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Use of Resonance Interaction in the Study of the Chain Folding of Insulin in Solution†

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ABSTRACT: Certain relationships of the chain folding within the monomer of bovine insulin in solution have been studied by covalently labeling the three amino groups of insulin with an optical probe. Three purified derivatives containing from one to three groups of the symmetric dye fluorescein isothiocyanate bound to the B-1; B-1 and A-1; B-1, A-1, and B-29 residues (Bromer *et al.* (1967), *Biochemistry 6*, 2378) were examined by absorption and circular dichroism spectroscopy between 400 and 550 nm. The results were interpreted according to the simplified exciton model. This approach was confirmed by the examination of three model compounds prepared by the covalent addition of the dye group to models of the insulin sequence at the sites of substitution and a fourth model compound prepared by disubstitution of L-lysine. These monosubstituted model compounds had absorption spectra similar to the free dye, maxima at 489–492 nm, were nearly optically inactive (400–550 nm), and had similar probe group pK values, average 6.46 ± 0.03. In contrast, disubstituted lysine was 54% hypochromic, revealed two new absorption bands at ±866 cm^{-1} about the monosubstituted maximum (*ca.* 20,320 cm^{-1}) with molar circular dichroism values of +15.4 and −18.0 M^{-1} cm^{-1}, respectively, had a single pK of 7.28 ± 0.06, showed mutarotation, was poorly fluorescent and revealed a maximum probe separation of 7.4 Å all in agreement with a dye–dye complex involving exciton formation. The insulin derivatives were shown to be monomeric below 5×10^{-6} M by comparison of sedimentation (10^{-2}–10^{-3} M) and Beer's law behavior (10^{-6} to 4×10^{-2} M). Monomeric di- and trisubstituted insulins revealed circular dichroism maxima at about ±620 and ±230 cm^{-1} about the monosubstituted maximum with molar circular dichroism of −10.0, +21.5, −32.0, and +91.5, respectively. The results revealed that the B-1 to A-1 and A-1 to B-29 dye group separations were at most about 7.6 and 10.5 Å, respectively. These results support certain tertiary relationships previously postulated as necessary for high immunological and biological activity (Arquilla *et al.* (1969), *Diabetes 18*, 193) and it is argued that much of the crystalline structure of rhombohedral two-zinc porcine insulin remains invariant in solution.

Previous investigations of the structure–function relationships of insulin by immunochemical, biological and physical techniques (Arquilla *et al.*, 1967; Bromer *et al.*, 1967; Mercola *et al.*, 1967, 1969; Morris *et al.*, 1968–1970a,b) have led to a model of certain chain relationships and conformational features necessary for high immunological and biological activity (Arquilla *et al.*, 1969). In an effort to refine these relationships we have explored a new use of a spectroscopic technique involving exciton formation and have initiated its application to insulin. The stimulus for this approach was the availability of the techniques for the preparation and purification of three insulin derivatives covalently labeled with one

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to three molecules of fluorescein isothiocyanate[1] at the three amino groups: B-1; B-1 and A-1; B-1, A-1, and B-29; mono-, di-, and tri-FTC insulins, respectively (Bromer *et al.*, 1967). In particular FITC is a symmetric dye and is therefore optically inactive. Further, the intense visible absorption and fluorescence emission bands are remote from the absorption and emission bands of insulin. Thus the optical properties, in particular induced optical activity, of the dye groups cannot be confused with the optical properties of insulin. Induced optical activity has been measured by circular dichroism. The origin of optical activity for each dye group has been identified as due to either dye–protein interactions or dye–dye (exciton) interactions. Dye–dye interactions represent, by far, the most interesting possibility since they may be described in terms of a simplified exciton theory (Tinoco, 1963) and their mutual separation in Ångstroms may be evaluated directly from the observed spectral properties.

The expected results due to the interaction of two or more identical groups containing strong electric dipole transitions have been summarized (Kasha, 1959, 1963; Bradley *et al.*, 1963). In the molecular exciton model, only the dipole–dipole intermolecular potential term is used, as an approximation, since, as in dye molecules, the electronically excited state is of the electric dipole type. The characteristic results for a dimer are the splitting of the monomer absorption band, ν_0, into two new bands, ν_+ and ν_-, symmetrically spaced about ν_0 with separation $(\nu_+ - \nu_-)$ that is proportional to the square of the monomer dipole transition moments, μ, and inversely proportional to the third power of separation, R

$$h(\nu_+ - \nu_-) = V = \frac{\mu^2}{R^3}G \qquad (1)$$

where V is the energy of interaction and may be assessed directly if ν_+ or ν_- are observable ($|\nu_+ - \nu_-| = |\pm(2\nu_0 - 2\nu_\mp)|$) and G is a dipole geometry factor (all in cgs units). Since the maximum possible value of G is 2 (*e.g.*, Bradley *et al.*, 1963) and since μ can be estimated from the resolved absorption spectrum (*e.g.*, Moscowitz, 1960), the maximum possible separation can be estimated from a measurement of ν_+ or ν_-.

This technique is distinct from the resonant transfer approach of Stryer and others (*e.g.*, Stryer, 1968) in that identical probes are used, it is independent of absorption–fluorescence spectral overlap, and it is insensitive to separations much greater than 10–15 Å. The use here of two or more bound probes has not previously been applied to globular proteins.

The quantities ν_+ and ν_- are not generally accessible to ordinary absorption measurements owing to the broad nature of solution spectra, changes in intensity upon complex formation, and limiting geometries relating the dipoles but may often be determined by recourse to CD measurements. The corresponding exciton contribution to optical activity (Moffitt *et al.*, 1957; Tinoco *et al.*, 1963; Bradley *et al.*, 1963) has been summarized in a simplified form for dimers (Tinoco, 1963). The expectations are the production of two CD bands of opposite sign centered at ν_+ and ν_-. Further, as a consequence of the sum rule, any physical variable leading to a change in one band is expected to be accompanied by a reciprocal change in the opposite signed band.

In order to assure that the qualitative expectations are reasonable and in order to obtain a semiquantitative confirmation of the theory in the case of the probe and conditions studied here, a series of four model compounds containing one or two dye groups was prepared and examined. Three of the compounds contained the dye group bound to models of the insulin sequence at the sites of substitution. The fourth compound was prepared by disubstitution of L-lysine at the α- and ϵ-amino positions. A number of physical criteria indicated that disubstitution led to van der Waals contact of the dye groups and the resultant optical properties were shown to support the exciton model. These concepts were applied to the monomeric forms of the FTC insulins and the results are discussed in terms of their meaning for the native structure of insulin in solution.

Materials and Methods

FITC isomer 1 was obtained from Baltimore Biological Laboratories (Baltimore, Md.) as an orange-green powder. Previous examination of FITC from this source by thin-layer chromatography showed that this preparation was chromatographically pure (Bromer *et al.*, 1967). The result was confirmed by examination of one lot (8011468) used extensively here by ascending paper chromatography according to a procedure modified from Corey and McKinney (1962). The molar absorptivity of FITC in 0.11 M Na_2HPO_4 was constant for pH values ≥ 8.5 and concentrations $<10^{-5}$ M and was found to be $8.5 \pm 0.08 \times 10^4$ cm^{-1} M^{-1} in good agreement with reported values (McKinney *et al.*, 1964; Klugerman, 1966).

Nonfluorescent aminofluorescein, used as a test of possible spectral artifacts of FTC group fluorescence, was prepared from FITC by dissolution in 0.25 N NaOH for 15 min followed by neutralization with HCl and precipitation with acetic acid and exhibited the appearance and spectral properties described (McKinney *et al.*, 1964; Klugerman, 1966).

The peptides[2] and amino acids used for model preparation were L-phenylalanyl-L-alanine (lot 87B-1780), glycyl-L-isoleucine (lot 105B-1818), and N^α-acetyl-L-ornithine (lot 106B-1470), Sigma Chemical Co. (St. Louis, Mo.), and were used as received. L-Lysine was obtained from Calbiochem (Los Angeles, Calif.).

d-10-Camphorsulforic acid (lot 1-618), J. T. Baker Chemical Co. (Cleveland, Ohio), analyzed, was stored *in vacuo*. Guanidine hydrochloride, reagent grade, was obtained from Sigma Chemical Co. Solutions were clarified by low-speed centrifugation before use. Urea, Ultra Pure Spectrograde, was obtained from Mann Chemical Co. Crystalline bovine insulin used for carbamylation was ten-times-recrystallized Novo insulin (lot 27667, 24.5 IU/mg) and was kindly supplied by Dr. J. Schlichtkrull, Novo Industries, A/S, Copenhagen, Denmark.

Preparation of the FTC Insulins. The preparation and characterization and the biological and immunological properties of the FTC-insulins studied here have been described in detail (Bromer *et al.*, 1967; Arquilla *et al.*, 1969).

[1] Abbreviations used are: CD, optical circular dichroism; DFL, N^α,N^ϵ-difluoresceinthiocarbamyl-L-lysine; FITC, fluorescein isothiocyanate isomer I; FTC, fluoresceinthiocarbamyl; FPA and FGI, fluoresceinthiocarbamyl-N^α-L-phenylalanyl-L-alanine, N^α-glycyl-L-isoleucine respectively; FAO, N^δ-fluoresceinthiocarbamyl-N^α-acetyl-L-ornithine. Correlation coefficient of best linear fit by the method of least squares, r; wave number, ν (cm^{-1}).

[2] Although phenylalanine and alanine are frequently occurring residues at positions B-1 and B-2, respectively, for known sequences of insulin, the peptide phenylalanylalanine does not correspond to a known sequence. The ornithine derivative was chosen over lysine because it is a commercially available N^α-protected derivative and because it has one less methylene group and therefore provides a somewhat more critical test for the influence of the asymmetric carbon atom.

Two modified procedures were employed for the preparation of tri-FTC insulin in high yield. In the first modification di-FTC insulin replaced insulin in a reaction mixture (10 mg/ml) containing a 3 molar excess of FITC and was incubated for 3 hr at 37° with gentle shaking. In the second method a solution of the FITC reactant was added sequentially to a 20-mg/ml solution of insulin in four steps separated by thirty minute intervals, bringing the initial mole ratio of 1:1 to a final ratio of 1:4 (insulin:FITC). All other conditions were as described (Bromer *et al.*, 1967). The fractional distribution of the FTC groups on the A and B chains (Bromer *et al.*, 1967) observed here were 0.47:0.53, 0.30:0.69, and 0.31:0.68.

