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Title STUDIES OF THE FUNCTIONAL TYROSYL RESIDUES OF INSULIN

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Acetylimidazole was used to acetylate the tyrosyl residues of insulin. The kinetics of acetylation were studied by termination of the reaction at several time intervals, isolation of acetylated insulin and determination of O-acetyltyrosyl groups by deacetylation with hydroxylamine. The deacetylation was also studied kinetically by means of the absorbancy change during the reaction. The degree of availability of the tyrosyl groups in native insulin, and of the O-acetyltyrosyl groups in the acetylated insulin was studied by the above kinetic studies. Two tyrosyl residues were acetylated at a rate sufficiently more fast than the remaining two so that a diacetyl derivative could be isolated. This preparation was fully active. The completely acetylated insulin was only thirty percent as active as native insulin in the mouse convulsion test. Native and acetylated insulin were inactivated by seventy percent on treatment with

hydroxylamine. These and other findings lead to the conclusion that one or both of the slowly acetylated tyrosyl residues of insulin are involved in the mechanism of action of the hormone.

In order to determine the location of the two acetylated tyrosyl residues, the diacetyl derivative of insulin was oxidized and the A and B chains isolated were assayed for acetyl groups. The diacetyl derivative was also digested separately with trypsin and chymotrypsin, and each core was also assayed for acetyl groups. From the results of these assays it was concluded that the diacetyl derivative of insulin was acetylated to the A19 and B16 tyrosyl residues, which are characterized as reactive, while the other two tyrosyl residues, A14 and B26 are non-reactive or bound. The fact that both tyrosyl B26, and asparaginyl A21 are bound and essential for hormone activity suggests a specific tyrosyl B26-carboxylate A21 interaction essential for maintaining the native conformation and the activity of insulin. The usefulness of the kinetic and structural studies employed here for evaluation of the degree of exposure of tyrosyl residues, selective blocking of tyrosyl groups, and determination of the location of reactive and non-reactive tyrosyl residues of insulin, for the purpose of structure-and-function studies on other problems, is discussed.

STUDIES OF THE FUNCTIONAL TYROSYL RESIDUES OF INSULIN

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INTRODUCTION

Correlation between the structure of protein molecules and their physiological function has been one of the endeavors in biochemistry since the crystallization and isolation of proteins in highly active forms. This challenge of expressing biological activity in chemical terms came to a stronger focus since the elucidation of the primary structure of proteins. The x-ray diffraction data reveal that in an enzyme not only its amino acid sequence but also the three-dimensional folding of the peptide chain(s) are precisely defined. The very fact that myoglobin has been resolved at the 1.5 angstrom level by x-ray analysis (Kendrew, 1962) is sufficient to indicate that each molecule of myoglobin is identically folded. Anfinsen and his coworkers (1961) first proposed the today widely accepted theory that the three-dimensional structure of a protein is determined by its primary structure. Identification of the role of each amino acid residue in maintaining the active conformation of a protein as well as on the specific action of enzymes on their substrates is the goal of structure-and-function studies. The chemical methods used for these studies can be classified in three general

approaches. Digestion with proteolytic enzymes has been used to reduce the active protein molecule up to a few amino acids from the N-, or C-terminal residues of each chain (exopeptidases), or specifically hydrolyze certain peptide bonds and split off whole peptides (endopeptidases). By means of this substractive method important conclusions about the significance of the removed amino acids or peptides for the activity of the protein were reached (White, 1954; Carpenter and Baum, 1962).

Another approach has been the production in the laboratory of peptide hormones which vary from the native ones by one or more amino acid residues, in order to identify certain structural features which are essential for biological function. This synthetic approach allows the highest degree of detailed study of peptide sequences, but it can only be applied in the case of relatively small peptide hormones, as oxytocin and vasopressin (Katsoyannis and Du Vigneaud, 1958; Du Vigneaud et al., 1960) and adrenocorticotropin (Li et al., 1963).

The third chemical approach to the structure-and-function studies is the chemical modification of proteins by site-specific selective reagents followed by examination of the properties of the modified proteins. Modifications which lead to unfolding of proteins should be avoided in this respect, and structural changes are detectable by physical measurements. The concept of an active site of

an enzyme which was deduced from simple geometric consideration of the interaction between enzyme and substrate was supported by digestion and modification studies on proteins. Vast regions of proteins proved unnecessary for enzyme action, while a small modification of one group inactivated the whole enzyme.

Apart from the chemical approaches used to correlate structure and function in proteins, certain physical methods have been applied successfully in measuring several important parameters of proteins' size and structure. Ultraviolet spectroscopy, optical-rotatory dispersion, and x-ray diffraction analysis have helped in elucidating structures, and monitoring changes in conformation of modified proteins.

Insulin was the first protein to be analyzed for its complete amino acid sequence by Ryle and coworkers (1955), and it was hoped that this achievement would lead quickly to an understanding of the relation between the chemical structure of the insulin molecule and its unique physiological function. The results of digestion and modification studies on insulin significantly advanced our knowledge on the essential amino acid residues of this hormone molecule.

Early studies (Du Vigneaud et al., 1931; Stern and White, 1937) showed that the integrity and shape of the molecule as a whole are essential for activity, because reduction of the disulfide bonds

led to progressive inactivation. Moreover, it was found that the biological activity of insulin was apparently destroyed to a much larger degree than the amount of reduced insulin present could justify. Thus, reducing only 10% of the total number of disulfide bonds per molecule resulted in drop of the activity by 87%. It was suggested (Lens and Neutelings, 1950) that the result could be explained if a physiologically active complex is made up of multiple subgroups, so that inactivation of a single disulfide bond inhibits the biological activity of all insulin in the complex. The above phenomenon would illustrate the numerous factors that must be considered which may influence the apparent biological potency of a modified hormone, since its evaluation involves comparison with the native hormone in assays using intact animals. These factors (Hofmann, 1963) include relative affinities of the hormone or modified hormone for plasma constituents and their rates of release at the receptor sites, the rate of transport from the locus of administration to the receptors, the rate of transport across cell membranes, the rate of breakdown, and probably many other unknown factors.

Reoxidation by air of the S-sulpho-derivatives of the two chains of insulin in mercaptoethanol (Du et al., 1961; Dixon and Wardlaw, 1960) resulted in regeneration of 5% or less activity of insulin, in contrast to almost 100% reactivation of reduced ribonuclease (White, 1961). It is concluded that the disulfide bonds are

essential for maintaining the three-dimensional structure and activity in insulin, but not in ribonuclease.

Treatment of insulin with formaldehyde under mild conditions resulted in no loss of activity (Fraenkel-Conrat and Fraenkel-Conrat, 1950) showing that amide and guanidyl groups are not essential in insulin.

Evidence from reaction with diazobenzene sulfonic acid, as well as specific iodination of insulin indicated (Fraenkel-Conrat and Fraenkel-Conrat, 1950) that a certain portion (one-third to one-half) of the phenolic and imidazole groups of insulin is not essential, but that more extensive substitution and coupling of the groups causes inactivation.

Acetylation of both α-, and the ϵ-amino groups of insulin did not affect activity (Stern and White, 1938). Glendening et al. (1947) showed that blocking all the aliphatic hydroxyl groups of insulin in the form of acid sulfate esters did not impair activity.

The importance of carboxyl groups for insulin activity was demonstrated by Charles and Scott (1931), and Fraenkel-Conrat and Fraenkel-Conrat (1950). Crystalline zinc insulin was inactivated by treatment with acid methyl alcohol. On treatment with sodium hydroxide they obtained partial reactivation. The reactivated insulin was crystallized and these crystals had the same microscopic appearance, isoelectric point, and physiological activity as the original

insulin crystals.

