Preparation and Properties of Fluoresceinthiocarbamyl Insulins*

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ABSTRACT: Fluorescein isothiocyanate was found to react very rapidly in nearly stoichiometric fashion with the two α-amino groups of insulin (A1, B1) at pH 9.1, room temperature; the ϵ-amino group of lysine (B29) reacted at a much slower rate. The order of reactivity of the three amino groups under these conditions was observed to be Phe > Gly $\gg$ Lys. Reaction mixtures were readily separated into mono-, di-, and trisubstituted components by chromatography on columns of DEAE-Sephadex in urea-containing buffers. Monofluoresceinthiocarbamyl (FTC) insulin was found to be substituted principally at N-terminal phenylalanine, di-FTC insulin at both N-terminal residues, and tri-FTC at all three amino functions. On the basis of the data a working hypothesis was proposed that substitution of phenylalanine was accompanied by a structural change in insulin such that the glycyl amino group became more available to the reagent; the slow reaction of lysine was attributed in large measure, but not entirely, to the fact that the ϵ-amino group was mainly in the protonated, unreactive form. Mono-FTC insulin was found to retain about 40% biologic activity and to react with a particular pool of insulin antibodies in a manner nearly indistinguishable from insulin. However, di-FTC insulin was found to retain only 4% biologic activity and to be effectively recognizable by only one-third of the insulin antibodies. Substitution of all three amino groups resulted in a derivative that was virtually devoid of biologic or immunologic reactivity.

Insulin has often been the subject of chemical and enzymic alteration with the aim of relating the effects of such changes to the function of the hormone. However, in the early studies methods were not available to allow adequate purification and characterization of the modified insulins. Also, comparatively few reports have been concerned with a quantitative measure of the interaction of insulin antibodies with purified derivatives of insulin of known covalent structure. Particular interest has been shown in FTC[1] insulin preparations for the purpose of studying the localization of the hormone in tissues (Berns *et al.*, 1962; Parker *et al.*, 1963; Maggi, 1966), and several investigators (Halikis and Arquilla, 1961; Tietze *et al.*, 1962) have prepared mixtures of FTC insulins which had reduced biological activity. Interestingly, Seidler *et al.* (1964) reported that an FTC insulin preparation free of unreacted insulin exhibited no hypoglycemic activity, casting considerable doubt on the suitability of such derivatives for localization studies. Tietze *et al.* (1962) showed indirectly that the terminal amino group of the phenylalanyl (B) chain was the most reactive to FITC, with some reaction occurring at the terminal glycyl residue of the A chain. On the other hand, Andersen (1954) using phenyl isocyanate and Christensen (1951) using phenyl isothiocyanate reported that the α-amino group of the glycyl residue was the more reactive. The questions of biologic and immunologic activity, of rates of reactivity of the amino groups, and of location of the fluorescein groups in FTC insulin required elucidation.

The purposes of the present report are to describe (1) the preparation and purification of FTC insulins which may also be useful in localization studies, (2) the relative rates of reactivity of the amino groups, (3) the localization of the covalent alterations in the insulin molecule, and (4) the effects of such alterations on some physical, biological, and immunological properties of the hormone.

Experimental Section

Materials

Preliminary studies were performed with FITC (lots 50771 and 50102) purchased from Calbiochem, Los Angeles, Calif. However, these preparations were separable by thin layer chromatography (12% methanol in benzene on Eastman Chromagram sheets, type K301R) into FITC isomer I (R_F *ca.* 0.2) and FITC isomer II (R_F *ca.* 0.3). Unless otherwise noted, the results reported were obtained with FITC (lot 510677, Baltimore Biological Laboratory, Baltimore, Md.) which was found to be chromatographically pure isomer I when observed under ultraviolet light. All other reagents and chemicals were of analytical reagent grade with the exception of sodium tetrathionate (lot 4536, 95–99% pure, obtained from K & K Laboratories, Plainview, N. Y.).

Insulin Preparations. Crystalline bovine zinc insulin,

* From the Department of Pathology, University of California, Los Angeles, California. *Received April 10, 1967.* Supported by a grant from Eli Lilly and Co., Indianapolis, Ind. A preliminary report of part of these data was presented at the 26th Meeting of the American Diabetes Association, Chicago, Ill., 1966.

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[1] Abbreviations used: FTC, fluoresceinthiocarbamyl; FITC, fluorescein isothiocyanate.

lot 795372 (25.6 IU/mg), was kindly supplied by the Lilly Research Laboratories, Indianapolis, Ind.

Methods

Preparation of FTC Insulin Mixtures. Initial studies were performed using the reaction conditions of Berns *et al.* (1962) and the procedure was later modified as follows. Crystalline beef insulin at 10–20 mg/ml was treated with FITC (molar ratio of reagent:insulin was varied from 1:1 to 4:1) at 22–24° for 1–8 hr in 0.05–0.2 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.1) containing NaCl at 0.05–0.13 M. The insulin was dissolved in an appropriate amount of 0.5 M $NaCO_3$–$NaHCO_3$ buffer and was diluted with 0.15 M NaCl. The FITC was sometimes added as a fine powder but preferably was dissolved in 1–2% in the same buffer and was immediately added to the insulin solution.

The reaction was terminated (A) by dialysis against 100 volumes of 0.1 M sodium acetate buffer at pH 4.5, or (B) by precipitation of FTC insulins (4°, 2–16 hr) through adjustment of the pH to 4.5 with HCl, or (C) by rapid passage of the reaction mixture at room temperature over a 2 × 27 cm column of G-25 Sephadex which was equilibrated and developed in 0.01 M sodium phosphate buffer (pH 7.4) containing NaCl at 0.15 M. In any case all reaction mixtures were subjected to both isoelectric precipitation(s) and gel filtration on Sephadex to remove low molecular weight impurities, with the final step being a precipitation and centrifugation at low speed. After removal of the fluorescent impurities, FTC insulins were found to precipitate quantitatively near pH 4.5, leaving no color in the supernatant liquid. The precipitate containing a mixture of FTC insulins was washed with a few milliliters of cold 0.01 M ammonium acetate at pH 4.5 and was dried *in vacuo*. The recovery of crude FTC insulins ranged from 85 to 97% of theory.