The mono-FTC insulins are known to be composed of a variable distribution of two monosubstituted isomers (Bromer *et al.*, 1967). The material studied here is characterized by 98 ± 4% (± indicates 0.95 confidence interval) phenylalanyl substitution and 5 ± 1% glycyl substitution (Bromer *et al.*, 1967).

The molar absorptivities of the FTC insulin derivatives were computed on the basis of semimicro-Kjeldahl total nitrogen determinations (Ballentine, 1957) of mono-FTC insulin using a figure of 14.6% nitrogen content and absorbance measurements in 0.11 M Na_2HPO_4 in the pH range of constant absorbance (≥pH 9.0) at concentrations that obeyed Beer's law. Triplicate determinations yielded an average value of 6.68×10^4 cm^{-1} M^{-1} with a maximum range of 4% in good agreement with the previous gravimetric determinations of 6.8×10^4 cm^{-1} M^{-1} (Bromer *et al.*, 1967). From the known ratio of absorbance of mono-:di-:tri-FTC insulin of 1:2:2.9 observed previously (Bromer *et al.*, 1967) and confirmed here for pH >9.0 the molar absorptivities of 13.4×10^4 and 19.4×10^4 cm^{-1} M^{-1} were adopted for di- and tri-FTC insulin respectively. Single Kjeldahl determinations for tri-FTC insulin supported these values. Several lots of FTC insulins used extensively here and not previously examined were tested for biological activity (Morris *et al.*, 1970b) and found to agree with previous values (Bromer *et al.*, 1967).

Models. The three models of the sites of substitution of insulin, FPA, FGI, and FAO, were prepared from the model peptides by a procedure similar to that for the preparation of the FTC insulins (Bromer *et al.*, 1967). The model compounds were purified by passage of the reaction mixture over Sephadex G-25 which was equilibrated and developed with 0.01 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. The first easily separated orange band was precipitated by dropwise addition of glacial acetic acid to a pH of 3.0, repeatedly washed with a minimum volume of 0.05 M ammonium acetate buffer (pH 3.0), and desiccated *in vacuo* to a bright yellow powder.

DFL was made from 1:1.9 mole ratio (lysine:FITC) reaction mixtures by the same procedures as for the other model compounds. The molar absorptivity of DFL at 472 nm was determined gravimetrically from solutions that obeyed Beer's law and was found to be 7.54×10^4 cm^{-1} M^{-1} for pH values >9.0. No weight loss was detected for 5- to 10-mg samples heated to 100–120° for 20–30 hr.

Purified model compounds were analyzed for the presence of unreacted FITC in order to determine their optical purity by electrophoresis. One to two millimicromoles of each model and FITC dissolved in 0.22 M sodium phosphate buffer (pH 7.4) were applied as 5-μl aliquots to separate lanes of cellulose acetate strips (Oxoid, Colab Laboratories, Inc., Chicago, Ill.) and electrophoresed with the same buffer for 90 min at 20 V/cm. The strips were exposed to concentrated NH_4OH fumes for several seconds which greatly enhanced their fluorescence (maximum *ca.* pH 10.4; Klugerman, 1966), placed directly on an ultraviolet light, and examined for fluorescence parallel to the slower migrating FITC. In this way it was possible to visualize 5–10 pmoles of free FITC. The absence of fluorescence corresponding to FITC yielded a clear indication of electrophoretic purity on the order of 99.5% (Mercola, 1969). In addition, the elution order, pH of precipitation, increased anodal migration rate, presence of fluorescence, and the absorption spectra of the products all agreed with the expected properties of the correct products.

Spectroscopy. CD measurements were made with two Jasco ORD/UV/CD-5 instruments with improved sensitivities, minimum scales of 10^{-4} ΔA/cm of chart (Mommaerts, 1968; Simmons *et al.*, 1969) and 3×10^{-5} ΔA/cm (Sproul Scientific Instruments, Boulder Creek, Calif.).

At high sensitivity and for highly absorbing optically inactive samples the first instrument produced a small but easily detectable base-line deflection—apparently due to interference in the high-voltage power supply (D. Sproul, personal communication). Base lines for samples measured with this instrument were generated with an equal optical density of an optically inactive model compound or were corrected by means of a linear standard curves generated with an alkaline solution of $K_2Cr_2O_7$ (Mercola, 1969).

For routine work a constant slit width of 0.09 mm was chosen (1.8-nm spectral band pass) based on the maximum observed signal and half-bandwidth values of the sample with the sharpest bands (tri-FTC insulin). A time constant of 16 sec and a scanning speed of 3 nm/min were used for all records presented here.

The standard used for calibration of the CD spectrophotometers was an aqueous solution of camphorsulfonic acid with a $\epsilon_l - \epsilon_r$ accepted as 2.09 cm^{-1} M^{-1} at 290 nm at ambient temperatures (*ca.* 25°). The wavelength accuracy was calibrated at 290 nm with camphorsulfonic acid and at 530 and 586 nm with a neodynium glass filter. Nonspecific absorption of insulin derivatives to cuvet surfaces during CD measurements of very dilute solutions (10^{-4} to 10^{-6} M) was avoided by the use of cylindrical Teflon cells (5–10 cm) fitted with 1.0-cm diameter quartz windows. CD results are expressed as molar circular dichroism: $\Delta\epsilon_M = \epsilon_l - \epsilon_r = A_l - A_r / l \times M$, where $A_l - A_r$ is the difference of absorbance of left and right circularly polarized light and is read directly, l is the optical path length in centimeters, and M is molarity as insulin or model compound.

Absorption spectra were made with a Cary 15 recording spectrophotometer equipped with a high-intensity visible source controlled at 80 V. The wavelength accuracy was checked with benzene vapor (J. T. Baker Chemical Co., analytical grade), a neodynium glass filter, and the hydrogen emission line at 486.13 nm. The observed values were well within the manufacturer-suggested tolerances.

Samples for CD and absorption measurements were placed at maximum distances from the photomultipliers (15 and 10 cm, respectively) to minimize fluorescence detection. All CD and absorption spectra presented here are independent of curvet position.

Sedimentation. Sedimentation velocity measurements were made with Beckman-Spinco Model E ultracentrifuges equipped with schlieren optical systems and rotor temperature control units. The rotor speed (nominal setting 60,000 rpm) was determined by means of the odometer. All measurements were made in 0.11 M sodium phosphate buffer (pH 7.4) and at the temperature (±0.1°) of the rotor at maximum speed (18–23°). Sedimentation coefficients were estimated by

TABLE I: Observed Critical Values.[a]

Derivatives	Absorbance Maximum (nm)	ϵ (10^4 cm^{-1} × M^{-1})	CD Extremum (nm)	$\Delta\epsilon$ (cm^{-1} × M^{-1})	Isosbestic Points (nm)
Models (FPA, FGI, FAO)	465 S	8.5	492 (FPA)	−0.65 (FPA)	518
	491				466
DFL	435 S				460
	472	7.5	472	+15.4	
	489		492 S		
			514	−18.0	
Mono-FTC insulin	465 S		465 S		470
	494	6.68	495	+4.3	518
Di-FTC insulin	465 S		474–6	−10.0	464
	492	13.4	508	+21.5	521
Tri-FTC insulin	460 S		479	−32.0	460
	489	19.4	497	+91.5	516

[a] Selected observed spectral values of the models and FTC insulins in 0.11 M HNa_2PO_4–H_2NaPO_4 buffer at the pH values of maximum absorbance and circular dichroism, optical path length, 5.0 cm. Circular dichroism values represent $\leq 5.0 \times 10^{-6}$ M. S indicates shoulder.

measuring the radial distance of the maximum schlieren ordinate value after the rotor had reached maximum speed and were corrected to $s^c_{20,w}$ by using the partial specific volume of 0.73 cm^3/g and the solvent corrections for 0.11 M Na_2HPO_4 (Svedberg and Peterson, 1940).

Titration. Spectrophotometric titrations (Gage, 1949) were carried out in 1.0- to 2.0-cm optical path-length cells in 0.11 M Na_2HPO_4 adjusted with 2.0 N NaOH or HCl. The pH was measured with a Radiometer pH meter (Model 22, Copenhagen).

Results

Models. The visible absorption properties of the three model compounds FPA, FGI, and FAO were very similar (Table I). An example of the spectra obtained in the pH range of the third fluorescein dissociation constant is shown in Figure 1. Above pH 8.5 (curve a) the spectral values were independent of pH and are characterized by a strong maximum at 491 nm and a shoulder at about 460–65 nm. This is in agreement with the spectal properties assigned to the dianion form of fluorescein (Figure 1) by Zanker and Peter (1958). In the pH range 5.5–8.5 families of curves were obtained and these curves were related by two isosbestic points at 466 and 518 nm (curves b–h). In the acidic range a new band appeared at about 455 nm with a shoulder at 430 nm. This is in agreement with equilibration of the monoanion forms (Figure 1) as assigned by Zanker and Peter (1958). Zanker and Peter observed only the one isosbestic point at 520 nm. The absence of the second isosbestic point near 466 nm probably reflects that their media contained a variable proportion of dioxane, a solvent known to decolorize fluorescein (Emmart, 1958; Nash, 1958).

The third pK values of FITC, the models, and the FTC insulins were studied on order to determine the pH range of single spectral forms, to examine the influence of the environment on the dye group pK values, and to assign the origin of optical activity with respect to the ionic forms of the dye. For FITC and the models at pH values much below 5.5 the 466-nm isosbestic point was violated and this was taken as an indication of interference by the more acidic equilibria. The absorbance value at 490 nm of such spectra were adopted at A_a for a first approximation and the data for families of curves (*e.g.*, Figure 1) were used to estimate the apparent pK (Figure 2). This procedure invariably yielded linear plots ($r \geq 0.98$) with slopes near unity, in agreement with the Henderson–Hasselbalch equation. The apparent pK values of FITC and the models were very similar, average 6.46 ± 0.03. This value was taken as an reference for comparison to the pK values of the FTC insulins.

