Preparation and Characterization of Diphenylthiocarbamyl-insulin and Des-Gly^{A1}-des-Phe^{B1}-insulin (Bovine)*

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ABSTRACT: The feasibility of using pH to control the relative rates of reaction of the amino groups in insulin with phenyl isothiocyanate (PhSCN) was tested by determining the rate of reaction of the model peptide, Ala-Val, with PhSCN as a function of pH. The study revealed, as expected, that the unprotonated form of the amine was the reactive species. The rates of reaction of the three amino groups of insulin (1 mg/ml) with a saturated aqueous solution of PhSCN at 40° was determined at pH 8.0 where the α-amines are largely unprotonated whereas the ϵ-amine of Lys^{B29} is largely charged. The half-times for the first-order reaction under these conditions were 0.5, 1, and 13 hr for the α-amine of Phe^{B1}, the α-amine of Gly^{A1}, and the ϵ-amine of Lys^{B29}, respectively. By limiting the time of reaction between insulin (1 mg/ml) and a saturated aqueous solution of PhSCN, a mixture of phenylthiocarbamylated-insulin (PTC-insulin) was obtained which consisted largely of diPTC-insulin. The latter was isolated by chromatography on DEAE-Sephadex in 7 M urea. Amino acid analysis of this product before and after exhaustive treatment with 1-fluoro-2,4-dinitrobenzene (FDNB) revealed that the two PTC groups were present almost entirely on the two α-amino groups and that no other functional groups of insulin, capable of reacting with FDNB, had been substituted. This conclusion was confirmed by reacting insulin with [^{14}C]PhSCN. The radioactive material, having the same chromatographic properties as diPTC-insulin, contained only two [^{14}C]PTC groups and both of these were lost upon completion of an Edman degradation by cyclization in anhydrous trifluoroacetic acid. This series of reactions was used to prepare des-Gly^{A1}-des-Phe-^{B1}-insulin. To demonstrate that the reaction conditions used to prepare diPTC-insulin as well as those used in its degradation to des-Gly-des-Phe-insulin, did not cause any unsuspected rearrangement, the same procedures were used to convert trimethionyl-insulin (Levy, D., and Carpenter, F. H. (1967), *Biochemistry 6*, 3559) back to Zn-insulin in crystalline form in overall yield of 50%. In the immunoassay for insulin all of these derivatives exhibited quite high activities (>50% of insulin). In the mouse convulsion assay, the hormonal response of diPTC-insulin (20% of insulin) was further decreased upon conversion to des-Gly-des-Phe-insulin (10% of insulin). New eluting buffers were developed which allowed the determination of the free and ω-DNP-amino acids on the amino acid analyzer.

The use of these systems revealed that attempts to improve the recovery of tyrosine in the acid hydrolysis of proteins by the incorporation of small amounts of phenol in the hydrolysis mixture gives rise to ninhydrin-reacting artifact(s). The latter appears to be a product of reaction between phenol and cystine.

The relationship of structure to biological activity of insulin has been the subject of numerous studies (for reviews, see Klostermeyer and Humbel, 1966, and Carpenter, 1966). Recently we have described the preparation of tri-Boc-insulin[1] and various triaminoacyl-insulins (Levy and Carpenter, 1966, 1967). In these derivatives reaction has taken place at each of the three amino groups of insulin (the two N terminals of the A and B chains and the ϵ-amine of Lys^{B29}). In the case of the triBoc derivative the amines were covered with a neutral residue to yield a product with reduced but still appreciable biological activity (25–50%) which upon treatment with anhydrous trifluoroacetic acid was converted back into insulin. In the triaminoacyl-insulins, various amino acid residues were added to each of the amino groups which in effect extended the peptide chains at the amino terminus as well as adding a side chain on the ϵ-amine of Lys^{B29}. Regardless of the addition of either neutral, acidic, or basic amino acid residues, the resulting derivatives were all about 50% as active as insulin. These results, which indicated that the peptide chains can be elongated at the amino end without unduly affecting the biological activity, raised the related question as to the effect of shortening the peptide chains from the amino terminus.

From the action of leucine aminopeptidase on insulin, Smith *et al.* (1958) concluded that the first six residues of the B chain were not essential for activity but that activity was decreased either by the removal of residues from the A chain or by hydrolysis past the interchain disulfide bridge. They

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[1] Abbreviations used are: Boc, *t*-butyloxycarbonyl; FDNB, 1-fluoro-2,4-dinitrobenzene; FSCN, fluoroscein isothiocyanate; FTC, fluorosceinthiocarbamyl; PhSCN, phenyl isothiocyanate; PTC, phenylthiocarbamyl; Cbz, carbobenzoxy; ω-DNP-amino acids, amino acids in which the functional groups other than the α-amino group are dinitrophenylated, *i.e.*, ϵ-DNP-Lys, *O*-DNP-Tyr, *S*-DNP-Cys, and *im*-DNP-His.

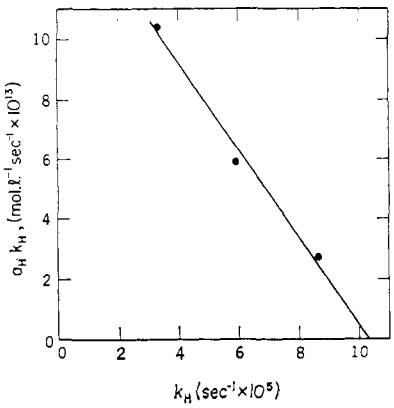

FIGURE 1: Graphic determination of the pH-independent rate constant and the ionization constant of the reacting amine by the method of Hill and Davis (1967). α_H is the hydronium ion activity and k_H is the experimentally determined pH-dependent rate constant.

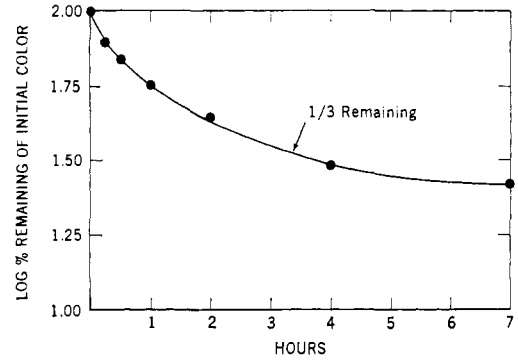

FIGURE 2: Overall rate of reaction of the primary amines of Zn-free insulin (1.0 mg/ml) with PhSCN at pH 8.0, 40° as measured by loss of ninhydrin color.

were unable to distinguish between these two possibilities. Recently in semisynthetic studies in which synthetic A chains were combined with natural B chains, very little biologically active material was produced from A-chain derivatives that were missing the N-terminal glycine residue (Weber *et al.*, 1967a,b). The interpretation of these latter results suffers from the fact that the low activity may have been due to poor efficiency in the correct pairing of disulfide bridges on recombination of chains rather than to a low activity of the correctly paired chains.

Our approach to the problem involved the application of a modified Edman-type degradation to the intact insulin molecule. This has led to the preparation of a diPTC derivative of insulin, having the PTC groups on the α-amino groups of the N-terminal glycine and phenylalanine, which upon cyclization in anhydrous trifluoroacetic acid yielded des-Gly^{A1}-des-Phe^{B1}-insulin. The degradative procedure was validated by using it to reconvert trimethionyl-insulin into insulin which was recovered in crystalline form. Independently similar degradative studies have been performed by Brandenburg and Ooms (1968) and Brandenburg (1969).