DEAE-Sephadex Chromatography. A modification of the method of Thompson and O'Donnell (1960) was employed to fractionate the crude FTC insulin mixture. The method has also been applied to the purification of desoctapeptide insulin (Bromer and Chance, 1967). All chromatographic buffers were 7 M in urea and 0.01 M in Tris (adjusted to pH 7.6 with HCl at 4°), with varying concentrations of NaCl. The buffers were prepared immediately prior to use and all succeeding steps were carried out at 4° except the spectrophotometric analysis of the fractions. Columns (1.5 × 90 cm) (Pharmacia Fine Chemicals, Inc.) containing DEAE-Sephadex (A-25) equilibrated in the buffer were prepared in the usual manner. Crude FTC insulin was applied to the column in starting buffer at 10 mg/ml and a linear NaCl gradient elution was begun immediately after a 2-ml wash was applied. Fraction volumes of 5–10 ml were collected automatically; flow rates of the columns were 25–30 ml/hr. Absorption of the fractions at 280 and 492 mμ was determined with a Beckman Model DB spectrophotometer. Pooled fractions were promptly dialyzed for 16 hr against 200 volumes (two or three changes) of 0.05 M sodium acetate buffer (pH 4.5) and the resulting precipitate was collected by centrifugation, washed, and dried *in vacuo*.

Electrophoretic Analysis. Electrophoresis was performed on 2.5 × 10 cm strips of Oxoid (Colab Laboratories, Inc., Chicago, Ill.) cellulose acetate either in the Shandon Universal cell or the Spinco Model R cell. Samples of 5 μl or less were applied using Drummond micropipets (Kensington Scientific Corp., Oakland, Calif.). For analytical separation of FTC insulins, electrophoresis was performed for 1 hr at 15 v/cm in 0.05 M Tris-HCl buffer (pH 7.0) containing urea at 7 M. The method is similar to that used by Carpenter and Hayes (1963); the presence of urea in the buffers is necessary to afford clean fractionation of the insulin modifications, presumably through minimization of intermolecular interactions. The best separations appeared to be obtained after the buffer had been used several times. Separation of the *S*-sulfonate chains of FTC insulins was accomplished by electrophoresis for 1.5 hr at 18–20 v/cm in a buffer (pH 3.2) containing 20 ml of acetic acid, 47 ml of water, and 48 g of urea. De Zoeten and De Bruin (1961) used this buffer for the separation of the chains of insulin by paper electrophoresis. Location of the protein on the strips was accomplished by staining with Ponceau S or, where applicable, by visualization under long ultraviolet light. FTC insulins or FTC insulin chains were sometimes fixed (with some loss) on cellulose acetate strips by rapid washing with 2 M $BaCl_2$, which permitted subsequent photography or viewing under ultraviolet light. This treatment was particularly important in the case of A chain, which was eluted by the common acidic stains.

For quantitative analysis insulin was stained with Ponceau S and determined according to Carpenter and Hayes (1963). FTC insulins and FTC insulin chains were determined by elution of the excised fluorescent band in 1 N NaOH immediately after electrophoresis; the strips were shaken gently in a Dubnoff bath for 15 min at 37° followed by a similar period of standing at room temperature; the absorption of the eluates was measured at 492 mμ. Oxidative sulfitolysis of the FTC insulins did not affect, within the limits of the method, the recovery of absorption at 492 mμ.

Purity of insulin and FTC insulins was estimated by a simple but effective method in which advantage was taken of the ready electrophoretic separation and high sensitivity of detection of these preparations on cellulose acetate strips. Aliquots (1 μl) of the preparations dissolved in the electrophoretic buffer (pH 7.0) were applied to the strips in a narrow band 8–10 mm in length and were subjected to electrophoresis. As little as 0.25 mcg of insulin (stained with Ponceau S) and 0.125 mcg of any of the FTC insulins (visualized under ultraviolet light) were readily observable. Simultaneous electrophoresis of 20 μg of a purified insulin derivative with 0.125 or 0.25 μg of the possible contaminants (mixed and separately) provided clear visual evidence of electrophoretic purity in the range of 99%. It should be emphasized that this estimation of purity is based principally on charge differences and provides no evi-

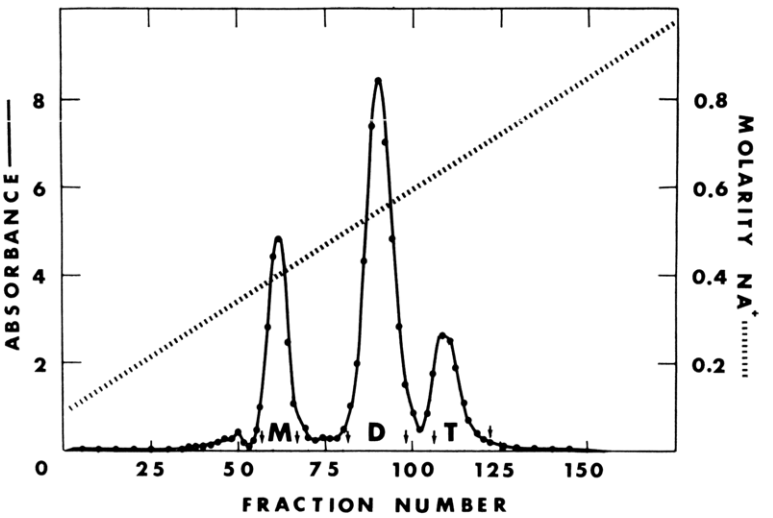

FIGURE 1: The chromatographic fractionation of about 30 μmoles of a 2:1 (molar ratio FITC:insulin) reaction mixture on a 1.5 $\times$ 90 cm column of DEAE-Sephadex (A-25). Flow rate was 25 ml/hr. Absorbancy of the 7-ml fractions was measured at 280 mμ. M = mono-FTC insulin, D = di-FTC insulin, and T = tri-FTC insulin. Arrows indicate fractions pooled for isolation.

dence of absolute purity for various FTC insulins which can be composed of three isomers.