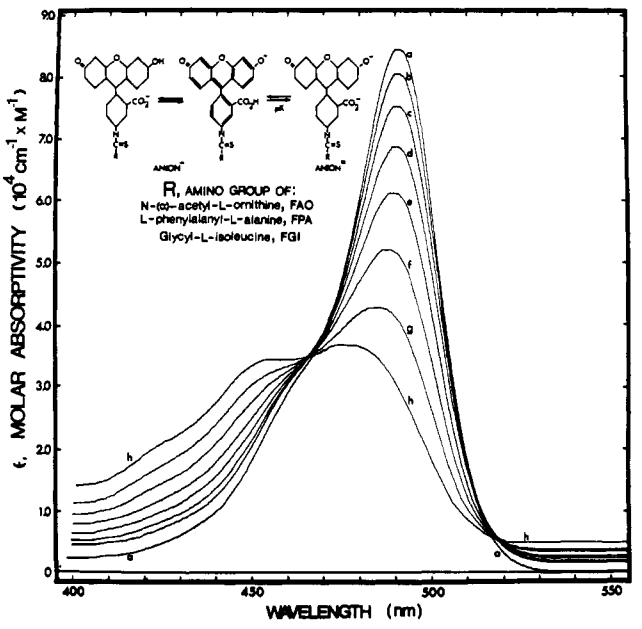

FIGURE 1: Examples of pH dependence of absorbance of the model compounds (here FPA, 5.7 × 10^{-6} M). Curves a–h at pH values of 9.0, 7.3, 7.15, 6.8, 6.6, 6.3, 6.0, and 5.5, respectively. Path length, 2.0 cm. Ionic equilibria of the dianion and two monoanion forms after Zanker and Peter (1958).

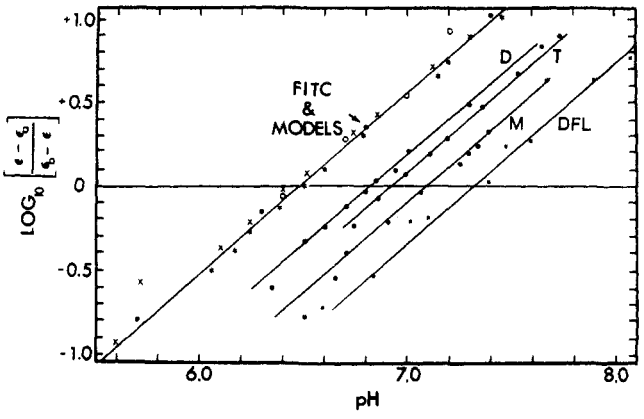

FIGURE 2: Examples of spectrophotometric titration at 490 nm of FITC, open circles; FGI, closed circles; FPA, stars; FAO, crosses; and mono-, di-, and tri-FTC insulin, M, D, and T, respectively. Average apparent pK (± standard error) for FITC and the models is 6.46 ± 0.03 (95% confidence interval is ±0.055; $r \geq 0.98$ for each). Average (at least triplicate) apparent pK ±0.02 for M, D, and T are 7.16, 6.83, and 7.02, respectively (95% confidence interval is ±0.04; for M, D, and T standard error and confidence interval based on quintriplicate data for tri-FTC insulin, $r \geq 0.955$ for all titrations of each FTC insulin). All calculations based on $Y > -0.5$, *i.e.*, the upper 76% of the titrated range only. A_a and A_b are the observed absorbancies for the most acidic and basic range spectra, respectively, that obeyed both isosbestic points (Table I). DFL: duplicate dichrometric titration as described in the text, pK = 7.28 ± 0.06, r = 0.995.

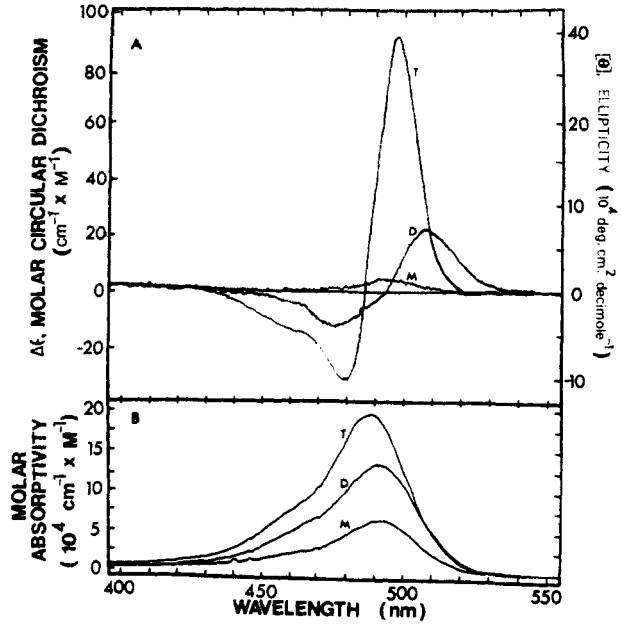

FIGURE 3: Equimolar (2.1×10^{-6} M) comparison of the circular dichroism (A) of mono-, di-, and tri-FTC insulin (M, D, and T, respectively) and the corresponding absorption spectra (B), obtained from the same instrument. All spectra obtained at pH 8.2. At this pH all derivatives approximate their maximum dichroism signals and are 92% in the dianionic form. Optical path length, 5.0 cm. The results are characteristic of five lots of di-FTC insulin and four lots of tri-FTC insulin.

The CD of the model compounds corresponding to the pH and spectral range of the absorption spectra was examined at higher sensitivity and absorbance combinations than used for the FTC insulins. No dichroism was observed for FGI and FAO (Mercola, 1969). The maximum peak-to-peak noise was 1.5×10^{-5} ΔA and within this limit FGI and FAO were judged to be optically inactive in the visible spectral region. Similar remarks held for nonfluorescent aminofluorescein, FITC, and FITC–insulin mixtures at a mole ratio of 3:1 (FITC:insulin). In basic media FPA produced a small ($\Delta\epsilon/\epsilon = -7.5 \times 10^{-6}$) negative band, that, as far as could be discerned, resembled the absorption spectrum. This signal was sixfold less in magnitude than the smallest FTC insulin signal.

By comparison of the spectral properties and structures of aminofluorescein, FITC, and the models it was judged that the potential complexities involved in thiocarbamyl bond formation, the proximity of asymmetric carbons and peptide substituents, or fluorescence emission did not lead to significant extraneous signals.

FTC Insulins Aggregation. The principal results obtained in this study, the CD spectra of mono-, di-, and tri-FTC insulin in the visible spectral region, are shown in Figure 3A. In order to assure that these results represent complete dissociation of aggregated states, the CD and absorption spectra were examined as a function of concentration at pH 7.4 and compared to their observed sedimentation properties and expected association behavior at the same pH. The expected behavior might be inferred from the observations of Jeffrey and Coates (1966a,b) where complete dissociation of insulin at pH 2.0, I = 0.2, net charge of 5.0, was observed in the range of 10^{-5} to 10^{-6} M. For the special case of pH 7.4 ($I \simeq 0.2$) the charge of mono-FTC insulin was computed (Tanford and Epstein, 1954a,b; Fredericq, 1954; Figure 2, curve M) to be 5.0.

The aggregation-dependent results are summarized in Figure 4. Concentration-induced aggregation led to a 2- to 3-nm blue shift and hypochromism of the major visible absorption bands (Table I), of each derivative. The optical density of the visible absorption maxima were found to be independent of concentration for all three FTC insulins in the range of $\leq 5 \times 10^{-6}$ M.

Concentration-induced aggregation also led to marked changes in the major visible CD bands of each derivative (Figure 4B). For mono-FTC insulin a large negative extremum developed at 502 nm, while for di- and tri-FTC insulin the major positive extrema (Figure 3) were diminished. These concentration-induced CD changes (Figure 4B) were clearly more sensitive to aggregation than ordinary absorption measurements over the concentration ranges studied. The general order of hypochromism and the CD measurements correlated well with the sedimentation constants and indicated that the order of aggregation was mono- > di- > tri-FTC insulin at any concentration.

Below 5×10^{-6} M the CD values of all of the derivatives were independent of concentration, implying the region of complete dissociation to monomeric forms. In addition the lowest observed sedimentation coefficients for di- and tri-FTC insulin correspond to values much less than the dimer value of insulin, *ca.* 1.85 (Moody, 1944; Fredericq and Neurath, 1950; Oncley *et al.*, 1952; Fredericq, 1956) confirming that the associated CD changes are sensitive to dimer dissociation. At higher pH values (Figure 3) the net charge of the FTC insulins is increased and aggregation is further depressed.

Mono-FTC Insulin. The visible absorption spectra of all the FTC insulins (Figure 3B) were qualitatively similar to those for the model compounds (Figure 1) and their characteristic values are listed in Table I. In the case of mono-FTC insulin, the maximum absorbance occurred above pH 9.0 and re-

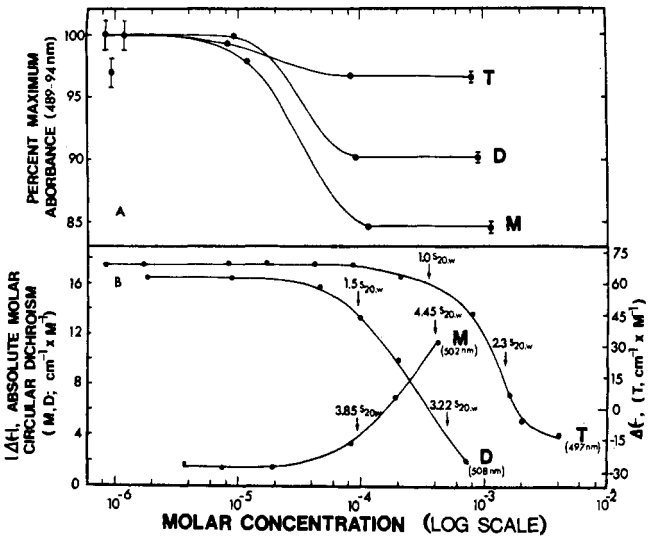

FIGURE 4: Concentration-dependence of absorbance (A) and circular dichroism (B) of the FTC insulins in 0.11 M sodium phosphate buffer (pH 7.4). A represents hypochromism only: difference (as per cent of maximum absorbance) determined from individual spectra. Brackets indicate spectrophotometric error for the measurement of a sample with an absorbance equal to the observed difference. Optical path lengths in order of increasing concentration 5.0, 1.0, 0.1, and 0.01 cm. (B) Absolute value of circular dichroism at indicated wavelengths. Concentrations were determined spectrophotometrically and corrected according to part A. Sedimentation constants were measured in the same solvent as for parts A and B. M, D, and T are mono-, di-, and tri-FTC insulin, respectively.