It is of interest that the various blocking groups used for modification studies on proteins should be small, because covering amino or hydroxyl groups with large molecules (Halikis and Arquilla, 1961; Tietze et al., 1962) was found to inhibit both the biological activity and the immunological properties of insulin, presumably by steric hindrance. Acetyl, amide, and formyl groups are considered as "physiological" (Hofmann, 1963), because these groups are normally present in the cells and there are metabolic pathways available for their attachment to, or elimination from, other molecules. Nevertheless, it seems that both the rat and the human organism possess at best only a very limited ability to convert the biologically inert protected material into the biologically active species (Hofmann, 1963).

The importance of the phenolic groups in insulin was investigated through iodination and acetylation studies. Fraenkel-Conrat and Fraenkel-Conrat (1950) showed that iodination of up to half the phenolic groups of insulin did not cause very extensive inactivation. They commented that iodination of the most reactive phenolic groups may cause less inactivation than their acetylation.

In earlier studies (Stern and White, 1938), acetylation of the tyrosyl residues in insulin was accomplished by acetic anhydride or

ketene, resulting in the inactivation of the hormone. It was commented, however, that these reagents are too drastic to be selective for phenyl hydroxyl groups only, and that other functional groups, or even the native conformation of the protein are probably affected (Fraenkel-Conrat and Fraenkel-Conrat, 1950; Riordan et al., 1965).

Springel (1961) was able to iodinate only the A chain of insulin under carefully controlled conditions. De Zoeten and Havinga (1961) iodinated crystalline zinc insulin with NaI^{131} under a set of different conditions, isolated the two chains, and hydrolyzed each one enzymatically. Each of the four fragments obtained was shown to contain one tyrosyl group, and the iodine content of each fragment was determined. It was concluded that the relative initial rates of iodination of glycyltyrosine and the four tyrosyl residues of insulin A14, A19, B16, and B26 were 1, 1, 1.6, 0.2, and 0.2. If the iodination reaction was carried out in the presence of 8 M urea the relative rates were 1, 1, 1.5, 0.8, 0.6 respectively. According to these results it seems that the reactive tyrosyl residues in zinc insulin are those on the A chain, while the ones on the B chain are unreactive.

In contrast to the above results are the conclusions by Kurihara et al., (1963) and Aoyama et al., (1966). They used cyanuric fluoride in order to specifically react with the tyrosyl residues of insulin. They found that two tyrosyl residues were

reactive and two non-reactive. On further structural studies they concluded that the reactive residues were the A14 and B16, while the non-reactive ones were the A19 and B26. The authors proposed that the lack of reactivity of the A19 and B26 tyrosyl residues is due to hydrogen bonding with B13 glutamyl, and B22 arginyl residues, respectively.

The disagreement between the results of iodination, and cyanuric fluoride reaction in establishing the location of the reactive tyrosyl residues of zinc insulin, prompted this investigation to use N-acetylimidazole for acetylation of insulin tyrosyl groups in order to study their reactivity, and their importance for biological activity of the hormone. Acetylimidazole was first used by Simpson et al. (1963) to acetylate the functional tyrosyl residues of carboxypeptidase. This reagent was found to be milder than acid anhydrides, and very selective, acetylating only the phenyl hydroxyl groups of carboxypeptidase, without unfolding the enzyme, as shown by O.R.D. measurements. It was later confirmed (Bethune et al., 1964) that acetic anhydride partially denatured carboxypeptidase, while no change in the enzyme conformation resulted on reaction with acetylimidazole. More recent studies (Riordan et al., 1965) on the specificity of acetylimidazole showed that the only groups acetylated under the conditions used were the phenylhydroxyl, sulfhydryl, and amino groups. The extent of amino group acetylation did vary with

each protein used, but it was lower than in reactions of acetic anhydride with all proteins tested. The fact that acetylation of tyrosyl groups of insulin caused more inactivation than iodination (Fraenkel-Conrat and Fraenkel-Conrat, 1950), as well as the fact that the acetyl group is small and "physiological" (Hofmann, 1963) in contrast to the cyanuric fluoride group, was another important reason for favoring acetylimidazole as a reagent in order to test the availability and functionality of the tyrosyl residues in insulin. Furthermore it appeared reasonable that acetylation of the phenyl-hydroxyl group of a tyrosyl residue was preferable to iodination when the hydrogen bond involving tyrosyl residues as hydrogen donors was to be investigated.

The participation of the B26 and one more tyrosyl residues in hydrogen bonds was demonstrated in the case of zinc insulin (Laskowski et al., 1956; Laskowski et al., 1960) on the basis of ultraviolet spectral shifts on trypsin digestion, and acidification of the tryptic core of insulin. As a result, in the proposed tyrosyl-carboxylate hydrogen bond the pK of the tyrosyl group involved increases above normal values, while the pK of the carboxyl group decreases. It was later estimated, however, that the free energy of formation of a tyrosyl-carboxylate bond in water was only -0.4 kcal/mole (Wetlaufer, 1962), and this interaction was too weak to explain the lack of reactivity of the hydrogen-bonded residues.

Furthermore, studies on the spectral shift of model systems in water solutions (Yanari and Bovey, 1960) did not appear to favor hydrogen bonds. Their interpretation of the spectral shift on denaturation of proteins was based on the change of environment of tyrosyl groups during unfolding of proteins.

The importance of tyrosyl-carboxylate hydrogen bonds for maintaining the native conformation of proteins was minimized by the above studies until very recently (Broomfield et al., 1965; Riehm and Scheraga, 1966), when it was revitalized as a specific tyrosyl-carboxylate interaction buried in hydrophobic regions of the proteins. Such regions of high refractive index would considerably strengthen the ion-dipole interaction to give a higher free energy of formation than in the case of a hydrogen bond in polar environment.

Ribonuclease was recently shown (Broomfield et al., 1965; Riehm and Scheraga, 1966) to contain such tyrosyl-carboxylate interactions, by specific blocking of all the other carboxyl groups except the ones bound to tyrosyl residues. Subsequent structural studies identified two aspartyl residues as counterparts of the two "buried" tyrosyl groups in ribonuclease.

In the light of these studies it would seem important to identify the "buried" tyrosyl residues of insulin as part of the studies to pinpoint possible tyrosyl-carboxylate interactions in insulin, and

study their importance in maintaining the native conformation and the biological activity of the hormone.

MATERIALS

Crystalline zinc insulin used was a product of Sigma Chemical Company (Lot Nos. 55B-1650, 74B-1020) with an activity of 24 I. U. per mg.

Amorphous insulin was purchased from Eli Lilly and Company (Lot Nos. W-3255 and 192-235B-188), or prepared from zinc insulin by dialysis against EDTA.

N-acetylimidazole was prepared by the method of Boyer (1952), and recrystallized from isopropenyl acetate to a melting range of 100-101°. Reported 101.5-102.5°. The reagent was stored over "Drierite" desiccant in a tightly closed jar, to prevent hydrolysis, since it is hygroscopic.

N, O-diacetyltyrosine was obtained from Mann Research Laboratories, Inc., and was used without further purification.

Trypsin recrystallized two times and lyophilized, salt free, was purchased from Worthington Biochemical Corporation (Lot No. TRL 6227). This enzyme was pretreated with 0.063 N HCl and 2 mM $CaCl_2$ for 24 hours at 37° to minimize chymotryptic activity.

Alpha chymotrypsin (Worthing Chymotrypsin I, Lot No. 6063) was a three-times-recrystallized product.