Rate of Reaction of FITC with Insulin. Aliquots (100 or 200 μl) of the reaction mixture were removed at various intervals and immediately subjected to gel filtration on 6 $\times$ 60 mm columns of G-25 Sephadex in 0.01 M sodium phosphate buffer (pH 7.4) containing NaCl at 0.15 M. Within 2 min the FTC insulins were separated from excess reagent. The first fluorescent peak was collected in a tube containing 0.4 ml of 0.5 M sodium acetate buffer at pH 4.5. Immediate precipitation of the FTC insulins occurred. Precipitation of the derivatives was judged to be complete (after about 1 hr at 4°) by the absence of yellow color in the supernatant liquid. After centrifugation, the water-clear supernatant fraction was decanted and the pellet was cautiously dried *in vacuo*. The FTC insulins were dissolved in the same tubes at 10–20 μg/μl in the Tris–urea buffer (pH 7.0), were separated by electrophoresis on strips of cellulose acetate, and were determined spectrophotometrically following elution from the strips. Removal of excess reagent was found to be necessary since the electrophoretic mobility of the reagent was only slightly greater than that of tri-FTC insulin. However, unreacted insulin was determined by direct electrophoretic analysis of the reaction mixtures, since it did not precipitate quantitatively under the above conditions.

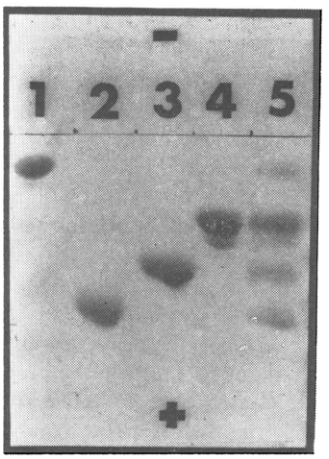

FIGURE 2: The separation of about 10 μg of FTC insulins by electrophoresis on cellulose acetate as visualized by staining with Ponceau S. Lane 1, unreacted insulin; lane 2, tri-FTC insulin; lane 3, di-FTC insulin; lane 4, mono-FTC insulin; and lane 5, crude FTC insulins from a 1:1 (molar ratio FITC: insulin) reaction mixture (supplemented with 1 mcg of tri-FTC insulin for photography). Line of application near cathode.

Sulfitolysis of FTC Insulin. The method of Bailey (1957) as modified by De Zoeten and De Bruin (1961) was used. FTC insulin at 10 mg/ml was reacted for 3–6 hr at 4° in a solution containing 0.03 M sodium tetrathionate, 0.075 M sodium sulfite, and 8 M urea adjusted to pH 7.6 with acetic acid. Aliquots were subjected to electrophoresis as above; sulfitolysis was found to be complete in about 3 hr as judged by electrophoretic comparison with nonreacted FTC insulins. However, trace amounts of fluorescent bands were sometimes observed migrating toward the anode with a mobility less than that of mono-FTC A chain. These possibly represented A chain with the intrachain disulfide intact and/or products of disulfide interchange resulting from incomplete oxidation with

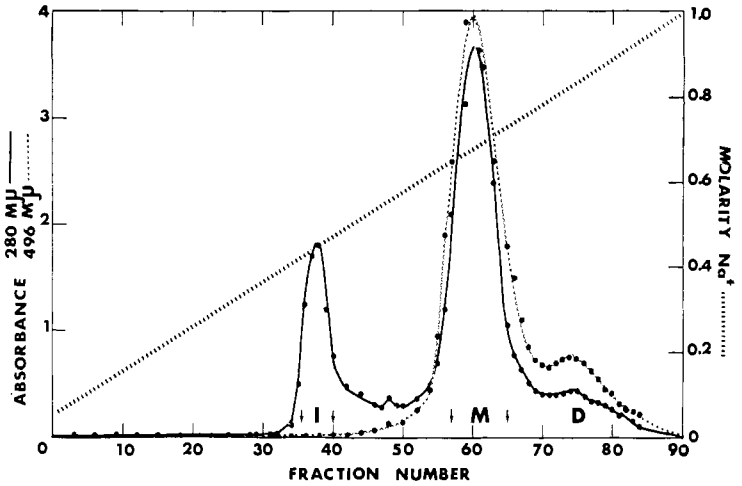

FIGURE 3: The chromatographic separation of about 17 μmoles of a 1:1 (molar ratio FITC:insulin) reaction mixture on a 2 × 45 cm column of DEAE-Sephadex (A-25). Flow rate was 20 ml/hr; 6-ml fractions were collected. I = insulin, M = mono-FTC insulin, and D = di-FTC insulin. Arrows indicate fractions pooled for isolation.

sodium tetrathionate (*cf.* Leach *et al.*, 1963). The latter reagent must be carefully protected from moisture prior to use.

Reaction of FTC Insulins with Trifluoroacetic Acid. Under conditions commonly used for the subtractive Edman degradation (Guidotti *et al.*, 1962), 0.1–0.5 μmole of an FTC insulin was treated with 0.5 ml of anhydrous trifluoroacetic acid at room temperature for 1 hr. The trifluoroacetic acid was removed *in vacuo* over KOH pellets and the yellow residue was dissolved in 0.5 ml of 0.2 N acetic acid. Split products were removed by extractions with 1 ml of ethyl acetate (equilibrated previously with 0.2 N acetic acid), until the organic phase contained no fluorescent material, and the remaining peptide material in the aqueous phase was dried *in vacuo*, hydrolyzed in 6 N HCl, and subjected to amino acid analysis.

Activity Determinations. Conventional mouse convulsion tests were kindly performed by the Lilly Research Laboratories. The immune hemolysis inhibition analyses were carried out according to the method of E. R. Arquilla (unpublished data). In this test five different dilutions (predetermined to result in a range between maximum to zero hemolysis) of pooled antiinsulin serum from mongrel guinea pigs were each preincubated with at least three different concentrations of standard insulin and of FTC insulins. An aliquot of a solution containing insulin-coated sheep red blood cells and guinea pig complement was then added to each tube, a second incubation was performed, and the amount of lysis (release of hemoglobin) was determined after centrifugation by measuring absorbance of the supernatant fluid at 570 mμ. Appropriate positive and negative controls were included in each experiment. In the absence of added insulin, the insulin antibodies combined with insulin fixed to the cells, activating the complement system which lysed the cell. In the presence of standard insulin or of native determinants still present in an FTC insulin, the antibodies were neutralized and hemolysis was inhibited. The amount of standard insulin and of FTC insulin necessary for the inhibition of 50% of the antibodies was determined and these values were used to calculate immunological activity.