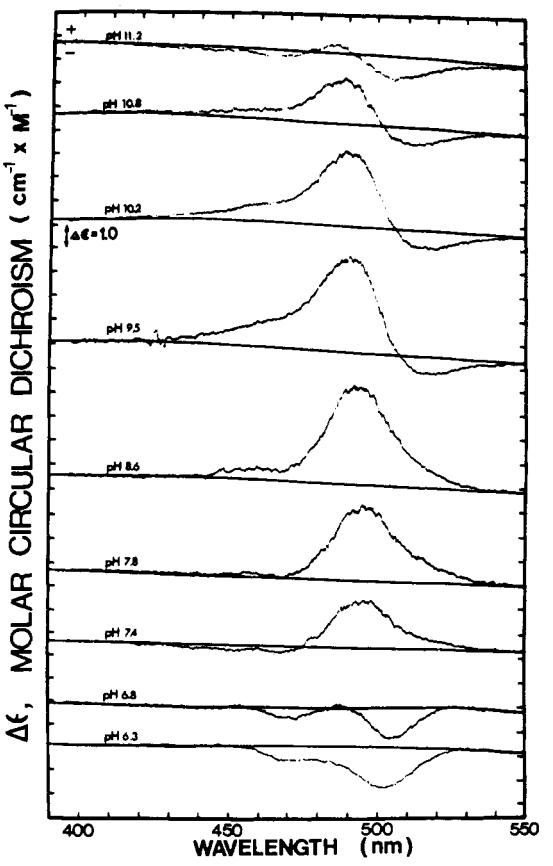

FIGURE 5: Example of molar circular dichroism of mono-FTC insulin (5.0×10^{-6} M) at the indicated pH values. Optical path length, 5.0 cm; maximum absorbance 1.7.

mained independent of pH even in 1 N NaOH. Throughout this range the maximum molar absorptivity was about 20% hypochromic with respect to the model compounds. Below pH 9.0 families of curves exhibiting two isosbestic points (Table I) were obtained. The average apparent pK estimated from such data was 7.16 (Figure 2) compared to 6.46 for FITC and the models.

Addition of the dye group to the B-1 phenylalanine of insulin was also associated with induced dichroism (Figures 3A and 5). Above pH 7.4 a positive extremum centered at 495 nm increased with increasing pH to a maximum $\Delta\epsilon$ of 4.3 cm^{-1} M^{-1} in the pH range 8.5–9.0. In this range the CD spectra resembled the absorption spectrum of the dianion form. However, unlike the absorption spectra, the CD values were remarkably sensitive to increased alkalinity and decreased with increasing pH above 9.0. The process was accompanied by the appearance of a shallow negative extremum near 520 nm that progressively shifted toward 494 nm. In 1 N NaOH spectrum was featureless.

In the more acidic range, discrete CD contributions suggestive of the monoanion form were not observed although the resemblance of the CD spectrum to the dianion absorption spectrum became increasingly distorted in the spectral region of monoanion absorption. Below pH 7.4 a negative extremum at 502 nm developed that resembled the concentration-induced CD band both in sign and position (Figure 4B) in agreement with the expected aggregation properties of this derivative in this pH range (charge <5).

DFL. The model for multiple substitution of insulin by FITC was DFL. The limiting factors influencing this choice were the realization that covalent linkage *via* thiocarbamyl bonds to even a one carbon bridge could lead to prohibitively large separations (>10 Å) of the xanthyl nuclei for a sensitive test and that a suitable dimer should not involve modification of the fused ring system. For the case of disubstituted lysine it was reasoned that the binding of the two dye groups to ends of a flexible aliphatic chain at a maximum separation far less than the mean separation of saturated solutions of the free dye (≃60 Å at 10^{-2} M) would constrain the dye groups to adopt the most state with respect to each other. For planar ring systems, this state is expected to involve stacking in van der Waals contact (*e.g.*, Scheibe, 1938; Hoppe, 1944; Shepard and Geddes, 1944; Zanker, 1952; Nash, 1958; Levchine and Baranova, 1958; Bradley and Wolfe, 1959; Stryer and Blout, 1961).

The resulting absorption spectrum of DFL is compared to an equimolar (as fluorescein) mixture of the two models representing the constituent substitution sites in Figure 6. The spectra represent the pH range of maximum absorbance (pH ≥9.3) for all three models. Simultaneous substitution at the α- and ϵ-amino groups of lysine led to striking hypochromism. In addition a new short-wavelength peak occurred at 472 nm and a long-wavelength shoulder was apparent above 514 nm. The shoulder at 460–465 nm characteristic of the mono-substituted models was not apparent in DFL but a short-wavelength shoulder was discernable near 435–445 nm. Finally, an absorption peak at 489 nm, close to the mono-substituted absorption maximum, was apparent.

In the pH range below 9.3 both DFL absorption bands decreased in concert. The resultant spectra were related by an isosbestic point at 461 nm in the pH range between 9.3 and 7.0 but the base and conjugate acid forms were only separated by about 0.3 absorbance unit per unit of DFL and titration was not feasible. However preliminary attempts revealed a com-

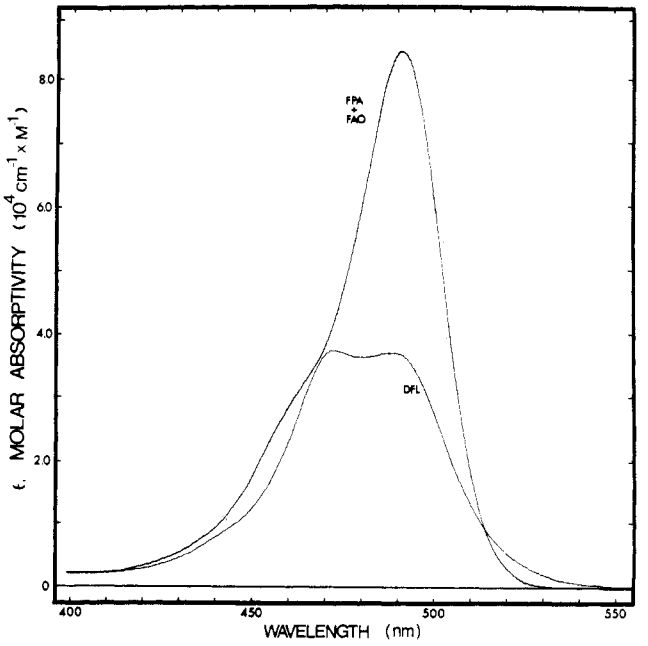

FIGURE 6: Equimolar (as total FTC groups, 5.9 × 10^{-6} M) comparison of the molar absorbance of DFL and a 1:1 mole mixture of FPA and FAO at pH 10.0. Optical path length, 2.0 cm. The ordinate is expressed as absorptivity per mole of FTC groups.

plex sigmoid curve that suggested at least two pK values existed, one above and one below pH 7.0.

The absorption properties of fresh solutions prepared from isoelectrically precipitated material and maintained near 0° prior to examination were stable within the time periods of the measurements reported here. However the absorption properties of sealed solutions left standing at ambient temperature displayed a mutarotation-like behavior. The absorption spectra slowly approximated the FPA-FAO mixture spectra over a period of several weeks. The process was dominated by an increase in absorbance of the 489-nm band which shifted to 491 nm, and after several weeks, approached about 80% of the model mixture absorbance. This process was greatly accelerated by warming.

In addition, unlike the brilliant green fluorescence characteristic of monosubstitued models, fresh solutions of DFL appeared nonfluorescent to the eye and exhibited a markedly reduced relative emission spectrum for excitation at 460 nm.

All of these properties may be understood in terms of an equilibrium between at least two conformers of DFL in aqueous media at ambient temperature. One extended with properties of the monosubstituted models (absorption band at 489 nm, low pK) while the other represents a true stacked state (two absorption bands at 472 and 514 nm, mutarotation, high pK, nonfluorescent). This interpretation is supported by observations of the rotatory properties of DFL (Figure 7). The CD spectrum is dominated by two extrema of opposite sign. The positive extremum at 472 nm corresponds well with the 472-nm absorption band and the negative extremum at 514 nm corresponds with the absorption shoulder near 514 nm. It is apparent by inspection alone that these extema may be described as symmetrically positioned at ±866 cm^{-1} about the monomer absorption maximum of the dianion form at 20321 cm^{-1} (*ca.* 492 nm). The observations are in excellent agreement with exciton theory for a dimer of degenerate transitions originating in the dianion form. Accordingly, these CD extrema would be expected to diminish reciprocally as the pH is lowered and not independently as might be expected for two noninteracting dye groups with unique environments and pK values. This effect is illustrated in Figure 8. Below about pH 9.0 families of spectra were obtained by decreasing the pH and these spectra were related by an isodichroic point at 498 nm. In particular the long-wavelength extremum vanished completely at pH 6.0 (Figure 8, curve a). In this system the disappearance of the couplet of reciprocally related extrema as the pH was lowered was not accompanied by the appearance of a new couplet that might be suggestive of interactions of the monoanion form.

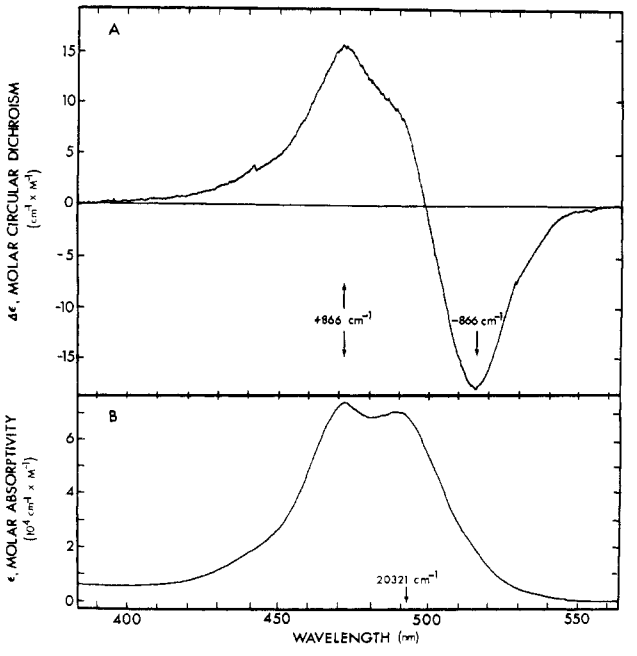

FIGURE 7: Molar circular dichroism (A) and absorptivity (B) of DFL (pH 9.3). (A) 5.0-cm optical path length, 4.1 × 10^{-6} M. (B) 1.0 cm optical path length, 4.7 × 10^{-6} M. CD extrema and short-wavelength absorbance maxima are located at 20,321 ± 866 cm^{-1} (20,321 cm^{-1} ≃ 492 nm).