EXPERIMENTAL

Acetylation of Insulin, Preparation of Ac_4-insulin[1] and Ac_2-insulin[1]

Acetylation of insulin was performed by adding a 25-fold excess of acetylimidazole per tyrosyl residue to a rapidly stirred solution of insulin (2.5 μmoles/ml) in 0.02 M sodium Veronal - 0.1 M KCl buffer, pH 7.0 at room temperature. The buffer was made 8 M with urea when required. The acetylation was allowed to proceed for two or three hours, during which the pH of the reaction mixture dropped to 6.5. The pH of the reaction mixture is stabilized at 6.5 due to the imidazole buffer formed by hydrolysis of acetylimidazole.

A few acetylations were carried out at constant pH 7.5 and 6.5 by means of a pH-stat (Radiometer, Copenhagen). Aliquots were withdrawn from the reaction mixture at time intervals just after the addition of acetylimidazole crystals (zero time for the acetylation reaction), and mixed with 10% trichloroacetic acid solution kept at 0°. Alternatively the acetylation reaction was terminated by isoelectric precipitation of the aliquots at pH 4.5. The precipitates

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[1] Ac_n-insulin designates insulin acetylated with acetylimidazole, where n is the number of O-acetyl-tyrosyl residues per insulin molecule as determined by deacetylation with hydroxylamine.

were centrifuged, washed with a few ml of distilled water and dissolved in 0.1 M sodium phosphate buffer, pH 6.6 (to give a concentration of about two mg insulin per ml). Zero time controls were treated in the same manner but without addition of acetylimidazole. These stock solutions of the Ac_n-insulin were kept in the deep dreeze, and used later after appropriate dilution for the assays referred to in the following paragraphs.

Insulin acetylated at all four tyrosyl residues, Ac_4-insulin, was prepared from amorphous or zinc insulin in 8 M urea under the usual acetylation conditions for 15 to 20 minutes. Ac_2-insulin, i.e., insulin acetylated at two of the four tyrosyl residues, was prepared from crystalline zinc insulin in 0 M urea solution, by stopping the reaction at ten minutes.

Deacetylation of Ac_n-insulin Preparations

The deacetylation of Ac_n-insulin preparations was followed kinetically by the increase of absorbance at 276 mμ of a solution of about 0.3 mg Ac_n-insulin per ml in 0.1 M sodium phosphate buffer, pH 6.6, made 0.07 M with hydroxylamine (at zero time) for four hours at 25°. Under such conditions no more increase of absorbance at 276 mμ was observed even by doubling the hydroxylamine concentration at the end of the four hours of deacetylation. The same deacetylation conditions were used for the degradation products of

Ac_n-insulin preparations. The number n_t of O-acetyl-tyrosyl residues per insulin molecule was that calculated according to Simpson et al. (1963).

Unmodified insulin (and its degradation products) were treated under the same deacetylating conditions in order to calculate the $n_{control}$ values, which are significantly higher than zero. The n_t minus the $n_{control}$ value was designated n and signified the number of O-acetyl-tyrosyl groups per insulin (or insulin fragment) molecule that are deacetylated by hydroxylamine. This n value is the one given as a subscript in the Ac_n- prefix of Ac_n-insulin.

Oxidation of Ac_2-insulin, Isolation of A and B Chains

In order to determine the position of the two O-acetyl-tyrosyl groups of Ac_2-insulin, it was necessary to split the molecule and isolate each chain under conditions that do not destroy either the tyrosyl residues or the O-acetyl-tyrosyl groups. The method by Craig et al. (1961), was used to oxidize Ac_2-insulin with performic acid. Preliminary experiments showed that under these conditions of oxidation there is no hydrolysis of the O-acetyl-tyrosyl ester bond. For isolation of A and B chains from the mixture, Fittkaw's ion exchange method (1963) proved inconvenient because the eluting hydrochloric acid partially hydrolyzed the O-acetyl-tyrosyl ester bonds.

Isoelectric precipitation was successfully used for the isolation of pure A and B chains from their mixture. The separation is based on the fact that the A chain is very soluble, while the B chain is quite insoluble in acid solutions.

About 100 mg of the lyophilized mixture of A and B chains were mixed with two ml distilled water in a centrifuge tube and N NH_4OH added dropwise with stirring until the pH reached a value between 2 and 4.5. The mixture was left in the deep freeze for about 30 minutes, and then centrifuged. The A chain was isolated from the supernatant by lyophilization. The precipitate containing the B chain was washed twice with two ml cold distilled water, and dried over P_2O_5.

Alternatively, the B chain could be quantitatively precipitated from a solution of the mixture of A and B chains by 20% trichloroacetic acid solution, while the soluble A chain could be isolated from the supernatant by dialysis in an 18/32 Visking dialysis tube against distilled water at 0° for about six hours. The trichloroacetic acid can be removed also by ether extraction.

Amino acid analysis of the A and B chains isolated by either of the above precipitation methods showed that the separation was complete. Furthermore, the methods were mild enough so that no hydrolysis of the O-acetyl-tyrosyl bond, or oxidation of the tyrosyl hydroxyl occurs. The above methods are applicable to the isolation

of the chains from both Ac_2-insulin or native insulin.

Digestion of Ac_2-insulin with Trypsin and Chymotrypsin, Isolation of the "Cores"

The following digestion conditions were modifications of the methods by Aoyama et al. (1965).

For the digestion with trypsin, about 10 mg Ac_2-insulin (or native insulin) were mixed with 3.0 ml 0.1 M sodium phosphate buffer pH 7.0. Drops of N NaOH were added until a clear solution was obtained at pH 7.5. A solution of one mg trypsin (treated as in Materials) per ml 0.063 N HCl 2 mM $CaCl_2$ is added to the Ac_2-insulin solution and the pH of the mixture adjusted to 7.0 with N NaOH. After six hours digestion at 25° about 2 ml 3 M cold trichloroacetic acid was added, and the precipitate separated by centrifugation, and mixed with 3 ml 0.1 M sodium phosphate buffer pH 7.0. The mixture was stirred well with a glass rod, and the pH adjusted to 7.0, if necessary, followed by centrifugation at 0° for about 20 minutes. This supernatant was shown by amino acid analysis (Table 6, page 53) to contain the tryptic core of insulin, with only traces of trypsin, which was shown to precipitate out during the second centrifugation.

The digestion with chymotrypsin was carried out as follows: In a 40 ml plastic centrifuge tube about 10 mg Ac_2-insulin (or native

insulin) were dissolved in 15 ml 0.1 M sodium phosphate buffer pH 7.0, and 4 mg α-chymotrypsin dissolved in 2.0 ml 0.1 M sodium phosphate buffer pH 7.0 were added. The digestion mixture was left for 10 hours at 25°, then precipitated by the addition of 4 ml 3 M cold trichloroacetic acid, and the precipitate separated by centrifugation at 0°. Three ml 0.1 M sodium phosphate buffer pH 6.8 were added to the precipitate, and the mixture was stirred well with a glass rod, adjusted to pH 6.8 with N NaOH, and centrifuged at 0° for about 20 minutes. The supernatant was shown to contain the chymotryptic core of insulin with only traces of chymotrypsin (Table 6, page 53), which after denaturation with trichloroacetic acid does not dissolve in 0.1 M sodium phosphate buffer pH 6.8.

Stability of O-acetyl-tyrosyl Ester

A series of control experiments with N, O-diacetyl tyrosine, Ac_4-insulin, Ac_2-insulin, Ac_2-A, and Ac_2-B chains of insulin were carried on in order to determine if the degradation conditions of Ac_2-insulin, and the isolation methods of its degradation products, hydrolyze the O-acetyl-tyrosyl ester bond, or oxidize the free tyrosyl group. The absorbance at 276 mμ of the above substrates under the incubation conditions used in this thesis was measured against appropriate blanks at several time intervals in order to detect

any splitting of the O-tyrosyl ester bond. Furthermore, amino acid analyses of the incubation mixtures were used to determine any destruction of free tyrosyl residues.