Amino acid analyses were kindly performed by the Lilly Research Laboratories using the method of Spackman *et al.* (1958) with the Beckman Model 120C analyzer. Hydrolysis was performed in 6 N HCl at 110° for 30 hr in sealed, evacuated ampoules. However, since some amino acids are destroyed and since some peptide bonds are resistant to hydrolysis, all data were corrected by multiplying residues found by factors derived from the mean of seven analyses of standard beef insulin under identical conditions. The factors are: lysine, 1.06; histidine, 1.06; ammonia, 0.99; arginine, 1.04; aspartic acid, 1.02; threonine, 1.10; serine, 1.23; glutamic acid, 0.99; proline, 0.88; glycine, 0.95; alanine, 0.95; half-cystine, 1.06; valine, 1.05; isoleucine, 1.25; leucine, 1.00; tyrosine, 1.12; and phenylalanine, 0.97.

Sedimentation Coefficients. Sedimentation velocity studies were performed on a Spinco Model E analytical ultracentrifuge using a schlieren optical system. Insulin preparations at 3 mg/ml were examined in 0.13 M $NaHCO_3$–Na_2CO_3 buffer (pH 9.1) containing 0.11 M NaCl.

Results

Preparation of FTC Insulins. Chromatography of a 2:1 (molar ratio FITC:insulin) reaction mixture showed the presence of three FTC insulin components that were judged from their order of elution to be mono-, di-, and tri-FTC insulins (Figure 1). This identification was confirmed by electrophoresis (Figure 2, lanes 2–4) and the relative purity of the fractions

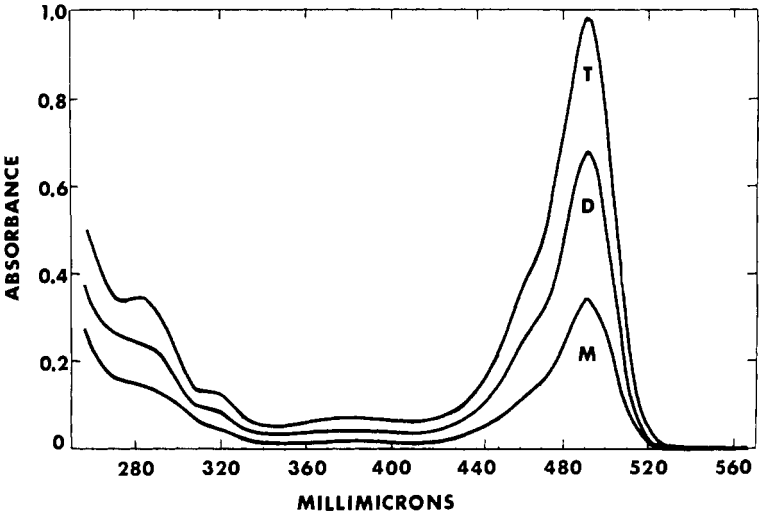

FIGURE 4: Absorption spectra of mono- (M), di- (D), and tri- (T)FTC insulins in 1 N NaOH.

was estimated by the electrophoretic method to be at least 99%. The minor component observable in mono-FTC insulin (lane 4) was judged to be monodesamido,mono-FTC insulin on the basis of its electrophoretic mobility, and was not considered to be an impurity for the purposes of this work. The starting insulin (lot 795372) contained about 10% monodesamido-insulin which may or may not be observed in the purified fractions, depending on the efficiency of the chromatography. The chromatographic fractionation (Figure 3) of a 1:1 reaction mixture (molar ratio FITC:insulin) resulted in the separation of two major fractions, unreacted insulin and mono-FTC insulin (Figure 2, lanes 1 and 4). A small amount of di-FTC insulin was observed; however, the trace amount of tri-FTC insulin known to be formed under these conditions was not found in this particular fractionation. Biologic and immunologic activities were determined for unreacted insulin and FTC-insulins isolated from two of the crude reaction mixtures (Table I). The unreacted insulin was of particular interest as an internal control on the possible effects of the conditions of chemical reaction and column chromatography on the hormone. The biologic and immunologic activities of the unreacted insulin were virtually indistinguishable from crystalline insulin (Table I). In addition, the unreacted insulin was also crystallized by the method of Schlichtkrull (1956) in high yield, forming regular rhombohedra characteristic of zinc insulin. These data suggest that insulin was not adversely affected by the above conditions and that structural changes in the FTC insulins may probably be ascribed to the effects of the substitution of the FTC groups in the insulin molecule.

TABLE I: Activity of Insulin Derivatives.[a]

Preparation	IU/mg ± Std Error of Mean	
	Mouse Convulsion Test	Immune Hemolysis Inhibn Assay
Unreacted insulin	21.6 ± 4.6	21.6
Unreacted insulin	22.9 ± 5.6	22.9
Mono-FTC insulin	10.1 ± 1.6	20.2 ± 1.1
Mono-FTC insulin[b]	7.8 ± 1.2	9.6
Di-FTC insulin	0.62 ± 0.1	6.9 ± 1.0
Di-FTC insulin[b]	0.88 ± 0.2	2.2
Tri-FTC insulin	0.1	0.76 ± 0.16
Tri-FTC insulin[b]	0.13 ± 0.07	0.46

[a] Not corrected for moisture. [b] Prepared with FITC which was a mixture of isomers I and II.

Absorption Spectra of FTC Insulins. Absorption spectra of FTC insulins in 1 *N* NaOH (Figure 4) were determined using a Cary Model 14 spectrophotometer. According to McKinney *et al.* (1964) such alkaline treatment would be expected to quantitatively release fluorescein amine. The molecular extinction coefficients (ϵ) at 492 mμ were calculated for mono-, di-, and tri-FTC insulins to be about 68,000, 135,000, and 195,000, respectively. The ratio of the coefficients was found to be 1.0:2.0:2.9, very close to the expected ratio of 1:2:3. It is evident that the spectra are qualitatively similar with other maxima at about 380, 317, and 283 mμ and a shoulder around 460 mμ. In 0.11 M sodium phosphate buffer (pH 7.5) the mono-, di-, and tri-FTC insulins provided $\epsilon_{19\mathfrak{E}}$ of about 55,000, 113,000, and 162,000, respectively. Other less intense maxima were noted at about 370, 321, and 272 mμ. The above data were not corrected for water content of the FTC insulins, which later was found to be about 15% or greater.