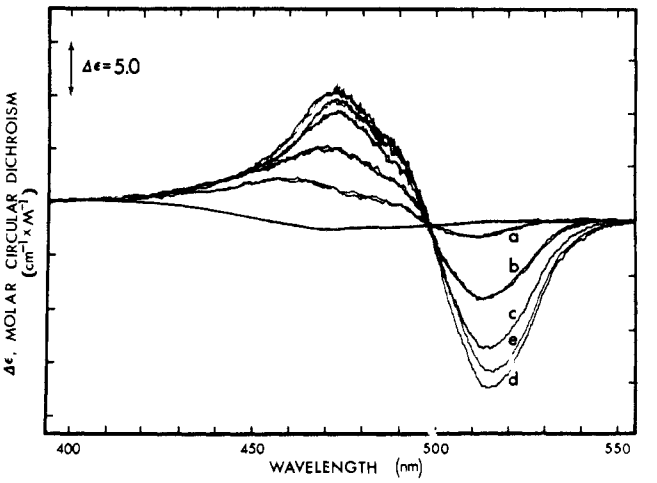

FIGURE 8: Example of the pH dependence of the molar circular dichroism of DFL (5.5 × 10^{-6} M). Curves a–e correspond to pH values of 6.5, 7.0, 7.5, 8.1, and 9.7, respectively. Optical path length, 5.0 cm. Base line, smooth curve, first instrument, corrected to the sample of highest absorbance (curve e) by means of a standard curve for the first instrument (see Methods and Materials).

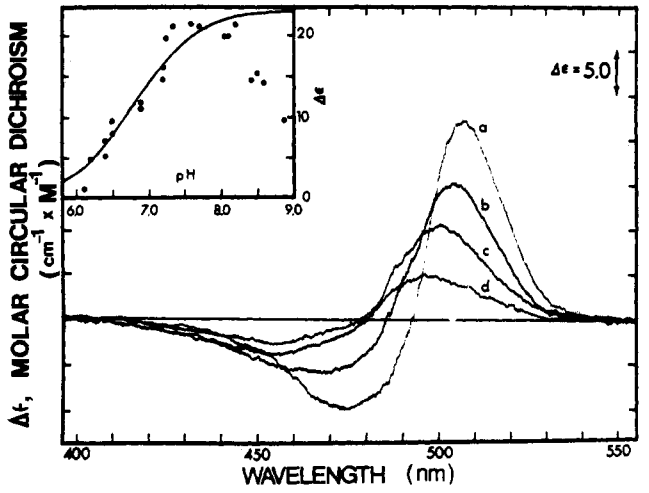

FIGURE 9: Example of the pH dependence of the molar circular dichroism of di-FTC insulin (3.0×10^{-6} M) in the range of the monoanion-dianion equilibrium. Curves a-d correspond to pH values of 8.2, 7.2, 6.7, and 6.2. Optical path length, 5.0 cm. Inset, solid circles, observed molar circular dichroism of the positive extremum; solid curve, calculated titration curve for the dissociation of one proton from a group with pK of 6.8.

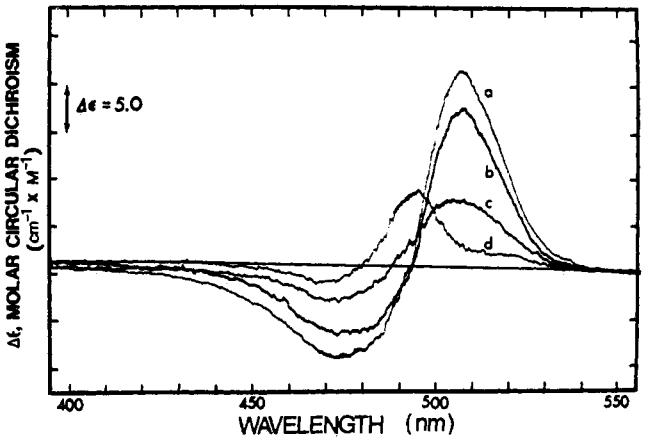

FIGURE 10: Example of the alkaline pH dependence of the molar circular dichroism of di-FTC insulin (3.0×10^{-6} M). Curves a-d correspond to final pH values of 8.2, 8.6, 8.9, and 10.5, respectively. Curve a was obtained by readjustment from pH 10.5 (curve d) to pH 8.2. Optical path length, 5.0 cm.

If it is assumed that the long-wavelength CD band is only a manifestation of dye–dye interaction, than the apparent pK of the dimer conformer can be obtained. For this purpose the fraction, α, of the observed to the maximum CD value at 515 nm ($\alpha = \Delta\epsilon/18.0$) is taken as proportional to the dianion form and $1 - \alpha$ as proportional to the monoanion form. Titrations obtained in this way (Figure 2) may be approximated by a linear curve with a slope of unity. The most alkaline pK of this complex is increased by over 0.8 unit with respect to the model (monosubstituted) values.

In addition, the CD spectra of DFL exhibited a positive shoulder near 492 nm. This shoulder also diminished with decreasing pH and a broad positive extemum near 455 nm corresponding to the monoanion absorbance (*cf.* Figure 1) appeared in the more acidic range. This is consistent with the existence of an extended conformer with CD and absorption properties approximating the monosubstituted models. Presumably the pK of this species is also similar to the monomer value and is in part responsible for the complex titration behavior.

Finally, it is noted that the mutarotation-like behavior of the absorption spectrum observed upon standing occurred in the pH range of complete dye group ionization and implicated the role of isoelectric precipitation during the preparative procedure as possibly mediating dye–dye interactions.

Di-FTC Insulin. Purified di-FTC insulin is characterized by essentially complete and exclusive substitution of the N-terminal amino groups of the A and B chains (Bromer *et al.*, 1967). Addition of the second dye group led to a twofold increase in absorbance in the pH range 8.8 through 1 N NaOH (Figure 3, Table I). Thus the pH-independent absorbance of di-FTC insulin is also about 20% hypochromic compared to the value based on the model compounds and this hypochromism also remained in strong alkali. Below pH 8.8 the spectra were related by two isosbestic points at 464 and 521 nm. The maximum difference between the molar absorptivities of the dianion and monoanion forms in the pH range of the two isosbestic points was about twice that of mono-FTC insulin indicating that both dye groups were titratable in this range. Spectrophotometric titrations yielded an average apparent pK of 6.83 (Figure 2)—intermediate between the pK values of the models and mono-FTC insulins. Correlation with a single titration with high precision (r = >0.998) extended virtually throughout the upper three-quarters of the titration range (Figure 2). Thus the existence of a separately titratable dye group with a pK near that of mono-FTC insulin was not detected.

Addition of the second dye group to the N-terminal glycine led to over a fivefold increase in the maximum observed CD and the production of two extrema of opposite sign centered near 475 and 508 nm or approximately symmetrically positioned about the dianion absorption maximum at 492 nm (Figures 3 and 9). The extrema were reciprocally diminished by decreasing the pH (Figure 9) and the decrease roughly paralleled the behavior expected for CD extrema originating in the dianion form (Figure 9, inset). However the pH-dependent behavior was complex. In the acid region (Figure 9, curve d) the CD spectra exhibited a shallow negative extremum centered near the absorption maximum of the mononaion form and a positive extremum shifted toward the absorption maximum of the dianion form. The observation suggests that the alkaline spectra may envelop at least one other contribution positioned at 492 nm as well as transitions responsible for the observed extrema. Further the actual maximum value occurred at pH 7.5 and remained roughly constant to pH 8.2.

The titration and CD results are suggestive of the expected properties of an exciton system. Thus it would be expected that the alkaline-labile nature of CD signal of the phenylalanine-boud dye group (Figure 5) would be manifested in di-FTC insulin as a reciprocal decrease in *both* major CD extema with increasing pH. An example of this effect is shown in Figure 10. Both CD extema rapidly diminished as the pH was raised above 8.2 (Figure 10). This alkaline-labile effect occurred at much reduced pH values compared to the analogous phenomena observed with mono-FTC insulin. Also in contrast to mono-FTC insulin, at pH values above 10.0 the CD spectra of di-FTC insulin were dominated by a 3.5-fold larger contribution at 495 nm (Figure 10). Thus this increase may represent alkaline-resistant properties of the second (A-1 glycine bound) dye group alone. The alkaline-labile effect is entirely reversible: curve a of Figure 10 is nearly identical

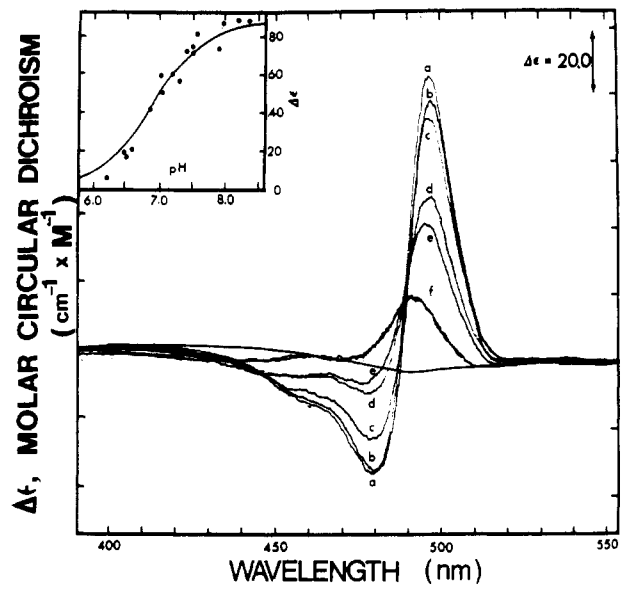

FIGURE 11: Example of the pH dependence of tri-FTC insulin (1.9×10^{-6} M) in the range of the monoanion–dianion equilibrium. Curves a and c–f correspond to pH values of 8.0, 7.4, 7.0, 6.85, and 6.5, respectively; curve b, pH 9.3. Base-line smooth curve, corrected for apparent dichroism based on the highest absorbing sample (curve b) by means of a standard curve for the first instrument (see Methods and Materials). Curve f below 455 nm represents the maximum apparent dichroism of the monoanion absorption. Inset, solid circles, observed molar circular dichroism of the positive extremum; solid curve, calculated titration curve for the dissociation of one proton for a group with a pK of 7.0. Optical path length, 5.0 cm. Curves b and e *non fide* in proof.

with curve a of Figure 9 but was obtained by adjusting the sample of pH 10.5 (curve d) to 8.2. In 1 N NaOH the CD spectra of di-FTC insulin were featureless.