Other Assays on Ac_n-insulin, and Its Degradation Products

Determination of the labile acetyl groups by the method of Balls and Wood (1956), at pH 7.5 and 25° for 20 minutes was used for the determination of O-acetyl-tyrosyl groups of the Ac_n-insulin preparations. The results from this method were compared with those according to Simpson et al. (1963).

The ninhydrin method by Moore and Stein (1954), as modified by Slobodian et al. (1962) was used for determination of the degree of amino-group substitution, using phenylalanine as a standard. The biological activity of the Ac_n-insulin preparations was determined by the mouse-convulsion assay of insulin as described by the General Medical Council (1953), and by Smith (1950), except that for each dose ten mice were used and the assay was repeated three times for each sample. Amorphous insulin dissolved in 0.1 M sodium phosphate buffer pH 6.6 was used as a control.

Determination of the Concentration of Insulin and Its Fragments

Insulin concentration was measured by the absorbance at 276 mμ. The molar absorptivity of the native amorphous insulin was

6.36×10^3 $M^{-1}cm^{-1}$ in 0.1 M sodium phosphate buffer pH 6.6, but after treatment with hydroxylamine under the deacetylation conditions (previously described), the molar absorptivity increases to 8.22×10^3 $M^{-1}cm^{-1}$. In general the treatment of insulin, the mixture of A and B chains, and of each chain separately, with hydroxylamine under the deacetylation conditions used in this thesis, results in a significantly different molar absorptivity than without hydroxylamine treatment. The $\epsilon_{276}^{Init.}$, and ϵ_{276}^{Lim}, corresponding to the molar absorptivity before the addition of hydroxylamine (zero deacetylation time), and at the end of the deacetylation assay, are given in Table 1. These values are averages of triplicate measurements varying from the average by 10% or less.

Table 1. Molar absorptivities of insulin and insulin chains.

	$\epsilon_{276}^{Init.}$ $M^{-1}cm^{-1}$	ϵ_{276}^{Lim} $M^{-1}cm^{-1}$
Amorphous insulin	6.36×10^3	8.22×10^3
A chain	3.31×10^3	2.80×10^3
B chain	3.37×10^3	3.50×10^3
A plus B chains	6.73×10^3	7.50×10^3

The concentration of Ac_n-insulin, Ac_n-A and Ac_n-B preparations were determined from their absorbance at the end of the deacetylation period and their ϵ_{276}^{Lim}.

Alternatively the concentration of insulin and its degradation products were measured by the method of Lowry et al. (1951), by weighing of dried preparations, and after amino acid analysis using a Beckman amino acid analyzer, Model 120B (Spackman et al., 1958). Hydrolysis of the protein samples was carried out by heating 6N HCl at 110° for 24 hours in evacuated and sealed ampules. The correction factors used were calculated on the basis of analysis of crystalline zinc insulin.

Determination of the Solubilities of Insulin, Ac_4-insulin, B Chain, and Ac_2-B Chain, at Several pH Values

Preliminary experiments were carried out in order to determine the pH of minimum solubility of insulin, Ac_4-insulin, B chain, and Ac_2-B chain, for the purpose of isoelectric precipitation of the above preparations. The solubilities were estimated by dissolving each preparation in solutions of given pH from 2 to 6, prepared by mixing portions of 0.1 M sodium phosphate buffer, pH 7.0, and N HCl. The saturated solutions were stirred well at 25° and the pH was measured, then were centrifuged at 25° for insulin and Ac_4-insulin or at 0° for B chain and Ac_2-B chain. The concentration of each preparation was determined spectrophotometrically at 220 mμ where both the unmodified and the acetylated preparations absorb equally.

Assays on Possible Ester Bond in Native Insulin

The fact that the absorbance at 276 mμ of native insulin solutions increases under the deacetylation conditions used, necessitated the use of assays for ester bonds in proteins.

Native insulin after treatment with hydroxylamine was assayed for bound hydroxamate according to the method of Balls and Wood (1956), modified in order to keep insulin in solution. The modification involved the substitution of ethyl alcohol for trichloroacetic acid in the reaction mixture. Furthermore, the methods of Blumenfeld and Gallop (1962) for detection of α - and β-carboxyl groups of aspartyl residue, and γ-carboxyl group of glutamyl residue, involved in ester bonds were used on native insulin after treatment with hydroxylamine.

RESULTS AND DISCUSSION

Calculation of n Values of the Acetylated Preparations

According to Simpson et al. (1963) $n_t = \frac{\Delta\epsilon_{278}}{\Delta\epsilon'_{278}}$, where $\Delta\epsilon_{278}$, and $\Delta\epsilon'_{278}$ are the increase of molar absorptivity at 278 mμ during deacetylation of carboxypeptidase, and N, O-diacetyl tyrosine respectively.

In the case of insulin, the increase in absorptivity at 276 mμ was used instead, because at this wavelength both the maximal absorptivity of insulin, and the maximal change in the difference spectrum of insulin-Ac_n-insulin occur. It was found necessary to correct the n_t value for the increase of absorptivity of native insulin (or its fragments) on treatment with hydroxylamine. The $n_{control} = \frac{\Delta\epsilon_{276}}{\Delta\epsilon'_{276}}$ was calculated and subtracted from the n_t value, to obtain the corrected $n = n_t - n_{control}$ value. This (corrected) n value represents more precisely the number of O-acetyl-tyrosyl bonds hydrolyzed by hydroxylamine per mole of insulin (or its fragments), than the uncorrected n_t value.

Another way of calculating n, even without actually deacetylating Ac_n-insulin is to estimate its molar absorptivity at 276 mμ. The molar absorptivity of native amorphous insulin, $\epsilon_{276} = 6,360$ $M^{-1}cm^{-1}$ cannot be accounted for by the four tyrosyl residues,

i.e. 4 x 1,367 = 5,468 $M^{-1}cm^{-1}$, and the three cystine residues, i.e. 3 x 141 = 423 $M^{-1}cm^{-1}$, where 1,367 and 141 are the molar absorptivities of tyrosine, and cystine respectively at 276 mμ (Beaven and Holiday, 1952). The difference between the observed, and the calculated molar absorptivity, i.e. 6,360 - (5,468 + 423) = 469 $M^{-1}cm^{-1}$ is the so-called residual absorptivity of insulin. This discrepancy is common to all proteins (Wetlaufer, 1962).

If we assume that the residual absorptivities of native insulin and Ac_n-insulin are equal, the molar absorptivity of Ac_n-insulin at 276 mμ can be calculated as a summation of n x 87 for the n O-acetyltyrosine residues, of (4-n) x 1367 for the free tyrosine residues, of 423 for the three cystine residues, and of 469 for the residual absorptivity, i.e. ϵ_{276} = n x 87 + (4-n) x 1,367 + 423 + 469 = 6,360 - 1,280 x n. This formula gives the molar absorptivity of any Ac_n-insulin preparation at 276 mμ as a function of the number of its O-acetyltyrosine residues n, and it can be used to calculate n from the ϵ_{276} of Ac_n-insulin which is experimentally determined by the absorbance of a solution of Ac_n-insulin, and the molarity of insulin in this solution. The n values calculated by this method for a series of Ac_n-insulin samples, were very close to the ones calculated by the original method of Simpson et al. (1963). Actually both methods are the same in principle, the difference being that the original method requires actual 100% deacetylation of the acetylated protein,

while the second method is based on the molar absorptivity of the acetylated protein calculated as a function of its n value, and compared to the absorptivity of the native protein.