Rationale. Our initial objective was to react PhSCN with insulin in such a fashion as to substitute the two α-amino groups without covering the ϵ-amine at Lys^{B29}. If this could be done, then the degradation would yield a derivative identical in charge properties with insulin but lacking the two amino-terminal residues. In order to secure selective reaction on the α-amino groups we planned to take advantage of the difference in pK_a values of α-amino groups (pK_a = 7.4) in contrast to that of the ϵ-amine of lysine (pK_a = 9.6) (Tanford and Epstein, 1954). It seemed reasonable to expect that only the unprotonated form of the amines would react readily with the PhSCN. If this were the case, then at a pH around 8 the α-amines would be largely unprotonated and reactive while the ϵ-amine would be largely protonated and unreactive.

Effect of pH on the Rate of Reaction of PhSCN with Ala-Val. To determine the effect of pH on the rate of reaction of amino groups as well as to determine the feasibility of performing the acylation reaction in water, the rate of reaction of a saturated aqueous solution of PhSCN with Ala-Val was determined as a function of pH at 40°. Since the solution was kept saturated with PhSCN, the reaction followed first-order kinetics. The resulting first-order kinetic constants were treated according to the method of Hill and Davis (1967) as shown in Figure 1. From the slope of the resulting line the pK_a of the amino group was calculated as 7.83, in good agreement with the value of 7.85 determined by direct titration at 40°. The graphic method assumes that only the unprotonated form of the amine reacts. If this were not so, the agreement between the kinetic data and the titration data would be impossible.

Rates of Reaction of the Amino Groups of Insulin with PhSCN at 40° and pH 8.0. The overall reaction of PhSCN with the amino groups of insulin at 40° and pH 8.0 was followed by the decrease in the ninhydrin color with time with the results shown in Figure 2. From this curve it is apparent that the overall reaction does not follow first-order kinetics. The rate falls off with time and becomes quite slow after four hours.

In order to determine the separate rate of reaction of the glycine and phenylalanine groups, aliquots were removed at various times, the reacted α-amino groups were removed by cyclization in trifluoroacetic acid and the unreacted α-amino groups were determined from amino acid analysis of an acid hydrolysate of the residual protein. This procedure depended upon determining the difference in amino acid composition between whole insulin and partially degraded insulins. Since the glycine residues decrease from 4 to 3 and the phenylalanine residues decrease from 3 to 2 in going from insulin to the completely PTC-insulin, the rate measurements, which depended upon determining small differences between rather large numbers, are only approximate.

In order to determine the unreacted ϵ-amine of Lys^{B29}, aliquots were treated with FDNB, subjected to acid hydrolysis, and the resulting ϵ-DNP-Lys was determined on the amino acid analyzer. When native insulin was treated with FDNB and the resulting product hydrolyzed and subjected to amino acid analysis, 92% of the lysine was recovered as ϵ-DNP-Lys. In the rate studies the analyses for ϵ-DNP-Lys on the partially PTC-insulins were corrected for this recovery value.

The results of these measurements (Figure 3) show, that in a saturated aqueous solution of PhSCN at 40° and pH 8.0, the N-terminal phenylalanine residue reacts the fastest with a half-time of 30 min. The N-terminal glycine reacts somewhat slower with a half-time of 1 hr while the ϵ-amine of

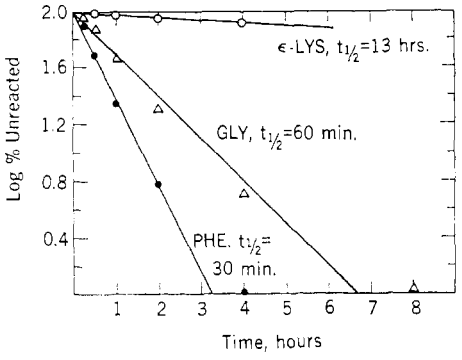

FIGURE 3: Individual rates of reaction of the primary amines of Zn-free insulin (1.0 mg/ml) with PhSCN at pH 8.0 and 40°. See text for details.

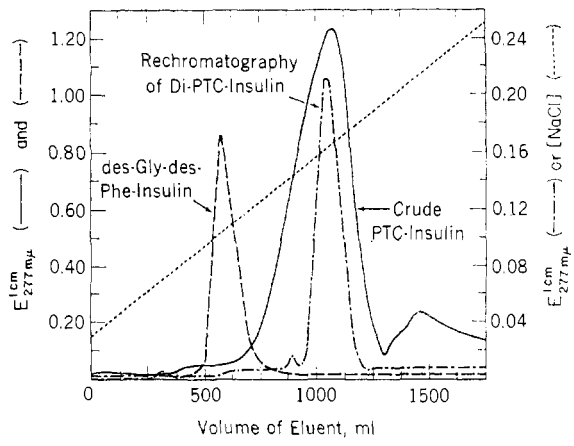

FIGURE 4: DEAE-Sephadex chromatography of insulin derivatives on a 2.5 × 30 cm column at 4° at 60 ml/hr flow rates in 0.01 M Tris (pH 7.9) buffers containing 7 M urea with a NaCl gradient. Crude PTC-insulin (—); rechromatography of the main peak of diPTC-insulin (-·-); des-Gly-des-Phe-insulin (— — —); NaCl gradient (---).

Lys^{B29} reacts very much slower with a half-time of 13 hr. This very large difference between the rate of reaction of the ϵ-amine of lysine and the two α-amino groups can be attributed to the difference in pK_a values of these groups and confirms the rationale for this experimental approach. However, the difference between the reactivities of the two α-amino groups deserves further comment.

The α-amino group of glycine reacts much slower than one would expect in view of the fact that Drobnica and Augustin (1956b) reported that free glycine reacted with PhSCN 1.5 × faster than any other amino acid tested. This low reactivity of the glycine N terminal may be attributed to one of two factors: (1) the pK_a of the Gly^{A1} amino group may be somewhat higher than that of the Phe^{B1}. If the pK_a value for the glycine amine were 8.2, then it would have the same pH independent rate constant as the phenylalanine amine with a pK_a of 7.45. A study of the rate of reaction of these two groups as a function of pH in a fashion similar to that performed on Ala-Val in this work or on the α chain of hemoglobin by Hill and Davis (1967) might resolve this question but has not yet been attempted. (2) An alternate explanation for the anomalous reaction rate of Gly^{A1} is that the α-amine is inaccessible to the reagent, either because of tertiary structure within the monomer unit or because of aggregation of monomers (quaternary structure).

Our finding that the amino group of phenylalanine reacts faster than the glycine amino group is just the opposite from that reported by Christensen (1951), using PhSCN, and by Andersen (1956), using phenyl isocyanate. Both of these investigators reported that the glycine residue reacted faster than the phenylalanine moiety. On the other hand, Tietze *et al.* (1962) and more recently Bromer *et al.* (1967), both using FSCN reported that the phenylalanine amino group was the most reactive to this reagent. Finally, Brandenburg (1969) in H. Zahn's laboratory[2] has found, that upon reaction of insulin in the presence of a limited amount of PhSCN, substitution occurred primarily on the phenylalanine amino group; a result which is in agreement with our kinetic data.