According to Tinoco *et al.* (1962) the interpretation of an exciton complex requires that the dye groups be rigidly held in a fixed orientation. The reversible pH dependence in the range of constant and *complete* dye group ionization suggested that the origin of the forces of putative complex resided in the protein structure and not in dye–dye attraction which, as one possiblity, may have been mediated in isoelectric media (purification) where the dye groups are nearly neutralized. Thus it was expected that exposure of di-FTC insulin under conditions of complete dye group ionization to solutes such as urea and guanidine hydrochloride which are known to denature protein interactions (Kauzmann, 1959; Tanford, 1962; Nozaki and Tanford, 1963; Lapanje and Tanford, 1967) and in particular insulin (Hashizume *et al.*, 1967; Tanford *et al.*, 1967) would lead to a largely reversible disruption of the putative complex. The CD of samples of di-FTC insulin in the presence of 8 M urea or guanidine hydrochloride were compared before and after dialysis. Urea led to about 40% reduction of both CD extrema and this effect was completely reversed by 6- to 10-hr dialysis. Guanidine hydrochloride nearly abolished the CD signal and this effect was 90% (*cf.* Figure 12) to 100% reversible (Mercola, 1969). When the urea concentration was increased to 9.5–10.0 M the signal was again lost. These results suggest that the visible CD spectrum is conformation dependent.

Tri-FTC Insulin. Purified tri-FTC insulin is characterized by virtually complete substitution of the three amino groups of insulin (Bromer *et al.*, 1967). As for the other FTC insulins the absorptivity (Figure 3) of this derivative is characteristically about 20% hypochromic in the pH range of the maximum absorbance, *i.e.*, >9.0. However in 1 N NaOH the CD spectra of all of the FTC insulins were featureless. Thus the characteristic hypochromism probably resides in the influence of the local protein environment and is independent of position or conformational integrity. Similar observations have been made by Tietze *et al.* (1962).

Below pH 9.0 the absorption spectra were pH dependent and were again related by a pair of isosbestic points (Table I). The maximum difference between the molar absorptivities of the dianion and monoanion forms in the pH range of the isosbestic points was about three times that of mono-FTC insulin and indicated that all three dye groups were titratable in this range. Spectrophotometric titrations yielded a single average apparent pK of 7.02 (Figure 2).

Addition of the third dye to the B-29 lysine was associated with dramatic changes in the CD spectrum (Figure 3). The CD spectra were dominated by a negative extremum at 479 nm and a very large extremum of opposite sign at 497 nm. Thus these extrema occurred at about a 14-nm narrower separation than for di-FTC insulin. Further the increased rotatory strength at 497 nm was *not* in simple superposition with the CD spectrum associated with the first two dye groups. The long-wavelength extremum of di-FTC insulin above 508 nm was not apparent as either a peak or shoulder in the corresponding region of the tri-FTC insulin spectrum. Finally, the maximum intensity of the two extema of tri-FTC insulin occurred on the alkaline side of the pK as for mono- and di-FTC insulin but decreased only slightly with increasing pH (*cf.* curves a and b, Figure 11). In particular, under conditions where the extrema of di-FTC insulin tended to minimal values (Figure 10), tri-FTC insulin retained 90% of the maximum signal (Figure 11, curve b).

Below pH 9.0, the CD extrema of tri-FTC insulin reciprocally decreased upon lowering the pH in a manner that roughly corresponded to optical activity originating in a single dianionic complex (Figure 11, inset). Equimolar comparisons of di- and tri-FTC insulin throughout this range also demonstrated the nonadditive effect of addition of the third dye group. Treatment of tri-FTC insulin with 8 M guanidine hydrochloride at the pH value of the maximum signal and over 90% ionization of the dye groups led to a loss in the signal which was essentially completely reversible upon dialysis for 6 hr (Figure 12).

Discussion

Mono-FTC Insulin. Covalent addition of a single dye to the N-terminal phenylalanyl residue led to substantial induced optical activity over that of the corresponding model compound. The pH dependence and shape of the CD spectrum identified the dianion form as the dominant optically active species. In the most favorable pH range, the CD and absorption spectra were roughly related by the ratio 6.5×10^{-5} ($\Delta\epsilon/\epsilon$) in the range 450–500 nm. The distortion from quantitative agreement increased in neutral and moderately alkaline media and probably represented the presence of the monoanion form and the alkaline-labile nature of the dianion signal, respectively. The qualitative correspondence between absorption and CD is in agreement with expectations for the induction of optical activity in strong electric dipole transitions by nondegenerate mechanisms (Moffit and Moscowitz, 1959). The observation corresponds to conditions of complete disaggregation of mono-FTC insulin and this strongly supports the previous reports (Blout, 1964; Yamaoka and Resnick,

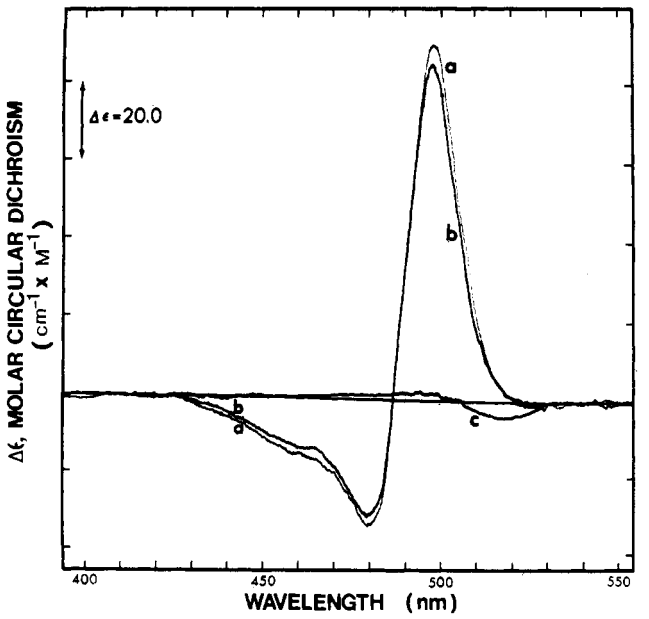

FIGURE 12: Comparison of the circular dichroism of equimolar (final concentration, 1.1×10^{-6} M) aliquots of the same sample of tri-FTC insulin without, a, after, b, and during (immediate) treatment which 8.0 M guanidine hydrochloride in 0.11 M HNa_2PO_4 (pH 8.2) optical path length, 5.0 cm. Aliquots a and b were dialyzed against two changes of 500 volumes of solvent at 4°. The analogous result holds for di-FTC insulin.

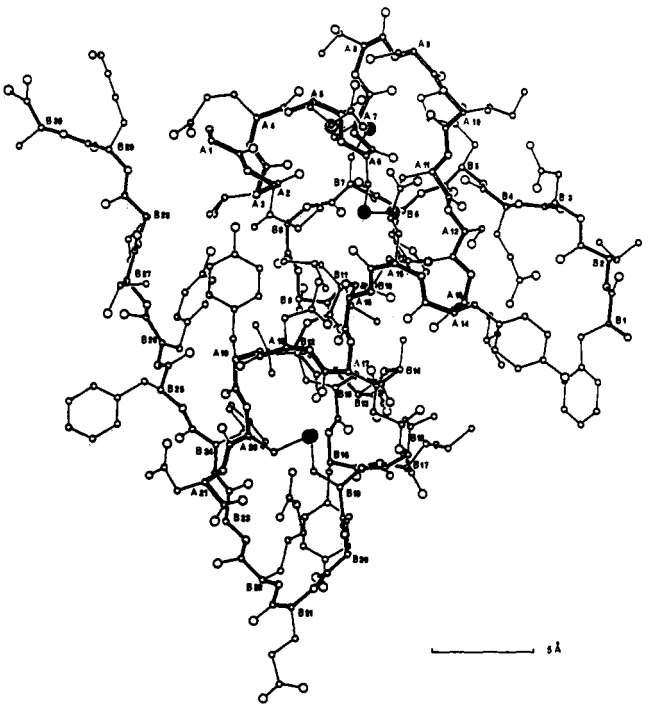

FIGURE 13: A monomer (1) of procine insulin as found in the rhombohedral two-zinc crystals viewed along a direction perpendicular to the threefold axis (Blundell *et al.*, 1971).

1966; Yamaoka, 1968; Coleman, 1968) that substantial optical activity can be induced in single symmetric dye groups bound to polymeric substrates.

The observed CD of all three derivatives is completely and reversibly lost in the presence of denaturants indicating that the observable CD almost entirely depends on the general integrity of the structure. The continuous decrease of the CD signal of mono-FTC insulin that occurred above pH 9.0 corresponded to the pH range of an increase of four or five negative charge units due to neutralization of the lysyl group and ionization of three to four tyrosine residues (Tanford and Epstein, 1954; Fredericq, 1954; Morris *et al.*, 1970). The continuous decrease in CD above pH 9.0 is most likely due to a sensitivity of the probe to local conformational changes. These changes may be related to repulsive electrostatic interaction with dianionic dye group. In alkaline media di-FTC insulin contained two additional negative charges due to the addition of the second dye group and the resulting exciton signal was much more sensitive to pH, the onset of the decreasing signal occurred at pH 8.0. This effect may also be identified with the behavior of the B-1 phenylalanine-bound probe. Above pH 10 a single CD band remained in di-FTC insulin that was consistent with induction of optical activity in a single dye group. The absence of this band in mono-FTC insulin at pH 10 identified this signal with the A-1 glycine-bound probe. This band is also conformation dependent, *i.e.*, absent in guanidine hydrochloride and FGI, and its presence in alkaline media under conditions where the signal of mono-FTC insulin is labile reflects the relatively more stable conformation determining the position of the group. According to this interpretation the loss of the excition nature of the signal of di-FTC insulin in base represents the behavior of the B-1-bound probe and suggests that the changes sensed by the probe occurred at least in part in the N-terminal portion of the B-chain. In addition the large difference in pH dependence of CD for these two derivatives again suggests that electrostatic repulsion rather than the possible "quenching" effects of a single vicinal charge are responsible for the pH-dependent effects. Consistent with this interpretation addition of a third dye group to the B-29 lysine residue led to further increase of three negative charge units at pH 9.0. The spectral properties of this derivative were independent of pH in the alkaline range presumably due to a further suppressiion of the pH region of stability of the B-1-bound probe (see below).

The interpretation of the N-terminal B-chain as exposed and marginally stable has previously been suggested. The order of reactivity of the three amino groups of insulin with certain reagents such as FITC (Bromer *et al.*, 1967) or phenyl isothiocyanate (Brandenburg and Ooms, 1968; Africa and Carpenter, 1970) is B-1 > A-1 > B-29. In particular, preliminary kinetic analyses of the reaction of FITC with aggregated insulin indicated that the reaction at the B-1 residue follows second-order kinetics with a rate constant nearly equal to that for the reaction of FITC with the model glycylisoleucine (Mercola, 1969), supporting the previous conclusion that the phenylalanyl residue "...must be freely accessible to FITC even in highly aggregated insulin (Bromer *et al.*, 1967)." Previous immunochemical and biological studies indicated that the integrity of the phenylalanyl residue is not required for either high immunochemical or biological activity (Arquilla *et al.*, 1969).