Finally the hydroxamate procedure by Balls and Wood (1956), gave results comparable with the above methods for the n values of several Ac_n-insulin preparations.

Acetylation Kinetics of Insulin

It has been found that for the acetylation reaction a pH between 6.5 and 7.0 is the best when the stability of acetylimidazole (Stadtman, 1954) and the O-acetyl-tyrosyl ester bond formed, are taken into consideration. Preliminary experiments with N,O-diacetyl tyrosine showed that at pH 5.0 to 7.0 the tyrosyl ester bond is very stable, but higher or lower pH's accelerate its hydrolysis. The hydrolysis of Ac_n-insulin at pH 7.5 is also faster than that at pH 6.5 (Figure 3, page 29). The acetylation reaction was quite fast under the conditions used, and the suspension of zinc insulin gave a clear solution in less than two minutes after the addition of acetylimidazole crystals.

Since both acetylimidazole (Stadtman, 1954) and Veronal buffer would interfere with the ultraviolet absorption spectrum of insulin, Ac_n-insulin must be separated from the reaction mixture. In preliminary experiments exhaustive dialysis against 0.01 M Tris

buffer pH 7.0, isoelectric precipitation at pH 4.5, and trichloroacetic acid precipitation were studied. It was found that both isoelectric and trichloroacetic acid precipitation were effective in quantitatively separating Ac_n-insulin from the reaction mixture without any significant hydrolysis of the tyrosyl ester bond, under the conditions used. Exhaustive dialysis on the other hand resulted in consistently lower n values (about 10%) than the ones by isoelectric or trichloroacetic acid precipitation for the same Ac_n-insulin preparation. Isoelectric or trichloroacetic acid precipitation proved to be very simple and convenient for instantaneously stopping the acetylation reaction (due to the lowering of pH) and isolation at the same time of the Ac_n-insulin from the reaction mixture. A 0.1 M sodium phosphate buffer at pH 6.6 was used to dissolve the trichloroacetic acid precipitates of Ac_n-insulin. The preparation was found not to hydrolyze if stored in the deep freeze for times up to two months. Ac_4-insulin on the other hand did not hydrolyze when left at ambient temperature in solution at pH 2 to 6.6 for ten hours. The isoelectric point for Ac_4-insulin was found to be pH 4.4. Figure 1 shows the solubility of Ac_4-insulin and native amorphous insulin as a function of pH at 25°.

Both amorphous and zinc insulin were acetylated with acetylimidazole under several conditions. Figures 2 and 3 show the effect of the acetylation time of amorphous and zinc insulin under a

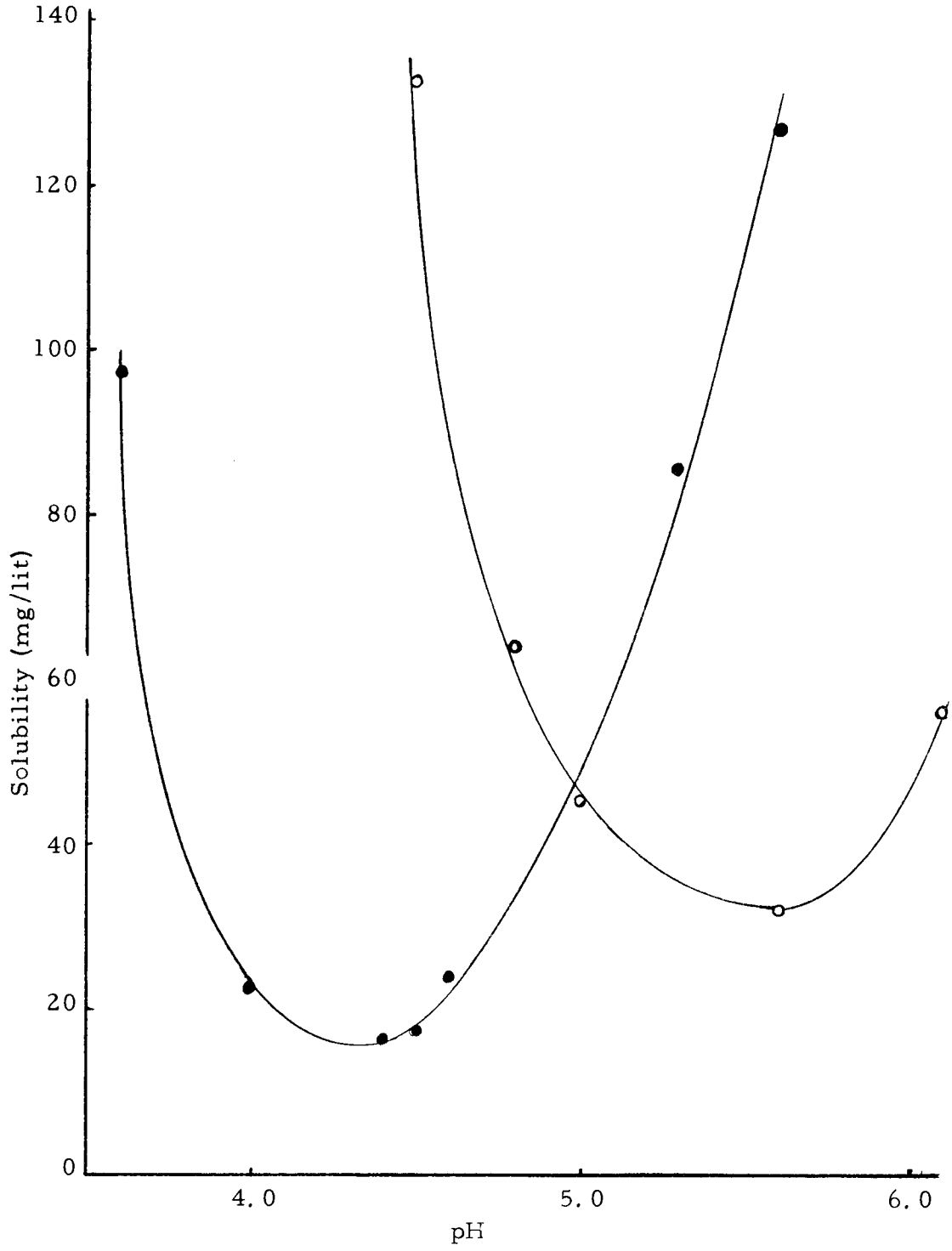

Figure 1. The effect of pH on the solubility of native amorphous insulin (o) and Ac_4-insulin (•) in mixtures of 0.1 M phosphate buffer and N HCl at 25°.