Electrophoretic Analysis of Insulin and FTC Insulins. The availability of highly purified FTC insulins per-

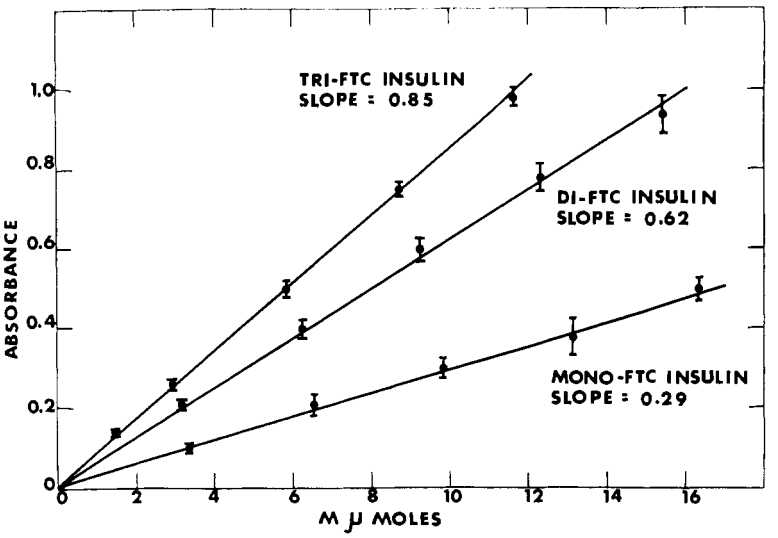

FIGURE 5: Recovery of purified mono-, di-, and tri-FTC insulins from strips of cellulose acetate. Absorbance measured at 492 mμ. Bars represent confidence interval at $P = 0.95$. Slope calculated from line of best fit.

mitted the determination of their recovery from strips of cellulose acetate (Figure 5). The ratios of the slopes of the linear plots of the recovery of absorbance from mono-, di-, and tri-FTC insulins was 1.0:2.1:2.9, showing reasonably close adherence to the theoretical values and to the 1:2:3 relationship observed in the absorption spectra at 492 mμ. Comparison of the absorbancies showed that a rather consistent loss of about 15% of the absorbance was incurred during the recovery experiments. Insulin stained with Ponceau S provided a linear recovery that compared well with the data of Carpenter and Hayes (1963).

Localization of the FTC Groups. Tietze *et al.* (1961) had found the terminal amino groups of the phenylalanyl and glycyl residues to be reactive to FITC. Treatment of FTC insulins with trifluoroacetic acid would be expected, in direct analogy to the Edman degradation, to result in the cyclization and loss of those N-terminal residues that had formed a thiocarbamyl linkage. Amino acid analyses of the remaining polypeptide material (Table II) showed the loss of about 0.7 residue of phenylalanine from mono-FTC insulin. About 0.8 residue of glycine and approximately one residue of phenylalanine were removed from di-FTC insulin. These data strongly suggested that the FTC group in mono-FTC insulin was located predominantly on the terminal amino group of the phenylalanyl residue, while both terminal amino groups were extensively modified in the di-FTC insulin. The low values for valine and isoleucine may probably be explained on the basis that hydrolysis of the preparations was inadvertently performed for 24 hr instead of 30 hr. The correction factors described previously were used as a basis for the best possible estimate of residues under the circumstances. Reasons for the small loss of tyrosine were unknown; however the finding was not too surprising in view of the treatment with trifluoroacetic acid and subsequent acid hydrolysis in the presence of small amounts of FTC and thiohydantoins. As considered later, these data most likely do not suggest that tyrosyl residues underwent appreciable reaction with FITC under these conditions.

Substitution by FITC of the ε-amino group of the lysyl residue in the penultimate position of the

TABLE II: Partial Amino Acid Analysis of FTC Insulins Following Treatment with Trifluoroacetic Acid.

Amino Acid	Number of Residues Found[a]		No. of Residues in Insulin
	Mono-FTC-Insulin	Di-FTC Insulin	
Aspartic acid	3.0	3.0	3
Threonine	1.0	1.0	1
Serine	2.9	2.9	3
Proline	1.1	1.0	1
Glutamic acid	7.1	7.0	7
Glycine	3.9	*3.2*	4
Alanine	3.1	3.0	3
Valine	4.6	4.6	5
Isoleucine	0.8	0.8	1
Leucine	5.9	6.0	6
Tyrosine	3.6	3.5	4
Phenylalanine	*2.3*	*2.0*	3

[a] Calculated on the basis that bovine insulin contains 13 residues of Glu + Leu; corrected as indicated previously although acid hydrolysis was inadvertently carried out for 24 hr instead of 30 hr.

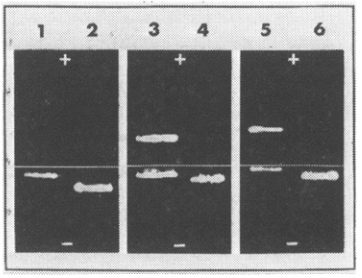

FIGURE 6: A photograph under ultraviolet light of the electrophoretic separation of the products of oxidative sulfitolysis of 20 mcg of FTC insulins. Line of application near center of strip. Lanes 2, 4, and 6 contain untreated mono-, di-, and tri-FTC insulins for purpose of determining completeness of sulfitolysis. Lanes 1, 3, and 5 contain mono-, di-, and tri-FTC insulins, respectively, subjected to oxidative sulfitolysis. Lane 1, mono-FTC B chain; lane 3, on cathode side is mono-FTC B chain with trace of di-FTC B chain adjacent and on anode side is mono-FTC A chain; and lane 5, on cathode side is di-FTC B chain and on anode side is mono-FTC A chain. Identification of the chains was made separately through comparison with the mobilities of purified A and B chains of insulin. Strip containing lanes 3 and 4 was inadvertently run for 1 hr instead of 1.5 hr in the case of the others.