A basis for these features may also be seen in the crystal structure of rhombohedral two-zinc porcine insulin (Adams *et al.*, 1969; Blundell *et al.*, 1971). The three N-terminal residues of the B chain do not interact with the body of the molecule but rather hand apart from the monomer (Figure 13) or dimer structure. The B-1 phenylalanine residue is also on the surface of the hexamer. In contrast, the A-1 glycine residue, while on the surface, is on the end of a distorted helix between A-2 and A-8 which leads into the structure. It seems likely

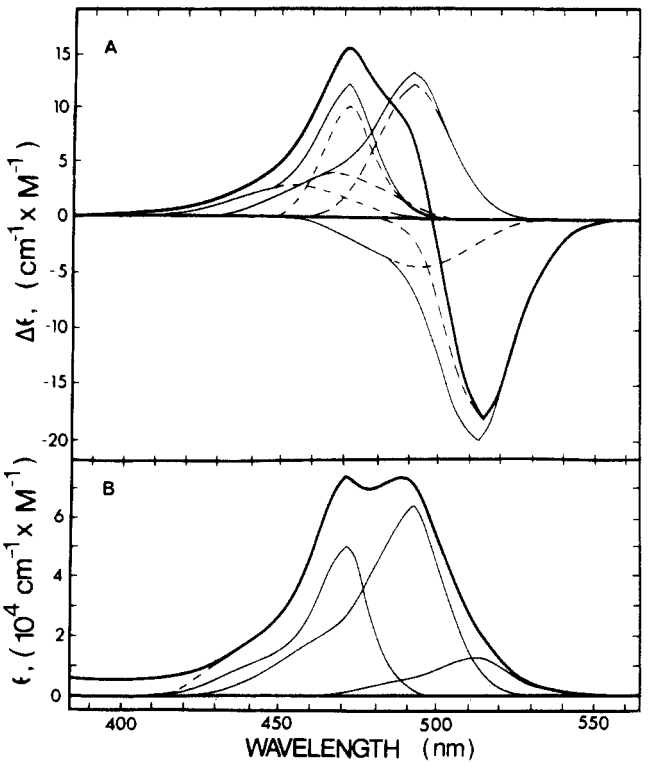

FIGURE 14: Resolution of observed spectra, broad solid curves, of DFL (Figure 8) into three skewed bands, thin solid curves, composed of two Gaussian bands each, dashed curves. This approach was used to derive the values listed in Table II. Omission of the long-wavelength dashed contribution led to negligible monomer (*ca.* 492 nm) CD contributions for di- and tri-FTC insulin (see Discussion).

that in solution the extended terminal B chain would change position and pack against the surface (Hodgkin and Mercola, 1971). The contrasting behavior of the A-1- and B-1-bound probes in solution provides support for the notion of a flexible N-terminal chain as judged from the rhombohedral crystals.

Multiple Substitution. Analysis. The essential requirements for the observation of exciton interaction have been stated as a need for identical energy levels and rigid geometry relating the interacting groups (Tinoco *et al.*, 1962; Stryer, 1962). The requirements arise because vibrations or rotations of the groups will blur the identity of the electronic levels and thus suppress interaction. These characteristics limit interaction to crystals and rigid polymers. The resulting expected optical properties in the ideal case according to the dipole approximation have been summarized in the introduction. The major finding of this study is that in all three cases of multiple substitution the CD spectra were dominated by pairs of extrema of opposite sign with observed or resolved (see below) separations and pH and solvent dependencies that are fully consistent with conformation-dependent resonance interaction based on the dianion energy levels.

In the case of DFL, the conclusion of resonance interaction as the dominant origin of optical activity is particularly favorable since a number of observations indicated that an appreciable fraction of the conformational species in fresh solutions formed a strong dye–dye complex. (1) Simultaneous substitution of the amino groups of lysine led to striking hypochromism. According to Tinoco and coworkers (Tinoco, 1960, 1961; Rhodes, 1961; Devoe and Tinoco, 1962; Tinoco *et al.*, 1962), hypochromism of a low-energy electric-dipole transition results from a dipole component in parallel alignment with a higher energy transition which "borrows" intensity. This model is derived in terms of dispersion forces and is consistent with the expected (*cf.* Results, DFL) mechanism of association in DFL. (2) The absorption maxima could readily be correlated with the extrema of the DFL CD spectrum in agreement with the expected properties (*e.g.*, Tinoco, 1963) of resonance interaction. (3) The increased pK of the strongly optically active species further suggested the existence of a dye group complex. The increased pK is not likely to be due to an extended conformer since the pK values of monosubstituted and carboxylate bearing models of graded side-chain complexity were very nearly the same as that of the free dye. (4) Similarly, the decreased fluorescence and mutarotation-like behavior of DFL are readily understood in terms of complex formation.

In order to obtain a semiquantitative test of the expectations of exciton theory as used here, the maximum observed dye group separation (eq 1) for the interacting conformer of DFL was estimated from the parameters derived by curve resolution using a Du Pont 310 curve analyzer according to the general procedures of Miles and Urry (1968) and has been described in detail (Mercola, 1969). The CD and absorption spectra were considered to result from a superposition of contributions due to resonance interaction (*i.e.*, bands centered at 472 and 514 nm) and a contribution resembling the monomer absorption spectrum. The observed spectra were resolved into Gaussian contributions with respect to wavelength as an approximation to Gaussian energy distributions since the maximum band separation here (DFL) was 42 nm. This approach alone did not satisfy the broad short-wavelength shoulders of the CD and absorption spectra near 445–455 nm (*cf.* Figure 7). If it is considered that the 465-nm shoulder of the monomer absorption spectrum represents a dominate vibrational level and that this band is manifested in resonance interaction by a closer separation than (492–465) nm (Kasha, 1963) then the existence of a contribution at 445–455 nm can be rationalized without the *ad hoc* inclusion of a new contribution. Accordingly, the correction for vibrational modes necessitated that *each* excition component be skewed by the addition of one short-wavelength subcomponent each about 18 nm below the parent excition bands (the subcomponent half-bandwidths were taken from the monomer absorption spectrum, Table II). This system with slight modification (Table II), readily fit the observed data for both the CD and the absorption spectra in the range 430–560 nm (Figure 14). The pH-dependent spectra could be generated by varying the magnitude of the three principal contributions (Figure 14, thin solid curves) while holding the positions and bandwidths constant and maintaining the subcomponent (Figure 14, dashed curves) relative peak heights roughly constant (for the spectra of the more acidic range the shape of the monoanion absorption spectra was taken into account as a minor contribution). However, even this system is known not to be unique. The long-wavelength skewed band could be replaced by a symmetric band (*i.e.*, omitting the subcomponent). The principal effects were large changes in intensities but all positions and bandwidths remained within 1–2 nm of the original positions.

Finally, the monomer dipole strength (*cf.* eq 1) for the principal transition centered at 492 nm was estimated by resolution of the observed absorption spectrum (Figure 1, Table II). The resolution was complicated by serveral observations indicating that a third unresolved transition existed within the observed envelope (*cf.* Figures 5 (pH 6.3) and 12c).

TABLE II: Resolved Critical Values.[a]

Derivative	Absorbance Maximum (nm)	Bandwidth (nm)	ϵ (10^4 cm^{-1} × M^{-1})	Dipole Strength (10^{-36} esu^2 cm^2)	$\Delta\epsilon$ (cm^{-1} × M^{-1})	Rotatory Strength (DM)	R_{max} (Å)
Models	466	23	3.05	21.0		~−0.06	7.4
	492	15	7.6	36.0			
DFL	+		4.6 (ws)		+11.5 (ws)		
	454	23	1.4	12.7	+2.75	+0.062	
	471 ≈ 21,231 cm^{-1}	12	3.5	15.0	+9.5	+0.118	
	465	23	1.8	14.5	+4.05	+0.085	
	492	15	6.0	29.8	+12.2	+0.162	
	−		1.2 (ws)		−19.5 (ws)		
	495	23	0.4		−4.5	−0.093	
	513 ≈ 19,493 cm^{-1} / 1,738 cm^{-1} = $\nu_+ - \nu_-$	15	0.9	4.1	−18.0	−0.023	
Mono-FTC insulin	467, 467	23, 23	2.45, 2.45	18.7, 18.7			
	495, 494	16, 15	6.1, 5.5	32.2, 27.2	~+3.0	~+0.04	
	504	15	0.95	4.6			
Di-FTC insulin	+				−11.0 (ws)		7.6
	456–457	23			−2.5	−0.055	
	476–477 ≈ 20,986 cm^{-1}	15			−10.0	−0.157	
	466–467	23			−1.7	−0.055	
	492	15			−7.0	−0.092	
	−				+25.0 (ws)		
	485	23			+5.5	+0.114	
	506–507 ≈ 19,743 cm^{-1} / 1,243 cm^{-1} = $\nu_+ - \nu_-$	15			+23.0	+0.295	
Tri-FTC insulin	+				−67.0 (ws)		10.5–13.3
	467	21			−24.0	−0.460	
	486 ≈ 19,743 cm^{-1}	11			−53.0	−0.524	
	−				+128.0 (ws)		
	479–480	22			+17.5	+0.350	
	497 ≈ 20,121 cm^{-1} / 455 cm^{-1} = $\nu_+ - \nu_-$	11			+115.0	+1.110	

[a] Selected computed spectral values for the components of the curve resolutions (*e.g.*, Figure 14 and Discussion). Dipole strength, D, for a single component is equal to the square of the transition dipole moment, μ, and was estimated from the relationship $D \cong 1.63 \times 10^{38} (\epsilon\Delta/\lambda)$, where ϵ is the resolved molar absorptivity and Δ is the half-bandwidth at ϵ/e for a component centered at wavelength λ (Moscowitz, 1960). The corresponding rotational strength was estimated from the relationship $R = 4.05 \times 10^{-39} (\Delta\epsilon \cdot \Delta/\lambda)$ (Moscowitz, 1960). One Debye magneton = 0.92732×10^{-38} cqs unit. R_{max} in Å = $[2D/(\nu_+ - \nu_-) \times 10^{40}]^{1/3}$ (see eq 1). Columns headed + and − represent high- and low-energy exciton components, respectively, for a given spectrum. Wave numbers have not been rounded for convenience and are accurate to about ±20 cm^{-1}. ws, total value without skewing; DM, Debye magneton.

Using the parameters of this third band, a three-band solution for the absorption spectrum of monomer was obtained. Comparison of the dipole strengths of the two- and three-band solution for the major contribution at 492 nm (Table II) shows that the difference in $D^{1/3}$ is small (*cf.* eq 1).