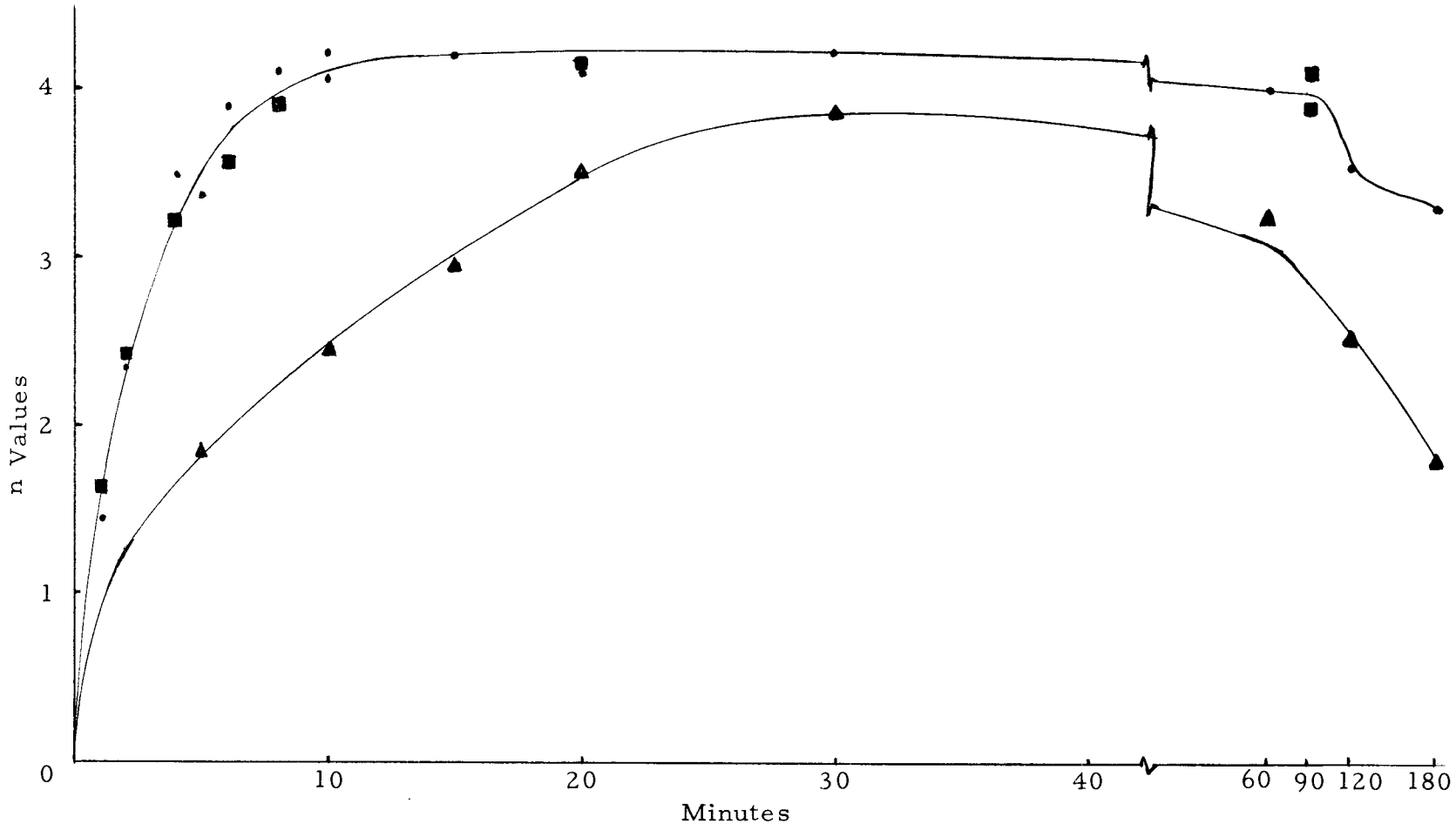

Figure 2. Time course of the reaction of acetylimidazole with the tyrosyl residues of amorphous insulin at pH 7.0 to 6.5, in 0 M urea (●), 8 M urea (■), and 2 M sodium chloride (▲). Insulin concentration 2.5 μmoles/ml; acetylimidazole concentration 250 μmoles/ml.

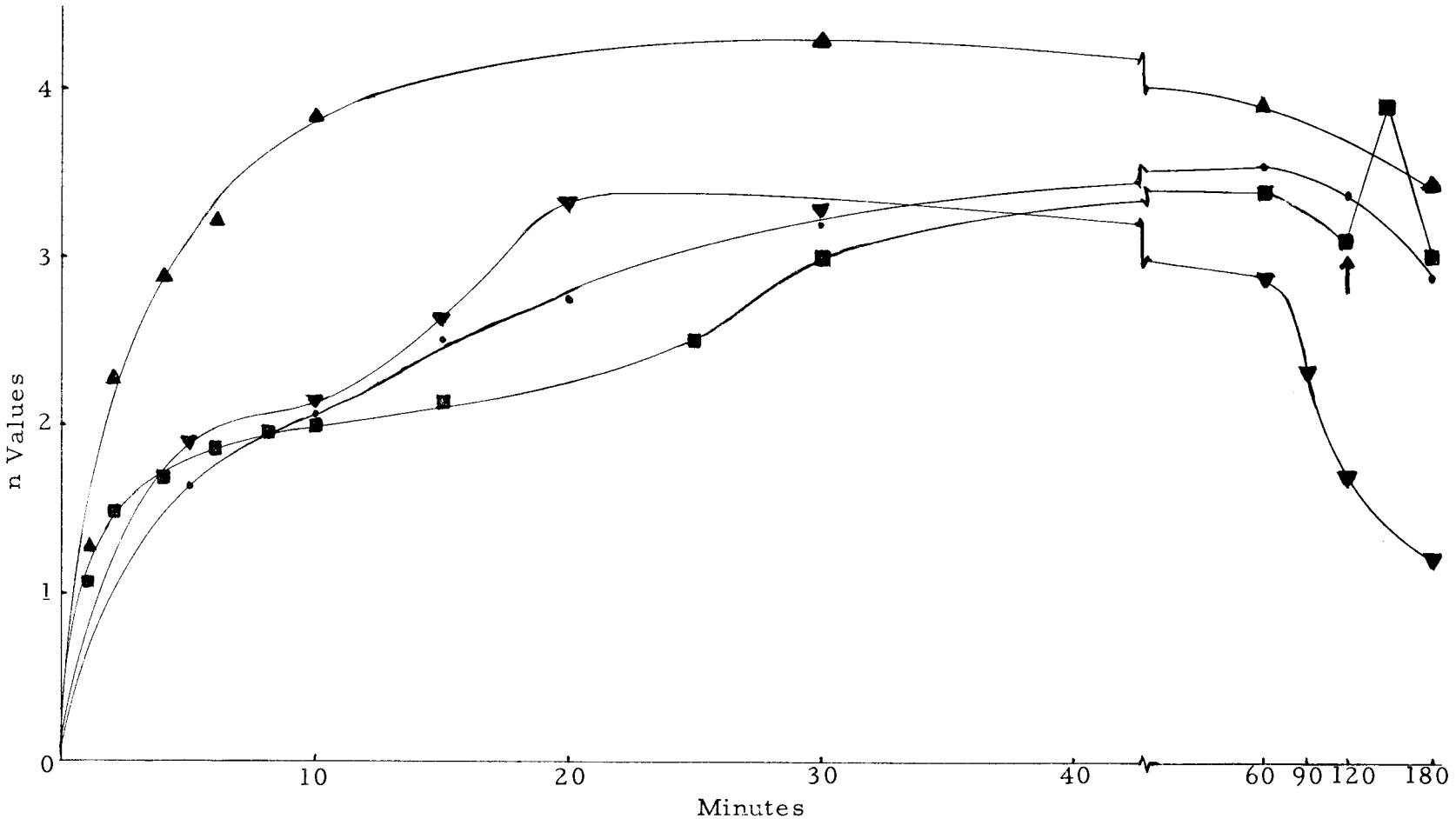

Figure 3. Time course of the reaction of acetylimidazole with the tyrosyl residues of zinc-insulin, in 0 M urea pH 6.5 (•), pH 7.5 (▼), pH 7.0 to 6.5 (■), and in 8 M urea pH 7.0 to 6.5 (▲). Insulin concentration 2.5 μmoles/ml; acetylimidazole concentration 250 μmoles/ml. The arrow indicates addition of 25% more acetylimidazole.

set of different conditions, on the n values of the Ac_n-insulin produced. The use of trichloroacetic acid precipitation of aliquots withdrawn from the reaction mixture at the specified time stops the acetylation reaction instantaneously. The precipitates are then deacetylated to give the n values of the Ac_n-insulin samples. It can be seen that acetylation of amorphous insulin in 0 or 8 M urea is completed in ten minutes, zinc insulin in 8 M urea is acetylated somewhat slower; still slower is the acetylation of amorphous insulin in 2 M sodium chloride, while the acetylation pattern of zinc insulin in 0 M urea under constant pH 6.5, 7.5, and pH 7.0 to 6.5, reveals that there are two distinct acetylation steps. The first step involves two tyrosyl residues, and the second step the remaining two tyrosyl residues, although complete acetylation of zinc insulin in 0 M urea requires additional acetylimidazole (Figure 3).