Diphenylthiocarbamyl-insulin. From calculations based upon the measured rate constants, it was decided to run the reaction of PhSCN with insulin at 40° and pH 8.0 for 4 hr at which time in theory 99.8% of the phenylalanine, 94% of the glycine, and 18% of the ϵ-Lys-amino groups would be carbamylated. The product of reaction was separated into its components on the basis of net charge by chromatography on DEAE-Sephadex in 7 M urea solutions at pH 7.9 with the results shown in the solid line in Figure 4. In this system insulin elutes at about 600 ml and desamido-insulin, which has one more negative charge, elutes at 850 ml (Levy and Carpenter, 1967). The main band, eluting at 1100 ml in Figure 4, has the chromatographic properties of an insulin derivative possessing two more net negative charges than insulin as expected for the diPTC-insulin. The material eluting at 1500 ml is presumed to be a mixture of triPTC-insulin and diPTC-desamido-insulin. When the main peak of Figure 4 was isolated and rerun in the same system, it gave a nearly symmetrical band indicative of a single component (Figure 4).

The purified diPTC-insulin was subjected to amino acid analysis before and after being treated with FDNB and compared with results obtained under similar conditions for insulin with the results shown in Table I. When insulin was treated with FDNB and hydrolyzed, there was a loss of one residue of glycine and phenylalanine, whereas with diPTC-insulin one obtained the same value for these groups before and after treatment with FDNB. This indicated that the glycine and phenylalanine residues in diPTC-insulin were unavailable for reaction with FDNB. On the other hand, when insulin or diPTC-insulin was treated with FDNB, there was a loss of one lysine which was replaced by ϵ-DNP-lysine, a loss of four tyrosines which was replaced by nearly a corresponding number of *O*-DNP-Tyr and a loss of two histidines which was replaced by *im*-DNP-His. These results indicate that the ϵ-amino group of lysine, the phenolic hydroxyls of tyrosine, and the imidazole groups of histidine were as free to react in diPTC-insulin as they were in insulin.

The above results indicated that the purified diPTC-insulin consisted largely of a derivative in which only the α-amino residues were substituted. Calculations based upon the rate constants indicate that a diPTC-insulin isolated from the

[2] We wish to thank Dr. H. Zhan for making this manuscript available to us prior to publication.

TABLE I: Amino Acid Analysis of Insulin and Insulin Derivatives before and after Treatment with FDNB.[a]

		Insulin after FDNB		diPTC-insulin after FDNB		Des-Gly-des-Phe-insulin after FDNB
Lys	1.00	*0.02*	1.01	*0.06*	0.97	*0.08*
His	1.88	*0.04*	1.96	*0.15*	1.68	*0.10*
Arg	1.00	1.00	1.00	1.00	1.00	1.00
im-DNP-His		1.60		1.26		1.49
ε-DNP-Lys		0.94		0.92		0.92
O-DNP-Tyr		3.45		3.24		3.54
Asp	2.94	2.95	2.96	2.96	3.05	2.98
Thr	0.95	0.84	1.02	0.88	0.98	0.91
Ser	2.50	1.91	2.60	2.01	2.57	1.95
Glu	7.06	7.06	7.04	7.04	6.95	7.02
Pro	0.90	1.01	0.91	0.99	0.91	0.98
Gly	4.09	*3.04*	3.52	3.59	3.03	3.06
Ala	3.00	2.85	3.09	2.82	2.95	2.87
Half-Cys	5.08	4.40	3.29	3.52	4.94	3.26
Val	4.74	4.85	4.89	4.50	4.61	*4.12*
Ile	0.80	0.98	0.87	0.98	0.79	*0.11*
Leu	5.85	5.86	5.96	5.96	5.96	6.02
Tyr	3.58	*0.04*	3.47	*0.06*	3.69	*0.03*
Phe	2.85	*2.04*	2.22	2.53	2.00	1.95

[a] Italics indicate those amino acid residues whose recoveries were decreased owing to reaction with FDNB. The decreased recoveries of serine and threonine in the FDNB-treated columns (hydrolyzed for 24 hr) as compared with the nontreated materials (hydrolyzed for 12 hr) is attributed to the increased time of hydrolysis. The low cystine values are attributed to the presence of phenol in the hydrolysis mixture used for analysis of the neutral and acidic amino acids (see Table V).

reaction mixture should consist of 98.1% (PTC-Gly)-(PTC-Phe)-insulin, 1.3% (PTC-Phe)-(ε-PTC-Lys)-insulin, and 0.6% (PTC-Gly)-(ε-PTC-Lys)-insulin. These latter two components may have been present in our purified diPTC-insulin but if so they were not present in amounts large enough to be detected by our analytical techniques or to be seriously detrimental to our procedures.

Rate of Conversion of DiPTC-insulin into Des-Gly-des-Phe-insulin. Since it is known that long exposure of insulin to trifluoroacetic acid can result in the incorporation of trifluoroacetic acid groups into insulin (Bak *et al.*, 1967), we thought it desirable to determine the rate at which the cyclization of the diPTC-insulin took place in trifluoroacetic acid in order to find optimum conditions for this conversion. An assay was developed which took advantage of the difference in the ultraviolet absorption spectra of diPTC-insulin and insulin or des-Gly-des-Phe-insulin as shown in Figure 5. By measuring the ratio of absorption at 255 mμ to that at 275 mμ the proportion of reactant and product in a mixture was determined. The reaction followed first-order kinetics with a half-time at 25° of 14 min. From this result we decided to use a treatment period of 2 hr for the cyclization in order to be assured of a complete reaction. This time of treatment is approximately twice as long as that recommended by Konigsberg and Hill (1962) for degradation of PTC-peptides in trifluoroacetic acid.

Des-Gly^{A1}-des-Phe^{B1}-insulin. DiPTC-insulin, which had been purified by DEAE-Sephadex chromatography, was treated with anhydrous trifluoroacetic acid to remove the N-terminal amino acids. The resulting product was subjected to ion-exchange chromatography with the results shown in Figure 4. The material has the chromatographic behavior expected for a substance with the same net charge as insulin. The symmetry of the elution curve is indicative of homogeneity. The des-Gly-des-Phe-insulin was isolated from the pooled chromatographic fractions and subjected to amino acid analysis before and after being treated with FDNB with the results shown in the last two columns of Table I. The analysis of des-Gly-des-Phe-insulin revealed that it was missing a residue each of glycine and phenylalanine as compared with

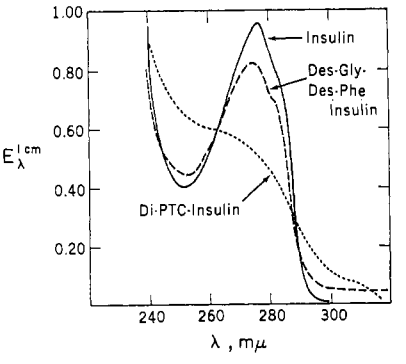

FIGURE 5: Ultraviolet absorption spectra of 1.0 mg/ml of insulin hydrochloride (—), 0.87 mg/ml of des-Gly-des-Phe-insulin (— — —), and 0.17 mg/ml of diPTC-insulin (---), all in 1-cm cuvets.