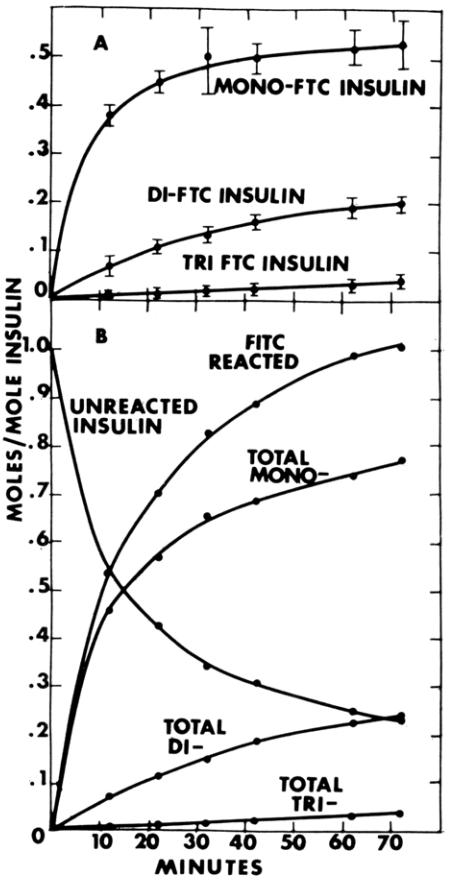

FIGURE 7: Rate of insulin formation and calculated results. (A) The rate of formation of mono-, di-, and tri-FTC insulins in a 1:1 (molar ratio FITC:insulin) reaction. Bars indicate confidence intervals at $P = 0.95$. (B) Results calculated from data in Figure 7A. Total mono-FTC indicates total monosubstitution occurring in reaction mixture and represents the sum of mono-, di-, and tri-FTC insulins determined. Total di-FTC indicates total disubstitution and was calculated on the same basis. Total tri-FTC represents total trisubstitution, which is the same as the rate of formation of tri-FTC insulin.

B chain had not previously been detected (*cf.* Tietze *et al.*, 1962). However, our finding of a tri-FTC insulin clearly showed that a third FTC group had been introduced into the molecule, and the ε-amino group represented a likely site of substitution. Several reports (*e.g.*, Harris and Li, 1952; Young and Carpenter, 1961) had provided ample evidence that the Lys–Ala (B29-30) bond is rapidly cleaved by trypsin, releasing free alanine. However, if the ε-amino group were treated with FITC, the bulky, acidic substituent would provide a block to the action of trypsin, in which case alanine would not be released. The results of such experiments (Table III) demonstrated that tryptic digestion resulted in the release of about 1 mole of alanine each per mole of control insulin, mono-FTC insulin, and di-FTC insulin. Only 0.11 mole of alanine was found in the tri-FTC insulin digest and this was considered to be a maximal figure in view of the small quantities involved and the difficulties of excluding traces of random amino acids from such a digest. Although the variability of the duplicate analyses (performed on a blind basis and separated by a month) precluded a precise quantitative interpretation, the data clearly showed that mono- and di-FTC insulins bore little or no substitution at the ε-amino group of lysine, while the tri-FTC insulin was probably substituted in this position to the extent of at least 90%.

TABLE III: Release of Alanine from Insulin and FTC Insulins by the Action of Trypsin.

Preparation	μmoles Reacted	μmoles of Alanine Found	Mean Moles of Alanine Released/ Mole of Insulin
Insulin	0.15	0.14, 0.15	0.97
Mono-FTC insulin	0.16	0.13, 0.18	0.97
Di-FTC insulin	0.14	0.12, 0.16	1.00
Tri-FTC insulin	0.13	0.01, 0.02	0.11

Confirmation of these findings was obtained by

TABLE IV: The Analysis of Separated FTC Chains Following Oxidative Sulfitolysis of FTC Insulins.

Preparation	FTC A Chain		FTC B Chain			
			Mono-		Di-	
	Mean	Confidence Limits[a]	Mean	Confidence Limits[a]	Mean	Confidence Limits[a]
Mono-FTC insulin	0.05	0.04–0.06	0.98	0.94–1.02	0	
Mono-FTC insulin[b]	0.23	0.20–0.26	0.77	0.73–0.81	0	
Di-FTC insulin	1.02	0.99–1.05	0.97	0.94–1.00	0.06	0.05–0.07
Di-FTC insulin[b]	0.83	0.77–0.89	0.95	0.90–1.00	0.14	0.11–0.17
Tri-FTC insulin	0.99	0.95–1.03	0		0.90	0.87–0.93
Tri-FTC insulin[b]	0.99	0.82–1.16	0.14	0.02–0.26	0.86	0.76–0.96

[a] $P = 0.95$. [b] Prepared with FITC reagent which was a mixture of isomers I and II.

oxidative sulfitolysis of mono-, di-, and tri-FTC insulins, separation of the chains by electrophoresis (Figure 6), and determination of the relative amounts of mono-FTC A and B chains, and di-FTC B chain in each FTC insulin (Table IV). In some preparations of mono-FTC insulin virtually all the fluorescence resided in the mono-FTC B chain (*cf.* Figure 6) and was accountable (from the above data) almost completely as substitution of the terminal phenylalanyl residue.

In other mono-FTC insulin preparations (particularly but not exclusively those made with reagent containing isomer II) about 0.2 mole of mono-FTC A chain and 0.8 mole of mono-FTC B chain were recovered (*cf.* Table IV). On the basis of the tryptic and acid degradation studies the A chain substitution was interpreted as substitution of the terminal glycyl residue. Possible reasons for the seeming variability in reactivity of the glycyl residue are manifold and are under study. In any case the terminal amino group of the phenylalanyl residue displayed in all cases a much higher rate of reactivity to FITC than the other amino groups.

In di-FTC insulin, essentially all the additional substitution was located in the A chain. Again, the only reasonable explanation involved reaction of the terminal glycyl residue. Tri-FTC insulin was considered to involve nearly full substitution at all three amino groups, giving about equimolar amounts of mono-FTC A chain and di-FTC B chain. Minor substitutions at arginine, histidine, and tyrosine were a possibility.

Rate of Reaction with FITC. Insulin was found to react remarkably fast with FITC at pH 9.1 (Figure 7A,B). Also of particular interest was the essentially complete utilization of the reagent in the presence of about 20% unreacted insulin. On the basis of the data previously described concerning the locations of the FTC groups in mono-, di-, and tri-FTC insulins, the kinetic data were best interpreted to mean that total monosubstitution (Figure 7B) represented primarily the rate of phenylalanine substitution, total disubstitution represented mostly the rate of substitution of the glycyl residue, and tri-FTC insulin represented the rate of substitution of the ε-amino group of the lysyl residue.