Reaction periods longer than 30 minutes actually lead to slow deacetylation of Ac_n-insulin at pH 6.5, and quite fast deacetylation in 2 M NaCl, or at pH 7.5.

Zinc insulin is not soluble in Veronal-KCl buffer pH 7.0, but this does not seriously affect the tyrosyl acetylation kinetics because solubilization occurs in less than two minutes after the addition of acetylimidazole. The solubilization is due to the acetylation of the free amino groups of zinc insulin, causing a lowering of the isoelectric point by two pH units (Figure 1). The possibility that zinc

insulin dissolves at pH 7.0 as a result of "extraction" of zinc from the zinc insulin dimer (Lindley and Rollett, 1955) by imidazole was excluded when imidazole added in the zinc insulin suspension did not solubilize it. The time course of the reaction of acetylimidazole with the free amino and tyrosyl groups of insulin is demonstrated by Figure 4, for the first 30 minutes of the acetylation reaction. The free amino groups of insulin are acetylated as fast as the tyrosyl groups in the rapid acetylation curves, and even as fast as the two tyrosyl groups in the stepwise acetylation curve. According to Jencks and Carriuolo (1959), acetylimidazole reacts faster with phenyl hydroxyl groups than with amino groups at neutral pH, but there is a general base catalysis due to imidazole which facilitates acetylation of amino groups. The relative rates of acetylation of amorphous or zinc insulin under the above conditions (Figures 2 and 3) were estimated from the slopes of the semilogarithmic acetylation graphs (Figures 5 and 6) plotted from the same data used in Figures 2 and 3. The 25-fold excess of acetylimidazole per tyrosyl residue makes the acetylation reaction a pseudomonomolecular one, so that one would expect first order kinetics for the change of concentration of free tyrosyl residues (4-n), with acetylation time t, i.e. $\log (4-n) = -kt$, where k is the acetylation constant under the conditions used (see Experimental). This would be the case if all four tyrosyl residues of insulin were equally available to the reagent

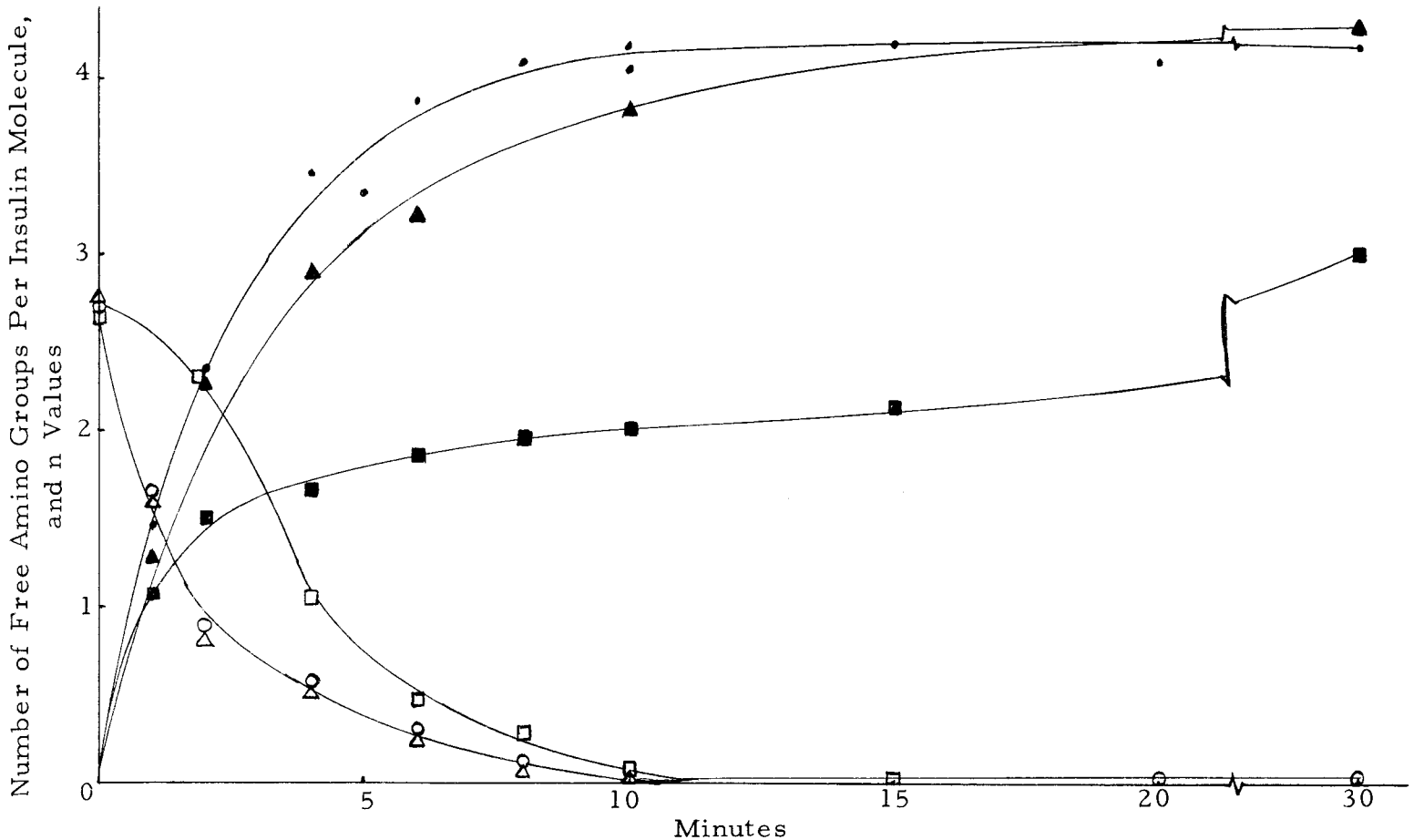

Figure 4. Time course of reaction of acetylimidazole with the tyrosyl (solid symbols), and free amino groups (open symbols) at pH 7.0 to 6.5, of amorphous insulin in 0 M urea (•,○), zinc insulin in 0 M urea (■,□), and zinc insulin in 8 M urea (▲,△). Insulin concentration 2.5 μmoles/ml; acetylimidazole concentration 250 μmoles/ml.

