tyrosine^{B26} (Figure 1). In order to detect the new C-terminal amino acid, the saponified, deaminated ester B as well as the other saponified derivatives were treated with carboxypeptidase A, and the amino acids released were determined (Table III).

Treatment of native insulin with carboxypeptidase A (Table III, column 2) released one residue of alanine (from the B chain) and one residue of asparagine plus aspartic acid (from the A chain), the latter resulting from the presence of a small amount of desamido-insulin in the preparation (Slobin and Carpenter, 1963). The same results were obtained by the action of the enzyme on the sodium carbonate treated insulin (Table III, column 3). This indicated that no internal peptide bonds had been cleaved by the mild alkaline treatment. When a mixture of insulin esters A and B which had not been treated with alkali were reacted with the enzyme, no amino acids were released, as expected (Table III, column 4). However, when the saponified, mixed insulin esters A and B were exposed to the enzyme, a residue of alanine and a residue of asparagine plus aspartic acid were produced (Table III, column 5). This result indicated that the mild alkaline treatment was sufficient to saponify the ester groups on the C terminals but that no other peptide bond had been broken. When deaminated and saponified ester A was treated with carboxypeptidase A, only alanine and an equivalent amount of asparagine plus aspartic acid were produced (Table III, column 6). This result indicated that in deaminated and saponified ester A, the free C-terminal amino acids are the same as those in insulin or in saponified insulin esters. However, when deaminated and saponified ester B was treated with carboxypeptidase A, there was again an equivalent amount of asparagine plus aspartic acid released but virtually no alanine was produced. Instead tyrosine, phenylalanine, and glycine were released in ratios approximating 1:2:1 (Table III, columns 7 and 8). Referral to Figure 1 shows that this is the expected result for an insulin ester which has undergone an *N*- to *O*-acyl shift involving threonine at position B27. Deamination followed by saponification of such an ester would liberate a modified tetrapeptide originating from the Thr-Pro-Lys-Ala sequence and expose a tyrosine residue at position B26. Treatment of this material with carboxypeptidase A would bring about the release of asparagine (plus aspartic acid) from the A chain plus the sequential release of tyrosine, two phenylalanines, followed by glycine from the B chain. Further cleavage is prevented by the presence of arginine at B22. It should be noted that during the isolation of the cleaved protein resulting from the base hydrolysis of the intrachain ester, the modified tetrapeptide is lost in the precipitation and/or acid-washing steps at pH 2.0. Hence, no alanine appears in the carboxypeptidase A digest of the deaminated, saponified product (Table III, columns 7 and 8).

The results of the carboxypeptidase A digestion on the deaminated and saponified esters A or B further support the occurence of a *N*- to *O*-acyl shift involving threonine at position B27 in the formation of ester B and also demonstrate a specific cleavage at the carboxyl side of tyrosine^{B26} by saponification of the intrachain ester bond in the deaminated ester B.

Discussion

The results of these studies have shown that when insulin is esterified in anhydrous hydrogen chloride–methanol two products are formed which can be separated by chromatography on CM-Sephadex in urea-containing buffers. The two materials have identical amino acid compositions, and furthermore, both products are fully esterified with the incorpora-

tion of six methoxyl groups. The separation of the mixture on CM-Sephadex could be attributed to products which differed in either charge and/or size. Although chromatography of these esters, either mixed or separately, on Sephadex G-50 in 2 N acetic acid indicated that they did differ slightly in size or molecular radius, the difference observed was not large enough to account for their separation on CM-Sephadex. Furthermore the difference was in the opposite direction from that predicted from their behavior on CM-Sephadex. In addition, when the esters, either mixed or separate, were chromatographed on Sephadex G-50 in 7 M urea they exhibited an identical elution behavior which was also the same as that of insulin. Thus the separation observed on CM-Sephadex in urea-containing buffers could not be attributed to differences in size and must be due to differences in charge. Since all of the carboxyl groups were esterified in both compounds, any difference in charge at pH 4.75 must be attributed to a different number of positively charged groups (presumably amino groups). Ester B, which was retained the longest on the CM-Sephadex, would be expected to have the largest number. However, no difference in the number of amino groups was detected between the two esters or insulin by the ninhydrin color reaction of Moore and Stein (1954). This fact was a stumbling block in the elucidation of the difference between esters A and B, but it can be explained by ester B being rapidly converted into ester A under the conditions of the ninhydrin reaction.

It was shown, using the ion exchange chromatography techniques, that the stability of insulin methyl ester B was quite sensitive to pH. At pH values of 2.2 or slightly below ester B was relatively stable. However, as the pH was raised, ester B was converted to insulin methyl ester A at a rate which depended on the pH (Figure 3). Although unstable in water at pH 4.5, in the presence of 7 M urea at pH 4.75, ester B remained unchanged for periods up to 36 hr.