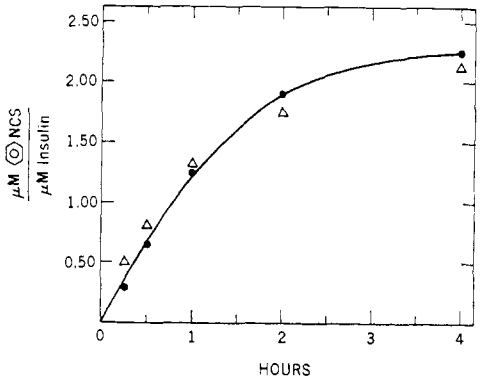

FIGURE 6: Reaction of [^{14}C]PhSCN with Zn-free insulin (1.0 mg/ml) at pH 8.0, 40°: (●—●) experimental points; (△—△) theoretical points calculated by assuming that only the amines of phenylalanine, glycine, and lysine reacted with PhSCN and that they reacted with the rates determined in Figure 3.

insulin. After treatment with FDNB followed by hydrolysis, the dinitrophenylated material was missing a residue of isoleucine and valine, indicating that these were the new N-terminal amino acids. This is the expected result for des-Gly-des-Phe-insulin. The loss of lysine, tyrosine, and histidine upon dinitrophenylation along with the appearance of ε-DNP-Lys, *O*-DNP-Tyr, and *im*-DNP-His, demonstrates the availability of these residues to react with FDNB.

Reaction of Insulin with [^{14}C]*PhSCN.* Although the above results on the action of FDNB on diPTC-insulin had indicated that the PhSCN had not reacted appreciably with ε-amino group of lysine, the phenolic groups of tyrosine, or the imidazole groups of histidine, it was conceivable that some unforeseen reaction had taken place with residues other than the two α-amino groups. In order to obtain a definitive answer to this question, [^{14}C]PhSCN was prepared from uniformly labeled aniline and was reacted with insulin under the same conditions as those used for the unlabeled compound. The rate of incorporation of the labeled isothiocyanate into insulin was followed with the results shown in Figure 6. In this figure the line which joins the solid circles represents the incorporation of labeled PhSCN into insulin. The open triangles are values that were calculated for the rate of incorporation based upon the rate constants for the reaction of each amino group as determined above. The reasonable agreement between the calculated points and the experimentally found curve indicates that there is no unsuspected reactive group.

After 4-hr reaction with [^{14}C]PhSCN, the labeled PTC-insulin was isolated and subjected to chromatography on DEAE-Sephadex with the results shown in Figure 7. The material was collected in two fractions as indicated on the figure. Peak I has the chromatographic properties of a diPTC-insulin. It contained 2.05 residues of [^{14}C]PTC/mole of insulin. When this material was treated with trifluoroacetic acid, all but 0.06 residue of the [^{14}C]PTC was removed. These results indicate that the material in peak I contained only two residues of PTC and that both of these were removed almost entirely upon completion of the Edman degradation in trifluoroacetic acid. On the other hand, the material in peak II contained 2.95 residues of [^{14}C]PTC/mole of insulin, indicative of a triPTC-insulin. Upon treatment of this material with trifluoroacetic acid, over 2 residues of PTC were removed but there remained 0.71 residue of [^{14}C]PTC attached to the protein. Presumably, this represents a residue attached to the ε-amino group of lysine which would be expected to be resistant to the action of trifluoroacetic acid.

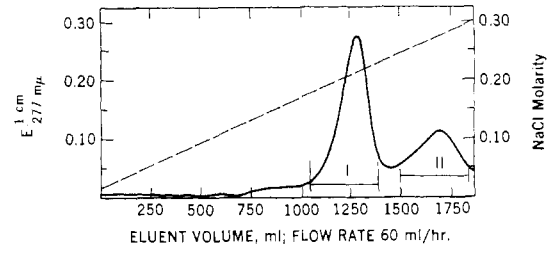

FIGURE 7: DEAE-Sephadex chromatography of [^{14}C]PTC-insulin. Same chromatographic conditions as for Figure 4.

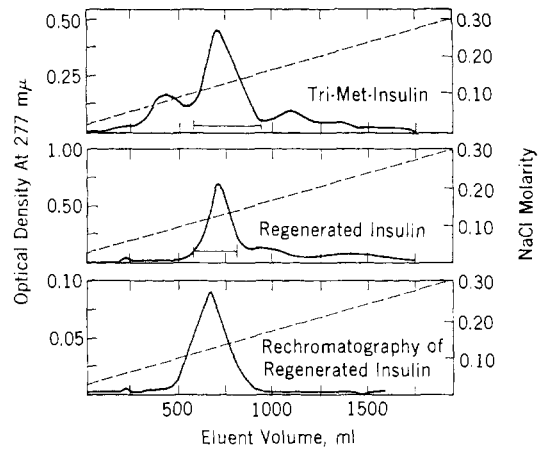

FIGURE 8: DEAE-Sephadex chromatography of triMet-insulin (top), of insulin regenerated from triMet-insulin (middle), and rechromatography of regenerated insulin (bottom). I—I indicates the fractions that were pooled for isolation. Chromatographic conditions are as for Figure 4.

Conversion of Trimethionyl-insulin into Insulin. The work described to this point indicated that it was possible to prepare an insulin derivative in which only the two α-amino groups were carbamylated and which upon treatment with trifluoroacetic acid was converted into a protein with the amino acid composition and with many of the free functional groups expected of des-Gly-des-Phe-insulin. However, the reactions had not ruled out the possibility of some undetected rearrangement of the molecule (such as disulfide interchange) brought about by the conditions used in the reactions. In view of the very low biological activity of des-Gly-des-Phe-insulin, it was important to establish that no unsuspected rearrangement had taken place. A possibility to establish this point was afforded by the availablilty of triaminoacyl-insulins, that is insulins in which an additional amino acid residue had been added to each of the two α-amino groups as well as to the ε-amino group of lysine (Levy and Carpenter, 1966, 1967). Reconversion of one of these derivatives into insulin would afford positive proof of the efficacy of the procedure.

Trimethionyl-insulin was selected for this work since the lack of methionine in bovine insulin makes it convenient to

TABLE II: Amino Acid Analyses of TriMet-insulin and of Insulin Regenerated from TriMet-insulin.

Amino Acid	TriMet-insulin	Regenerated Insulin
Lys	0.97	0.95
His	1.89	1.85
Arg	1.00	1.00
Asp	3.04	3.00
Thr	0.95	0.97
Ser	2.62	2.62
Glu	6.96	7.01
Gly	4.08	3.88
Ala	2.99	3.02
Half-Cys	5.18	5.46
Val	4.71	4.80
Met	3.14	0.08
Ile	0.84	0.82
Leu	6.02	5.84
Tyr	3.68	3.70
Phe	3.00	2.84

TABLE III: Biological Activities of Insulin and Insulin Derivatives.