Sedimentation Studies. Zinc insulin, zinc-free insulin, and mono-FTC insulin were examined under the conditions of pH and ionic strength used in the reaction with FITC, and their $s_{20,w}$ values were compared with previous data (Table V). It was interesting that under these conditions mono-FTC insulin was found to exhibit a lower $s_{20,w}$ than the zinc insulin from which it was made. This finding was explainable partly on the basis that subsequent purification of the derivative had resulted in a loss of zinc. However, such an explanation was not acceptable since the $s_{20,w}$ found for zinc-free insulin was higher than for mono-FTC insulin. Evidently, substitution of the terminal phenyl-

TABLE V: Svedberg Constants ($s_{20,w}$) of Insulin and Derivatives.

Compound	pH	Concn (mg/ml)	$s_{20,w}$ This Study	$s_{20,w}$ Slobin and Carpenter (1966)
Zinc-free insulin[a]	9.1	3.0	1.64	–
Zinc-free insulin	9.0	3.4	–	1.65
Zinc insulin	9.1	3.0	3.32	–
Mono-FTC insulin	9.1	3.0	1.22	–
Desalanine-des-asparagine-insulin	9.0	5.1	–	1.17

[a] Another component ($s_{20,w}$ = 2.40 S) was also observed in this preparation, suggesting that all the zinc had not been removed.

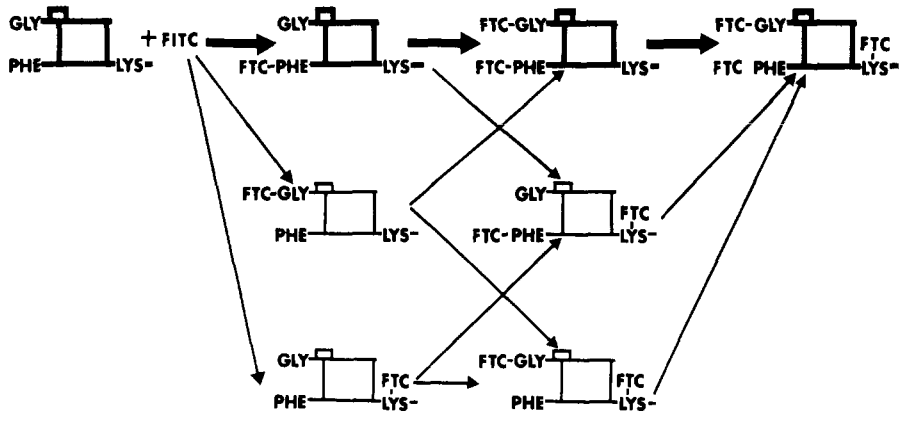

FIGURE 8: Possible routes of reaction of FITC with the amino groups of insulin. Major observed products indicated by bold figures.

alanyl residue with FITC was accompanied by a decrease in intermolecular interactions.

Discussion

Reaction of FITC with Insulin. Aside from the expected reaction of FITC with amino groups, substitution could have occurred on the side chains of tyrosyl, histidyl, and arginyl residues. However, Tietze *et al.* (1962) found no evidence for such side reactions. Furthermore, since each such substitution would have increased the net negative charge of the protein, appreciable side reactions should have been readily detectable through the chromatographic and electrophoretic methods used in the present study. Particularly pertinent was the fact that no FTC insulins having an anionic electrophoretic mobility exceeding tri-FTC insulin were detected, even when a 4:1 molar ratio of FITC:insulin was reacted for 8 hr, forming tri-FTC insulin as a predominant product. The consistency of the observations provided evidence that little, if any, substitution occurred at functional groups other than the amino positions.

The possible routes of reaction of FITC with the amino groups of insulin are depicted in Figure 8, with the major observed pathway indicated by the bold figures. Of the three possible isomers of mono-FTC insulin, the product of substitution of the phenylalanyl residue was found to predominate; this was in agreement with the findings of Tietze *et al.* (1962). Several explanations are possible in regard to this finding that the terminal amino group of the phenylalanyl residue was much more reactive to FITC than were the amino groups of the glycyl or lysyl residues.

It is pertinent to note that titration and thermodynamic studies (Tanford and Epstein, 1954; Gruen *et al.*, 1959) had established that the three amino groups of insulin ionized in a normal manner and that the lysyl amino group was not involved in strong interactions (as H bonding) with neighboring groups. On this basis all three amino groups would be free to react with FITC.

Therefore, if one considered, as did Klugerman (1966), that the reaction probably involved an electrophilic attack of a positively charged isothiocyanate group on the amino group of highest electron availability, then clearly the pH of the reaction and the pK's of the amino groups were important factors. The pH of the reaction mixture was held relatively constant at 9.1 through use of a buffer. From titration studies (*e.g.*, Tanford and Epstein, 1954; Gruen *et al.*, 1959; Fredericq, 1954) the pK's of the α- and ϵ-amino groups were estimated at 7.5 and 9.6–11.1, respectively. Therefore, the slow reaction of the lysyl residue with FITC may probably be explained largely on the basis that the ϵ-amino group was mainly protonated at pH 9.1. However, steric or other factors probably contributed to the relative lack of reactivity. Such factors could involve the size and ionic character of the reagent as well as the environment in the protein around the lysyl side chain.

If both α-amino groups have the same intrinsic pK, the slower reaction of the glycyl residue cannot be explained on the basis of a lower electron availability. It is of interest to note that Christensen (1951) reported that phenyl isothiocyanate reacted more readily with the amino-terminal glycyl residue rather than with the phenylalanyl residue in insulin. Apparently stereochemical factors play a role in the reaction. However, the present data suggested another possible interpretation for the selectivity. The finding that certain purified mono-FTC insulin preparations were modified almost exclusively at the N-terminal phenylalanyl residue implied that the rate of reaction at this site was so much more rapid than at the other two sites that a nearly stoichiometric reaction would be expected between FITC and the N-terminal phenylalanyl residue in a 1:1 (molar ratio reagent:insulin) reaction mixture. The observed composition of such a reaction mixture and the kinetic data (Figure 7B) clearly showed that the reaction at phenylalanine was not stoichiometric

under these conditions, but that appreciable amounts of di- and tri-FTC insulins were formed and about 20% of the insulin was not reacted. A reasonable explanation for this result is that modification of the phenylalanyl residue resulted in an increase in the rate of reactivity of the glycyl residue, perhaps to the same order of reactivity as the phenylalanyl residue. The rate of substitution of the lysyl residue (*cf.* Figure 7B) was found to be slow and essentially linear, and was apparently unaffected by substitution of the terminal amino groups. This finding was also consistent with the idea that the lysyl amino function was poorly reactive because it was largely in ionized form.