The maximum observed center-to-center (xanthyl group) dye group separation estimated with the aid of the resolved parameters (Table II) was about 7.4 Å. When compared to a minimum possible separation of about 4.0 Å (Pauling, 1960) and a maximum possible separation of 27 Å as judged by a Pauling–Corey–Koltun space-filling model of DFL, the derived range of 4.0–7.4 Å was judged to be in good agreement with the physical indications of complex formation.

The same approach was used to analyze the CD spectra of di- and tri-FTC insulin and readily yielded satisfactory fits (Table II). Thus all three CD spectra were satisfied by the same or nearly the same set of skewed bands together with a monomer contribution by adjustment of position alone. In the case of di-FTC insulin the solution satisfactorily explained the pH-dependent behavior for the range $pK \leq pH \leq pK + 2.0$. In the case of tri-FTC insulin, all the CD spectra were satisfied on the basis of exciton interaction alone.

In all of these cases it was observed that the sum of the rotational strengths (Table II) for the pairs of resolved exciton components was never zero. This does not necessarily represent a violation of the sum rule since contributions due to coupling with higher energy transitions by nondegenerate mechanisms and local conformation-dependent asymmetry (Tinoco *et al.*, 1962) were not taken into account.

For di- and tri-FTC insulin the dipole strength used for the

estimate of maximum separation was obtained by resolution of the mono-FTC insulin absorption spectrum (Table II). As all the FTC insulins were nonspecifically 20% hypochromic this step provides a reasonable adjustment of the index of refraction.

The maximum separation for di-FTC was estimated to be about 7.6 Å (Table II). In the case of tri-FTC insulin, several observations indicated that the B-1 dye group could not be considered to participate in exciton formation in this derivative. Addition of the third dye group to the B-29 lysine residue led to a strong CD contribution that was not in simple superposition with the CD spectrum of di-FTC insulin and the resulting exciton band system exhibited a markedly reduced band separation. Both observations are indicative of a new system of greater separation and/or different dye group geometry (*cf.* eq 1). Further, unlike mono- and di-FTC insulin, the alkaline-sensitive nature of the B-1 dye group CD contribution was not manifested in the CD spectrum of tri-FTC insulin. The result strongly suggests that the B-1-bound group is not rigidly positioned in this derivative. The new system is then due to interaction between the A-1- and B-29-bound probes alone. The absence of interaction with the B-1 probe had been attributed to the effects of successive addition of charged groups at A-1 and B-29 on a flexible N-terminal B chain. This conclusion is consistent with and indeed follows independently from the requirement of rigidity in an exciton system. Estimation of the maximum separation based on this identification yielded a value of 10.5 Å.

Estimates of dye group separations based on a geometry factor (eq 1) for a random orientation led to somewhat smaller values for all probe separations. However the absence of conformation-dependent hypochromism in di- and tri-FTC insulin indicated that there was little if any dye group overlap and that the maximum separation was in fact more realistic.

Two final factors raised by the examination of the multiple substituted derivatives are the absence of significant CD associated with monoanion forms of the dye groups and the possibility of an equilibrium of conformers of the FTC insulins. The absence of participation of the monoanion forms in resonance interaction considerably simplified the identification of the origin of pairs of CD extrema although the exact reason for this phenomenon is not known. The result is most probably not due to pH-dependent changes in mutual dye group geometry or separation because the CD extrema of two of the multiple-substituted derivatives decreased with decreasing pH at constant wavelengths (constant band separation). Equation 1 indicates that band separation is a function of both geometry and distance while the full expression for rotational strength (Tinoco, 1963, eq 6) has a different dependence on geometry and distance. Thus while the decreasing rotational strength of the extrema with decreasing pH is consistent with altered separation and geometry, alterations in these two variables would have to cancel each other in eq 1 in order to be compatible with the constant band separations. The resolution of the relevant spectra (*cf.* Figure 8 and 11) may be identified to within 1–2 nm and implies that such cancelling would have to be precise. One speculative explanation may reside in the multiple forms of the monoanion (*cf.* Figure 1). In addition to those indicated, the hydroquinoid form possesses a symmetric tautomer. The molecular orientation of the transition dipole may be different in some of these forms. Accordingly, for a given dimer geometry, there are up to 3! possible mutual dipole orientations. Perhaps excitation of such a population leads to no net CD signal due to averaging.

The observation of conformers and mutarotation in DFL raises the possiblity that, for a given set of conditions, an equilibrium between distinct conformational states exists for the FTC insulins. There is no direct evidence presented here bearing on this problem although several observations argue against the possiblility. (1) The visible CD signals of both di- and tri-FTC insulin were nearly completely restored after exposure to guanidine hydrochloride—a treatment that might be expected to facilitate equilibration with competing forms in the absence of a dominant specie (Teiple and Koshland, 1971). (2) Addition of a third dye group to the B-29 lysine eliminated any manifestation of the long-wavelength CD band of di-FTC insulin. If not all substitutions at the lysyl residue led to an identical effect, it might be expected that a reduced shoulder corresponding to the di-FTC insulin CD spectrum would be resolvable. In the limited spectral region of interest (*ca.* 520 nm) the maximum CD signal of di-FTC insulin could be increased to 50×10^{-5} ΔA by means of highly absorbing samples, whereas the signal for an equimolar preparation of tri-FTC insulin remained flat. Judging from a minimum detectable signal of 1.5×10^{-5} ΔA, the comparison indicated that over 95% of all additions at the B-29 lysyl residue led to the same effect. (3) The magnitudes and positions of the CD and absorption bands were invariant for at least 24 hr and so mutarotation-like behavior as seen in DFL was not apparent. (4) The requirements for a rigid geometry relating the dye groups have been emphasized and suggests to us that under a set of conditions where interaction is observed significant conformational mobility could not be expected. Nevertheless, it clearly remains a critical assumption that the observed results for a given set of conditions represent a homogeneous population.

Structure of Insulin in Solution. Di-FTC Insulin. The relatively primitive analysis of the dye group separations described in this initial application and the probability of local distortion (Arquilla *et al.*, 1969) precludes detailed arguments about the terminal chain positions however major restrictions have been derived from a simple comparison of maximum separations. The maximum possible separation of the two labeled *amino groups* of di-FTC insulin was readily estimated from space-filling models of the two thiocarbamyl dye groups arranged in colinear but opposite directions (*i.e.*, as in two T's with one inverted and on top of the other) and separated by 7.6 Å and was found to be 20–22 Å. For comparison, the maximum possible amino group separation consistent with the primary structure of insulin was estimated from a space-filling model by extending the A and B chains in opposite colinear directions from the A-7–B-7 disulfide bridge. The configuration of the chains was that of the chains in silk fibroin pleated sheets (Pauling, 1960). Measurements from a complete model, yielded estimates in the range 60–65 Å. Thus the upper two-thirds of all possible amino-terminal group separations in the monomer of di-FTC insulin in solution are ruled out.

The crystallographic analysis of insulin has revealed that the amino-terminal group separations within the two monomers of the asymmetric unit are in the range 19–22 Å (Figure 13; Blundell *et al.*, 1971). Conversely, the closest center-to-center approach for two dye groups adapted to the Kendrew–Watson model of the crystallographic structure in a way that is consistent with general van der Waals contacts with the adjacent surface of the molecule lie in the range 6–9 Å. In the crystal structure model both amino groups are on or near the surface of the molecule and thus the two dye groups can be made to lie totally on the surface. The consistency between

the crystallographic determination and the solution estimates implies that the termainal amino groups in the monomeric state and in neutral solution retain positions that are similar to the crystalline state. However the combined experience with the N-terminal B chain suggests a limitation in that this region of the molecule is particularly susceptible to change. Thus the apparent consistency may reflect the influence of a particular affinity of the B-1-bound dye group that tends to favor the crystal structure orientation or may reflect a new amino group separation that only approximates the separation in the crystalline state.

Tri-FTC Insulin. For the A-1 and B-29 residues there is no simple analysis restricting the separation of the amino groups in the insulin other than the observation that a separation sufficient to allow intervening back bone chains would most likely preclude dye group proximity. Thus the spectral data imply that the B-29 and A-1 *amino groups* may be very close compared to the overall dimensions of the monomer. This conclusion is consistent with the chemical and crystallographic evidence. Zahn and Meienhofer (1958) using a bifunctional reagent identified two cross-links, one between the A-1 and B-1 and one between the A-1 and B-29 amino groups (but not between the B-1 and B-29 amino groups). The observations were interpreted with the assumption that only intramolecular cross-links were formed and it was concluded that the C-terminal B chain must be folded back on itself leading to a close proximity of the B-29 lysine residue and N terminus of the A-chain (Zahn, 1964; Klostermeyer and Humbel, 1966). These results have recently been supported by the demonstration of the intramolecular nature of the cross-link formed by reaction with a bifunctional Edman reagent (D. Brandenburg, personal communication). The range for the A-1 and B-29 amino group separations for the two monomers of insulin in the crystallographic asymmetric unit is 9–11 Å (whereas the B-1 and B-29 amino group separations are in the range 22–29 Å; Blundell *et al.*, 1971). In this case the closest approach of two dye groups adapted to the Kendrew–Watson model is van der Waals contact. If the A-1-bound probe is confined to the position considered for di-FTC insulin the separation tends to 10 Å depending entirely on the position of the ϵ-amino bound group.

Previous studies of a series of insulin derivatives have led to a model of certain tertiary relationships necessary for high biological and immunological activity (Arquilla *et al.*, 1967, 1969). In this model the C-terminal B chain folds back on itself principally in the region B-19 to B-23 and leads to a C-terminal heptapeptide (B-24 to B-30) that nearly spands the length of the monomer core and complements the formation of a hydrophobic center (Mercola *et al.*, 1967; Arquilla *et al.*, 1967, 1969). This conformation may be critically stabilized by an interaction of the A-21 asparagine with the residues B-21 or B-22 (Morris *et al.*, 1970a) and is probably involved in dimer formation in solution (Morris *et al.*, 1968). About 24% of the residues of insulin have been estimated to be in helix configurations which are presumably located outside of the C-terminal B chain (Mercola *et al.*, 1967). The folding of the C-terminal B chain in this model leads to a close association of the immunoreactive and biologically sensitive amino groups at A-1 and B-29 (Arquilla *et al.*, 1967, 1969). Thus this relationship is supported by the spectral results presented here. Finally it is noted that a basis for all of these relationships can be readily recognized in the rhombohedral crystal structure (Figure 13) (Adams *et al.*, 1969). Thus the consistency strongly suggests that many features of the crystalline structure remain invariant in solution.

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