These observations led us to believe that the conditions for esterification had brought about a nitrogen to oxygen shift at the threonine and/or serine residues to yield ester B. This was in line with the previous experience of Chibnall *et al.* (1958) who had reported acyl migration in insulin during similar treatment. Under suitable pH conditions ester B underwent a reverse shift (O → N) to form insulin methyl ester A. A comprehensive review of this subject has been published (Iwai and Ando, 1967). The nitrogen to oxygen shift would introduce a positive charge into ester B accounting for its stronger retention on CM-Sephadex. The fact that the reversion of ester B to ester A at pH 4.75 is severely inhibited by the presence of 7 M urea is puzzling. Since the rate of this reversion is dependent on the concentration of the nonprotonated amine, the urea simulates an effect comparable with lowering the pH of the medium or increasing the pK of the amino groups. Whatever the mechanism of action of the urea, its effect made it possible to separate the two esters by chromatography on CM-Sephadex. Without this fortuitous effect, ester B would have reverted to ester A at the pH of chromatography and would not have been detected.

To establish an N → O shift and to locate its position(s), deamination was carried out on insulin methyl ester B with nitrous acid in order to trap the product in the intrachain ester form (*O*-acyl form) (Ramachandran and McConnell, 1955) and prevent the reverse shift by substituting a hydroxyl group for the liberated amino group. The same reaction was carried out on insulin methyl ester A for comparison. The amino acid analyses showed the loss of one threonine residue in methyl ester B, while there was no change in the composition of methyl ester A. The selective cleavage of the newly introduced ester bond at threonine-27 of the B chain was accomplished on the deaminated product by mild base treatment to yield a large fragment and a modified tetrapeptide, Thr*-Pro-Lys*-Ala (Figure 1). The modified tetrapeptide derivative was removed during the isolation of the large fragment. The position of cleavage in the saponified product was determined by detecting the newly formed C-terminal residues on the large fragment with carboxypeptidase A. Exposure of the large fragment for 8 and 20 hr to the enzyme gave similar results, indicating that complete reaction had occurred at all newly exposed carboxyl groups. As shown in Figure 1, after base cleavage of the deaminated ester, tyrosine-26 of the B chain is exposed. This amino acid is then followed by two phenylalanine residues and a glycine residue in the primary sequence. These amino acids also are susceptible to attack by carboxypeptidase A as each previous amino acid residue is cleaved by the action of the enzyme. The liberation of these amino acids, as well as asparagine, from the C-terminal end of the A chain indicated that deaminated insulin methyl ester B had been selectively cleaved at only one position in the molecule. In a similar treatment, insulin methyl ester A remained uncleaved.

These results thus confirm the hypothesis that insulin undergoes a nitrogen to oxygen shift when exposed to hydrogen chloride–methanol solution and that in the purified insulin methyl ester B this shift occurs primarily at the threonine residue of the B chain. It should be noted that in other previous studies which made use of anhydrous, strongly acidic media, serine as well as threonine has been shown to undergo this rearrangement in insulin (Iwai and Ando, 1967). Furthermore, Chibnall *et al.* (1958) found that upon esterification of insulin in methanolic hydrogen chloride under conditions similar to those used in this work, and subsequent treatment with nitrous acid, there was a decrease in both serine and threonine residues, although the decrease was greater for threonine. They attributed this result to an N to O migration involving both the serine and threonine residues. Our data indicate that ester B is composed almost entirely of a product in which the migration took place at only the threonine residue. Two possible explanations for this discrepancy are as follows. (1) If migration occurs much more rapidly at threonine than at serine, then any product having appreciable migration at serine would have two or more new positive charges (one for threonine and one or more for each serine) and as a result would be separated from ester B on the CM-Sephadex chromatography. (2) Any migration involving a serine residue might go back to a peptide bond by reverse reaction during the chromatography in 7 M urea at pH 4.75 whereas that involving the threonine residue is stable to this condition. The data do not exclude the possibility that migration occurred at the serine residue during esterification. If so, such products were either removed during the isolation of ester B or underwent a reverse migration during the isolation procedures. In any event it will be of interest to determine whether the selectivity exhibited by the threonine residue in these experiments can be demonstrated in other proteins. If so, it offers the possibility of developing a method of cleavage of peptide chains at the threonine residue.

Although ester B appears to be the first described protein derivative which was formed by a specific N → O shift involving a *threonine* residue, Gold and Fahrney (1963) have presented evidence for the occurrence of a specific N → O shift involving the active-site *serine* residue of chymotrypsin. In this latter case, elimination of the phenylmethanesulfonyl group in acid solution from phenylmethanesulfonyl-α-chymotrypsin apparently resulted in an N → O shift involving the serine residue at the active site.

Because of the reverse oxygen to nitrogen shift, insulin methyl ester B is most certainly converted into insulin methyl ester A when subjected to biological assay. The complete loss of biological activity of insulin ester A might arise as a result of the chemical modification of one or more of the carboxyl groups needed for binding of the hormone to its receptor site. Studies concerned with the selective modification of only one or two carboxyl groups with a variety of reagents are in progress.

Optical rotatory dispersion studies on insulin, insulin methyl ester A, and insulin methyl ester B (Figure 5) indicate that esterification effects a conformational change between insulin and insulin ester A. The *N*- to *O*-acyl shift effects yet another conformational change between the two ester products. Thus a second cause for the loss of biological activity may be the result of a conformational change induced by the complete modification of the carboxyl groups.

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