The manner in which the FTC phenylalanyl substitution could influence the rate of reaction of the other N-terminal group is of interest. The sedimentation studies (Table V) showed that zinc insulin (under the usual conditions of the FITC reaction) was highly aggregated, zinc-free insulin was less aggregated, and mono-FTC insulin was probably in monomer form ($s_{20,w}$ = 1.22 S). Also of pertinence were preliminary studies indicating that the rates of reactivity of the two terminal amino groups were about the same when the reaction was carried out in 7 M urea. A possible interpretation of these data is that the glycyl amino group in aggregated insulin was oriented in such a manner that the approach of FITC was hindered; however, derivatization of the phenylalanyl residue was accompanied by dissociation, which may have been related to a structural change making the glycyl amino group more readily available to the reagent.

A working hypothesis of the reaction consistent with our data would suggest that the initial attack of the reagent is at the phenylalanyl amino group, which must be freely accessible to FITC even in highly aggregated insulin; the first substitution leads to a structural alteration, perhaps mediated by disruption of intermolecular forces that render the glycyl amino group more reactive; the ϵ-amino function reacts very slowly with FITC either because it is largely protonated or because of steric hindrance. Aspects of the hypothesis may be tested, and are under investigation. For example, the rates of reaction of FITC with glycyl and phenylalanyl peptides are being studied.

Structure–Function Relationships (*cf. Table I*). Substitution of the terminal amino group of the phenylalanyl residue altered the structure of the hormone in such a manner that about half the biologic activity remained. Maintenance of a precise conformation of the N-terminal portion of the B chain was clearly not vital to the function of the hormone; however, this structural alteration may have resulted in a lower binding affinity for a receptor. It is interesting that insulin antibodies scarcely distinguished this derivative from insulin, suggesting that this particular pool of guinea pig antibodies was directed mostly toward determinants located at a distance from the N-terminal phenylalanyl residue. Conversely, the amino-terminal end of the B chain may be either poorly antigenic or simply not on the surface of the antigen. This could be in part a consequence of the fact that a highly aggregated antigen (alum-precipitated insulin suspended in Freund's adjuvant) was used, which may have resulted in a "masking" of the terminal amino group. However, such a generalization could only be partly correct since strain 2 guinea pigs produced antibodies that were directed toward the amino-terminal end of the molecule (E. R. Arquilla, unpublished data). Also, additional substitution of the amino-terminal glycyl residue (as in di-FTC insulin) diminished immunological activity. In any case, mono-FTC insulin would appear to be more suitable for localization studies since considerable biologic activity and immunologic competence are retained.

Factors that cannot be ignored when attempting to relate structure and function are the bulkiness and hydrophobic character of the largely planar fluorescein and the increase in anionic character that accompanies each substitution. These factors alone could be responsible for changes in biologic and immunologic reactivities.

When both amino terminals were substituted, as in di-FTC insulin, a small but definite biologic activity remained. Presumably the active site remained intact, but the structure was altered sufficiently that hormonal action was very inefficient. The effect of disubstitution on interaction with antibodies is quite interesting; in effect two-thirds of the antibodies no longer recognized the molecule. A possible interpretation is that the N-terminal portion of the A chain is in close proximity to a major determinant.

Substitution of the ϵ-amino group of the lysyl residue in addition to the N terminals resulted in a molecule which was essentially devoid of biologic activity and which, in effect, was recognizable by only 3% of the antibodies. This finding could be a consequence of the increased negativity of the molecule, or it may also be attributed to disruption of structure in a particularly vital part of the molecule. Other studies have shown that relatively minor alterations of C-terminal structures of insulin lead to hormonal inactivation, *e.g.*, removal of the two C-terminal residues (Slobin and Carpenter, 1963) or of eight residues from the C terminus of the B chain (Young and Carpenter, 1961).

Acknowledgments

The authors are grateful to Dr. Alexander N. Glazer, Department of Biological Chemistry, for use of the Cary spectrophotometer, and to Mr. Douglas Brown of the same department for the ultracentrifugal analyses.

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Isolation of a New Glycoprotein-a and a γG-Globulin from Individual Cow Milks*

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ABSTRACT: A new protein, tentatively named glycoprotein-a, has been isolated from bovine milk. It shows a single band by gel electrophoresis at pH 4.3; however, on electrophoresis at alkaline pH, several closely spaced bands, attributable perhaps to genetic polymorphs, are evident. Glycoprotein-a has a sedimentation coefficient of 4.0 S and a minimum molecular weight of the order of about 48,000 based on the presence of a single methionine residue per molecule. A milk γG-globulin has also been isolated and characterized. Although it is closely related to milk pseudoglobulin in most of the chemical and serological properties investigated, differences in behavior in gel electrophoresis were observed. Comparisons of properties of γG-globulins prepared from bovine colostrum and blood indicate virtual identity of these proteins; minor chemical and immunological differences between these and the milk γG-globulin and pseudoglobulin were found.

Immune proteins from cow's milk and colostrum were isolated by classical methods of fractionation with ammonium sulfate by Smith (1946, 1948). Smith prepared and characterized euglobulin and pseudoglobulin from colostrum and milk and compared the properties of the purified proteins with those of immune globulins isolated from bovine blood. He found that all of these globulins were closely related, immunologically, but that they were not identical in physical and chemical properties.

We have applied the more refined methods now available for fractionating proteins to a reinvestigation of the proteins of the γ-globulin[1] fraction of cow's milk. In so doing, a new glycoprotein was found in

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[1] We follow the nomenclature for human immunoglobulins proposed in *Bull. World Health Organ. 30*, 447 (1964). γG-Immunoglobulin (or γG-globulin) denotes that class of immunoglobulins often designated as 7S γG-globulin.