Sample	Mouse Convulsion (units/mg)	Immunoassay
Insulin control	18.1 ± 2.4[a]	22.2
PTC-insulin		
Crude	3.5 ± 0.7	14.3
di-PTC-insulin		
Chromatographed	5.2 ± 0.2	13.8
des-Phe-des-Gly-insulin		
Crude	2.6 ± 0.5	14.3
Chromatographed	2.0 ± 0.3	14.2
Zn-insulin (lot PJ-7462)	21.7 ± 2.8	25.1
TriMet-insulin	2.8 ± 0.4[b]	2.7
Insulin regenerated from triMet-insulin		
Crude	16.4 ± 2.6	21.6
Crystallized	19.3 ± 1.9	22.2

[a] The ± refers to one standard deviation from the mean. [b] The mouse convulsion assay on this preparation of triMet-insulin was considerably lower than that obtained on two other preparations which assayed at 12.3 ± 1.7 (Levy and Carpenter, 1967) and 11.2 ± 2.0 units per mg.

follow the course of the reactions by amino acid composition. The trimethionyl-insulin was prepared by the procedure of Levy and Carpenter (1967) and then purified by chromatography on DEAE-Sephadex as shown in the top panel of Figure 8. The main fraction, eluting at about 500 ml, was collected and used for these studies. It had an amino acid composition as shown in Table II. All of the amino groups in trimethionyl-insulin should have about the same pK_a values and the same reactivity to PhSCN as the N-terminal amino groups in insulin. To ensure complete reaction, the derivative was treated with PhSCN for 6 hr (instead of the usual 4) at 40° and pH 8.0. The resulting PTC-methionyl-insulin was isolated and, without subjecting it to further purification, the dry material was treated with trifluoroacetic acid. The protein isolated by this treatment was subjected to DEAE chromatography with the results shown in the middle panel of Figure 8. The main peak, which had the expected chromatographic behavior, was pooled as indicated and the protein was isolated. A portion of this was rechromatographed with the results shown in the bottom panel of Figure 8. The symmetry of the curve is indicative of homogeneity. Another portion of the material was subjected to amino acid analysis with the results shown in Table II. It was practically free of methionine and had the expected composition of bovine insulin. A third portion of the material was subjected to several isoelectric precipitations in the presence of zinc. On the second precipitation, crystals were obtained which were recrystallized to give a 50% yield (based on the starting trimethionyl-insulin) of crystalline bovine insulin with nearly full biological activity.

The above series of reactions offer an unambiguous proof that no unforeseen rearrangements are taking place to any large extent during the reactions involved in the modified Edman degradation as used in this work. The results also validate the synthetic reactions used in preparing the trimethionyl-insulin. It will be recalled that most of the triaminoacyl-insulins prepared by Levy and Carpenter (1967) possessed about 50% of the activity of insulin. The fact that one of these derivatives can be reconverted in good yield into insulin of high potency indicates that no unforeseen rearrangements took place in the synthetic reactions and that the decreased activity of these products is a property of the molecule and not due to some unknown side reaction. In this connection, it should be noted that the trimethionyl-insulin used in these studies assayed at 2.8 units/mg in the mouse convulsion test, a value somewhat lower than that (12.3 units/mg) reported by Levy and Carpenter (1967). Despite this low activity, the material was still reconverted into insulin of good potency (20 units/mg) (Table III).

Biological Activity of Insulin Derivatives. The various insulin derivatives were subjected to two biological tests, a mouse convulsion assay performed by a technique similar to that described by Smith (1950) in which 150 mice were used for each assay and an immunoassay.[3] The top section of Table III shows the results obtained on a series of samples involved in the preparation of des-Gly-des-Phe-insulin. The insulin control refers to a sample of insulin that was put through all of the solvents and manipulations involved in the preparation of des-Gly-des-Phe-insulin with the exception that the PhSCN as well as the chromatographic purification was omitted. This control exhibited an activity about 10% below that of the crystalline Zn-insulin (PJ-7462, see bottom section) from which it was made. Since this difference is within the error of the assay, it is apparent that the solvents and manipulations had little effect on the biological activity of the material.

[3] The assays were performed at the laboratories of Eli Lilly and Co., Indianapolis, Ind.

TABLE IV: Elution Times Obtained with Pyridine Buffers at 70-ml/hr Buffer Flow Rates.

Amino Acid	Buffer 1 (min)	Buffer 2 (min)
im-DNP-His	35	
S-DNP-Cys	56	
Lys	62	
His	71	
ε-DNP-Lys	89	
NH_3		58
O-DNP-Trp (not resolved)	112	
		100
Arg	140	64

TABLE V: Effect of Presence of Phenol in the Acid Hydrolysis on Recovery of Cystine, Tyrosine, and Artifactual Peaks.

Amino Acid Peak	Insulin (residues/mole)		Cystine (μmoles recovd)	
	−Phenol	+Phenol	−Phenol	+Phenol
Half-Cys	5.70	3.71	0.375	0.227
Tyr	3.68	4.01		
Peak at 29 min[a]	0.00	0.34	0.00	0.034
Peak at 31 min[a]	0.00	1.34	0.00	0.124

[a] Assumed to have the same color value as leucine.

The crude PTC-insulin refers to material in the whole reaction mixture which, as shown by Figure 4, contains several components including di- and triPTC-insulins. In the mouse convulsion assay, the crude material exhibited somewhat less activity than the purified diPTC-insulin obtained on chromatography of the mixture. Both crude and purified material assayed about the same and at a much higher value in the immunoassay than in the convulsion assay. These results indicate that the antigenic determinants of the molecule were not affected nearly as much by the substituti on of the amino group as was the hormonal activity. When the diPTC-insulin, which possessed about 20% of the hormonal activity of insulin, was converted into des-Gly-des-Phe-insulin there was a further drop in this activity. The latter compound whether crude or chromatographed exhibited about 10% of the hormonal activity of insulin but again the response to insulin antibodies was quite high, being about 60% of that of insulin.

The bottom section of Table III shows the results obtained in the control experiment which demonstrated the efficacy of the procedures used to synthesize triaminoacyl-insulins as well as those used in the degradation of insulin. When crystalline Zn-insulin was converted into trimethionyl-insulin by the procedure of Levy and Carpenter (1967), the resulting derivative exhibited a quite low activity (about 10% of insulin) in the one mouse convulsion assay performed on this particular sample. Several other samples of trimethionyl-insulin prepared in this laboratory have assayed much higher at about the 50% level. Unfortunately not enough of this particular sample was retained to allow a reassay, so we are uncertain as to the significance of this low activity. Be that as it may, when this sample was put through the modified Edman degradation, the crude material before chromatography possessed about 75% of the activity of the starting insulin. After this was chromatographed the material in the main peak was isolated in crystalline form in an overall yield of 50% from the triMet-insulin and with a hormonal activity comparable with that of the starting insulin.

Ion-Exchange Chromatography of ω-DNP-amino Acids. The use of the amino acid analyzer with ninhydrin color development for the analysis of ω-DNP-amino acids in hydrolysates of DNP-proteins presents several problems. The ω-DNP-amino acids must be resolved from all the other amino acids, preferably in such a manner that all of the amino acids can be determined. If elution from the sulfonated polystyrene resin is attempted with the normal salt and pH gradients, the ω-DNP-amino acids tend to elute as very broad, slowly moving peaks. Presumably this is caused by strong interaction between the resin and the DNP groups. Nishakawa *et al.* (1967) suggested incorporating *p*-hydroxybenzoic acid into the buffer to reduce this absorption. In our hands, their procedure resulted in a broad ε-DNP-Lys peak which overlapped the adjacent ammonia and arginine peaks to such an extent that accurate determinations were precluded.

The system reported here incorporates pyridine into the buffers in order to decrease the interaction of the DNP group with the resin. Although quite sharp peaks are obtained, it was necessary to run two columns with different buffers to resolve all of the commonly occurring ω-DNP-amino acids from the basic amino acids. Buffer 1 (0.21 M pyridine–0.05 M sodium citrate at pH 5.28) resolved *im*-DNP-His, *S*-DNP-Cys, Lys, His, ε-DNP-Lys, and Arg (Table IV). In this buffer ammonia and *O*-DNP-tyrosine overlapped. In order to analzye for the latter, it was necessary to run a new column with buffer 2 (0.4 M pyridine–0.05 M sodium citrate–0.18 M NaCl adjusted to pH 5.28). Brief attempts to combine the two buffers in either a gradient or a stepwise elution pattern so that all components would be resolved in a single analysis were unsuccessful.

Artifacts Arising from the Use of Phenol in Acid Hydrolysis. Following the suggestion of Benisek *et al.* (1967), it has been the custom in this laboratory to add 2–3 mg of phenol to acid hydrolysis tubes before sealing. This has resulted in raising the routine recoveries of tyrosine residues in insulin from 3.6 in the absence of phenol to 3.9–3.95 in the presence of phenol. The pyridine chromatographic system revealed an unfortunate side reaction which makes this addition of phenol unsatisfactory for general use. When insulin, casein, egg-white lysozyme, or chymotrypsin were hydrolyzed in the presence of phenol and the hydrolysate analyzed on the short column in pyridine buffer 1, two unknown peaks appeared at 29 and 31 min. These two peaks effectively obscure any *im*-DNP-His (elution time of 31 min) in a DNP-treated protein. The artifactual peaks were absent when phenol was omitted from the hydrolysis mixture. Their origin appears to be due to an interaction between phenol and cystine as shown in Table V. In the standard accelerated analysis for basic

amino acids (Spackman, 1963), the two artifactual peaks emerge as a single peak at 30 min, and might be confused with chlorotyrosine or tryptophan. Despite these handicaps the addition of phenol to a hydrolysis mixture can still be very useful in critical analyses for tyrosine.

Discussion

In many cases one of the principal problems causing ambiguity in correlations between chemical modifications with biological activity is due to the lack of reagent specificity which gives rise to heterogeneous products. The results discussed above indicate that, at least for the diPTC-insulin and the des-Gly-des-Phe-insulin, problems of reagent specificity and product heterogeneity have been solved and that the two derivatives differ chemically from native insulin only in the defined manner in which the N-terminal residues of the two peptide chains have either been blocked or removed.

The diPTC-insulin possessed about 20% of the hormonal and 50% of the immunological activity of native insulin. It is interesting to note that the triBoc-insulin prepared by Levy and Carpenter (1967) had essentially the same activity. One might infer from this that the ϵ-amino group of lysine has little importance in maintaining a biologically active molecule. Bromer *et al.* (1967) reacted insulin with FSCN and separated the components on the basis of charge into mono-, di-, and triFTC-insulins. The monoFTC-insulin, estimated to be at least 70% FTC-Phe^{B1}-insulin, possessed 50% of the hormonal activity and 100% of the immunological activity of insulin. In the diFTC-insulin, which was judged to be substituted primarily on the N-terminal glycine and phenylalanine residues, the hormonal activity had dropped to about 3% and the immunological response to 30% of that of insulin. The triFTC-insulin was virtually inactive in both assays. The difference in response between the diPTC-insulin and diFTC-insulin and between the triBoc-insulin and triFTC-insulin can probably be attributed to the difference in steric size of the substituent groups; the bulky, planar, and hydrophobic fluorescein group exhibits a much greater effect than the PTC or Boc residues.

Des-Gly-des-Phe-insulin has the same net charge as native insulin and it also lacks additional groups which might contribute an effect of their own to insulin's three-dimensional structure, *e.g.*, by sterically preventing a preferred structure or increasing hydrophobic interaction and thereby leading to a new structure or aggregation pattern. The fact that going from diPTC-insulin to des-Gly-des-Phe-insulin produced a further substantial decrease in the biological activity, indicates that the integrity of one or both of the N-terminal residues is essential for a fully active molecule. In recent work Brandenburg (1969) was able to prepare a monoPTC-insulin which was largely substituted on the N-terminal phenylalanine. When this was cyclized he obtained a des-Phe^{B1}-insulin in crystalline and fully active form. Thus the large loss in activity in going from insulin to des-Gly-des-Phe-insulin, as noted in this work and as also reported in a note by Brandenburg and Ooms (1968), must be attributed to a loss of glycine from the N-terminal residue of the A chain. As noted above this glycine reacts abnormally slowly with PhSCN and this fact may be related to the unknown function which glycine performs in maintaining the biologically active form of the hormone. It should also be recalled that in all the species of insulin investigated so far the N-terminal residue on the A chain has been glycine (Smith, 1966). Thus the N-terminal glycine on the A chain like the C-terminal asparagine (or aspartic acid) on this chain (Slobin and Carpenter, 1963) appears to play an important role in defining the hormonal action.

The fact that des-Phe-des-Gly-insulin possesses some, albeit quite low, hormonal activity, makes it unlikely that the missing amino acids represent "active-site" residues in the sense that this term is applied to enzymes. Conceivably these residues are needed to maintain the three-dimensional structure characteristic of the fully active molecule. In order to test this hypothesis the derivatives reported here as well as a number of those previously reported (Young and Carpenter, 1961; Slobin and Carpenter, 1963; Levy and Carpenter, 1967) have been subjected to a series of physical–chemical studies including optical rotatory dispersion, circular dichroism, ultracentrifugation, and zinc binding. These results will appear in a forthcoming communication.

Experimental Section

Materials. FSCN, FDNB, trifluoroacetic acid, and *N,N*-dimethylformamide were all obtained from Distillation Products Industries. PhSCN was purified according to Dains *et al.* (1958). The purified reagent was stored in small glass ampoules, which were refrigerated until opened as needed. Trifluoroacetic acid, obtained in 100-g bottles, was used without further purification. The bottle was flushed with nitrogen and stored in a desiccator after each use. Dimethylformamide was deionized by passing 500 ml through a column containing 20 g of mixed-bed resin (Bio-Rad Laboratories, AG 501-X8 20–50 mesh). The deionized dimethylformamide was then mixed with 25 g of calcium hydride (Metal Hydride Co.) and distilled under a water-aspirator vacuum at 52°. The distillate was collected and stored at 4° over molecular sieves (Linde 3A). FDNB was used without further purification (Matheson, Coleman & Bell). Whenever insulin or its PTC derivatives were treated with ether, the ether was obtained from a freshly opened can of Squibb USP peroxide-free ether. Pyridine was prepared free of ninhydrin-positive components according to Dixon (1956). L-Ala-L-Val (lot 656), L-alanine (lot 131018), and L-Val (lot 106593) were all obtained from Mann Research Laboratories. Under the standard conditions of amino acid analysis, each sample showed only one ninhydrin-positive component.

[^{14}C]PhSCN was synthesized from uniformly labeled [^{14}C]aniline with an activity of 5 μCi/μmole (New England Nuclear Corp.) by a micro-scale adaptation of the procedure of Dains *et al.* (1958). The product was purified by high-vacuum distillation across a 4–5-mm gap between the bottom of a sublimator and a cold finger (Kontes Bantamware) which was filled with Dry-Ice–acetone. The final product had an activity of 24×10^3 cpm/μmole and showed only a single component migrating identically with an authentic sample of PhSCN upon thin-layer chromatography on silica gel G in the following four solvent systems: benzene, acetone–water (9:1), ethyl acetate–ether (4:1), 1-butanol–acetic acid–water (60:15:25).

Boc-MetpNp was prepared from Boc-Met (Cyclo Chemicals Co., lot 800635) as described by Levy and Carpenter (1966).

The ester was crystallized from ether–petroleum ether (bp 30–60°) and had a melting point of 94–95°.

ε-PTC-Lys was prepared from α-Cbz-L-Lys (mp 220–221°) obtained from Mr. Thomas Pillsbury of this laboratory. Acylation of the latter compound in pyridine–H_2O with PhSCN yielded a noncrystalline, acid-insoluble material. The carbobenzoxy group was removed by HBr in acetic acid (Ben-Ishai and Berger, 1952) and the product was purified by chromatography on a 15 × 0.9 cm column of type 50A ion-exchange resin (Beckman-Spinco) using an eluent of 0.25 M pyridine-acetate buffer at pH 5.2. The solvent was removed from the material eluting at 44–76 ml, the residue was dissolved in water, and the solution was brought to pH 6 with dilute NaOH. The solvent was again removed and the resulting solid was crystallized from ethanol–ether (mp 202–205° dec). Amino acid analysis on the short column used for basic amino acids of a solution of these crystals showed only one peak at about 35-ml elution volume. After acid hydrolysis at 120° for 24 hr, only lysine and ammonia were obtained as ninhydrin-positive materials. The ultraviolet spectrum was typical of a PTC-amino acid (Ilse and Edman, 1963) with an $\epsilon_{242\ m\mu}$ of 14,700.

Anal. Calcd for $C_{13}H_{19}N_3O_6S$: C, 55.40; H, 6.76; N, 14.93; S, 11.40. Found: C, 54.76; H, 6.55; N, 14.90; S, 10.88.

Bovine Zn·insulin (Lots OLVOO, PJ7463) was a gift from the Eli Lilly Co.; it was converted into the Zn-free insulin hydrochloride by the method of Carpenter (1958).

Trimethionyl-insulin was prepared by the procedure of Levy and Carpenter (1966) without modification.

Standard Solutions of ω-DNP-amino Acids. The concentration of a solution of ε-DNP-Lys (Mann Research Biochemicals) was obtained using an $\epsilon_{365\ m\mu}$ of 17,700 (Fraenkel-Conrat *et al.*, 1955). The concentration of a solution of *O*-DNP-Tyr (Mann Research Biochemicals, K1164) was obtained using an $\epsilon_{350\ m\mu}$ of 2900 (Sanger, 1949). *S*-DNP-Cys was obtained from Nutritional Biochemical Corp., it should have been colorless (Hille, 1960) but was yellow. The solution gave only one ninhydrin-positive component when chromatographed with pyridine buffer 1. It was used as a positional but not as a quantitative standard. A noncrystalline imidazole-DNP-histidine was prepared by the method of Siepmann and Zahn (1964). Upon chromatography with pyridine buffer 1, only one ninhydrin-positive component was detected. The material was used as a positional standard. For quantitating the recoveries of amino acids in hydrolysates, the color value of *im*-DNP-His was taken to be 80% that of free lysine.[4]

Methods. Absorption spectra were obtained using a Cary 15 recording spectrophotometer. Whenever the absorption at a single wavelength of a series of samples was to be measured, a Zeiss spectrophotometer (Model PMQII) equipped with a Gilson Medical Electronics automatic transferator was used. Melting points were determined in unsealed capillary tubes, using a Thomas Hoover apparatus, and are uncorrected. Elemental analyses were performed by the Department of Chemistry, University of California, Berkeley. Nonbuffered aqueous solutions were maintained at a given pH with a pH-Stat (Automatic Titrigraph, Type TTT 1C, Radiometer, Copenhagen).

Counting of ^{14}C-labeled samples was performed in a Packard Tri-Carb scintillation counter at 4°. Each scintillation vial contained 0.5 ml of aqueous sample, 1.2 ml of 2-ethoxyethanol, and 5.0 ml of a scintillation fluid containing 4 g/l. of 2,5-diphenyloxazole and 0.1 g/l. of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 70% toluene–30% 3-ethoxyethanol (v/v) (Hall and Cocking, 1965).

Reaction of Insulin and PTC-insulin with FDNB was performed by a modification of the method of Schroeder and LeGette (1953). The whole procedure was performed in the ignition tubes in which the derivatives were subsequently hydrolyzed. Insulin or PTC-insulin (1 mg, 1.7 μmoles) and sodium bicarbonate (15 mg, 180 μmoles) were dissolved in 1 ml of water and reacted with 2 ml of a 2% FDNB in ethanol (v/v) for 6 hr at room temperature while being stirred magnetically. The ethanol was largely removed by reducing the volume by two-thirds on a rotary evaporator, the solution was acidified with concentrated HCl and extracted three times with 4-ml volumes of ether. The DNP derivatives were pelleted by centrifugation, the aqueous phase was removed, and the residue was hydrolyzed in 2 ml of 5.7 N HCl as detailed below.

Amino Acid Analyses. Samples were hydrolyzed in 5.7 N glass-distilled HCl at 120° in a forced draught oven. Hydrolysis tubes were cleaned, and samples were degassed, as detailed by Moore and Stein (1963). Amino acid analyses were made on a Beckman-Spinco amino acid analyzer (Model 120B) according to the method of Spackman (Spackman *et al.*, 1958, 1963). DNP-insulin samples were hydrolyzed for 24 hr (Africa, 1968); all other samples were hydrolyzed for 12 hr (Carpenter and Chrambach, 1962). Analytical data are presented as moles of amino acid per mole of insulin. For the neutral and acidic amino acids, the amount of insulin was taken to be (moles of aspartic acid plus moles of glutamic acid)/10. The recoveries of basic amino acids are normalized to 1.00 for arginine. For some of the work reported here, 2–3 mg of phenol was added to the hydrolysis tubes. Its presence improves the yields of tyrosine and *O*-DNP-Tyr but it can lead to some artifacts eluting close to *im*-DNP-His. Critical analyses of tyrosine and *im*-DNP-His were performed in the presence and absence of phenol.

Chromatography of ω-DNP-amino Acids. Hydrolysates of DNP-insulin were analyzed on a 22 × 0.9 cm column of Type 50A ion-exchange resin (Beckman-Spinco) at 53° and flow rates of 70 ml/hr. Two pyridine buffer systems were used for complete analyses. Buffer 1 contained 0.21 M pyridine and 0.05 M sodium citrate adjusted to pH 5.28 with HCl. Buffer 2 contained 0.40 M pyridine–0.05 M sodium citrate–0.18 M NaCl, adjusted to pH 5.28 with HCl. Representative elution times obtained with the two buffers are given in Table IV.

Determination of the Concentration of PhSCN in Solution. Suspensions of PhSCN in the various solvents were stirred vigorously for 15 min at the required temperature, aliquots were removed, and the ultraviolet absorption was recorded in a Cary 15 spectrophotometer at the same temperature using the appropriate solvent as a blank. A value of 6900 for the molar extinction at 265 mμ was calculated from the solubility data of Drobnica and Augustine (1965a) and the absorption of the saturated solution at 25°. Using this value for the ϵ_{265}, the following concentrations of PhSCN were found in the various saturated solutions at 40°: water, 1.36×10^{-3} M; 0.05 M $CaCl_2$, 0.88×10^{-3} M; and 1 mg/ml of PTC-insulin, 1.12×10^{-3} M.

[4] Personal communication from A. H. Nishikawa.

The rate of the reaction of PhSCN with Ala-Val was determined in unbuffered aqueous solutions of the peptide (2.5 μmoles/ml) to which was added 5 μl (42 μmoles) of PhSCN/ml of solution. The vigorously stirred reaction mixture was maintained under a nitrogen barrier at 40° in a water-jacketed vessel and at the desired pH by a pH-Stat. At various time intervals, 1-ml aliquots were removed and added to 3 ml of 0.005 N $NaHCO_3$ and 3 ml of benzene. After vigorous shaking the emulsions were broken by centrifugation. The benzene extractions were repeated twice and the aqueous solution was concentrated to dryness in a rotary evaporator. The thoroughly dried PTC peptide was treated with 3 ml of trifluoroacetic acid for 2 hr. The trifluoroacetic acid was removed on a rotary evaporator and the residue was dissolved in 4 ml of 0.1 N HCl. The aqueous solution was extracted three times with 4-ml portions of ethyl acetate and then concentrated to dryness. The residue was dissolved in a known volume of pH 2.2 citrate buffer (Spackman *et al.*, 1958) and an aliquot was placed on the 60-cm column of the amino acid analyzer to determine the proportions of Val and Ala-Val in the mixture. In order to decrease the length of time involved in the chromatography, the buffer change from pH 3.28 citrate to pH 4.25 citrate was made at 0.5 hr. This gave complete resolution of Val and Ala-Val in 3.5 hr at a buffer flow rate of 40 ml/hr.

Rate of reaction of PhSCN with the amino groups of insulin was determined at pH 8.0 and 40°. A solution of insulin hydrochloride (1 mg/ml) was brought to pH 8.0 and maintained there by a pH-Stat under a nitrogen barrier. PhSCN (2 μl or 16.8 μmoles/ml) was added and the reaction was stirred magnetically. At various time intervals, aliquots were removed and extracted with benzene as described for the reaction with Ala-Val. An aliquot of the aqueous solution was removed for the ninhydrin reaction and the remainder was concentrated to dryness in two portions. One portion was treated with trifluoroacetic acid in a procedure similar to that detailed for Ala-Val to remove the amino terminals which had reacted with PhSCN, and the resulting degraded protein was subjected to hydrolysis and amino acid analysis. The other portion was treated with FDNB to detect the unreacted amino groups and then subjected to hydrolysis and amino acid analysis (see above).

DiPTC-insulin was prepared by allowing the reaction of insulin hydrochloride (1 mg/ml) with PhSCN (2 μl/ml) to proceed for 4 hr at 40° and pH 8.0. The excess reagent was extracted with ether and the aqueous solution was lyophilized. The residue was dried over P_2O_5 and stored at 4° under vacuum. The diPTC-insulin was separated from other reaction products by chromatography on DEAE-Sephadex as detailed below.

DEAE-Sephadex chromatography of insulin derivatives was performed by a procedure similar to that of Bromer and Chance (1967). The column (2.5 × 30 cm) was packed with DEAE-Sephadex A25 (Pharmacia, lot 891) equilibrated with 0.03 M NaCl–0.01 M Tris in 7 M urea solution with a measured pH of 7.9 at 4°. After addition of the sample (up to 200 mg at 20 mg/ml), elution was performed at a flow rate of 60 ml/hr with a linear gradient from 0.03 to 0.30 M NaCl in the Tris–urea solution obtained by connecting 1-l. reservoirs of each solution. In order to minimize the formation of cyanate (Stark *et al.*, 1960), the urea-containing buffers were made up fresh just before use. The initial buffer was made by the addition of 420 g (7 moles) of urea to enough (about 700 ml) of 0.01 M Tris–0.03 M NaCl at pH 7.5 at 25° to give 1 l. of solution. The final buffer was prepared in a similar fashion. The pH of these solutions as measured directly with the glass electrode at 4° was 7.9.

Desalting of Chromatographic Fractions. The pooled fractions obtained from the chromatography on DEAE-Sephadex in urea-containing buffers which had volumes of 200–350 ml were desalted by gel filtration on 6.5 × 35 cm column of Sephadex G-25 (Pharmacia) containing about 220 g of dry Sephadex. The column was developed at 4° with 0.01 N ammonium acetate at pH 8.5 at a flow-rate of 75 ml/hr. The solvent and ammonium acetate were removed from the protein by lyophilization.

Kinetics of the Cyclization of PTC-insulin in Trifluoroacetic Acid. Small amounts (1.0 ● 0.1 mg) of diPTC-insulin were placed in separate ignition tubes. Each sample was treated with 0.5 ml of anhydrous trifluoroacetic acid for a given length of time at room temperature (25°). At the end of that time, the acid was removed in about 30 sec in a strong stream of nitrogen; each sample was immediately extracted twice with 2 ml of ether, and the residual protein was dissolved in 1.0 ml of 10^{-5} M NaOH. The absorption spectrum of each solution was recorded from 320 to 230 mμ. The extent of the reaction was determined by the ratio of absorption at 255 mμ to that at 275 mμ. For diPTC-insulin this ratio is 1.21 while insulin has a ratio of 0.55.

Des-Gly^{A1}-des-Phe^{B1}-insulin was prepared in quantities of 30–150 mg by treatment of the corresponding purified diPTC-insulin (6 mg/ml) with anhydrous trifluoroacetic acid for 2 hr at room temperature. The trifluoroacetic acid solution was reduced to 10–12 ml, cooled in an ice bath, and the des-Gly-des-Phe-insulin was precipitated by the addition of two to three volumes of cold ether (USP). The precipitate was collected by centrifugation, washed three times with ether, and dried under vacuum. In order to convert the trifluoroacetic acid salt into the isoelectric form in the presence of zinc, it was dissolved in 0.5 M acetic acid containing 0.1 mg/ml of zinc acetate dihydrate (Mallinckrodt Chemical Works) to give a solution containing 7–10 mg/ml of protein. The solution was slowly brought to pH 5.8 by the dropwise addition of 5 N ammonium hydroxide and allowed to stand at 4° overnight. The precipitate was collected and washed with water, acetone, and ether by centrifugation and then thoroughly dried before storage at 4°. In some cases the trifluoroacetic acid salt was further purified by chromatography on DEAE-Sephadex before being converted into the isoelectric form.

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