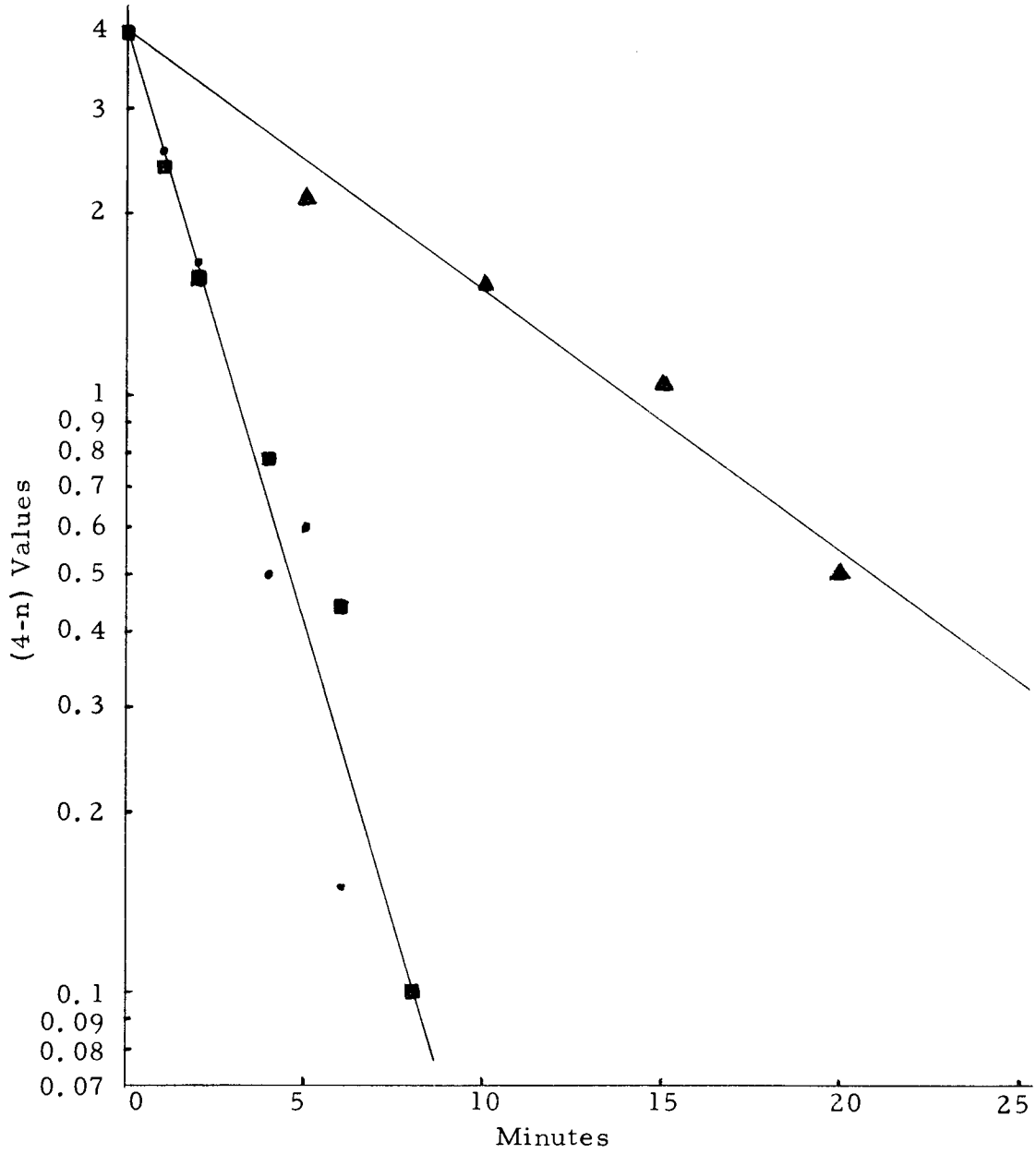

Figure 5. Acetylation of the tyrosyl residues of amorphous insulin at pH 7.0 to 6.5, in 0 M urea (•), 8 M urea (■), and 2M sodium chloride (▲). Insulin concentration 2.5 μmoles/ml; acetylimidazole concentration 250 μmoles/ml.

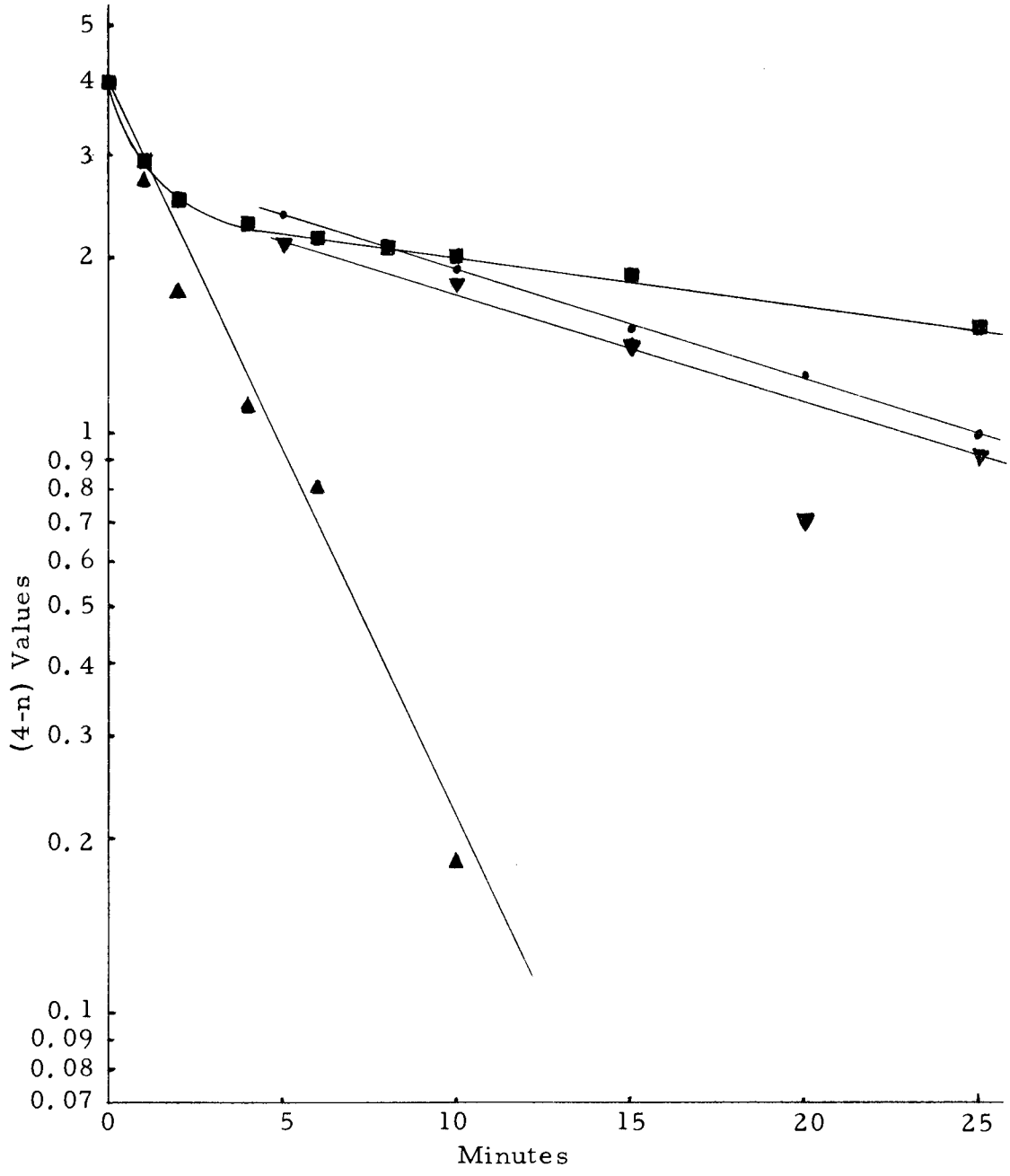

Figure 6. Acetylation of the tyrosyl residues of zinc insulin at pH 7.0 to 6.5 in 8 M urea (▲), in 0 M urea (■), and at constant pH 6.5 (•), and 7.5 (▼) in 0 M urea. Insulin concentration 2.5 μmoles/ml; acetylimidazole concentration 250 μmoles/ml.

acetylimidazole. Figures 5 and 6 give single straight lines for amorphous insulin in 0 and 8 M urea, and 2 M sodium chloride, as well as for zinc insulin in 8 M urea, while for acetylation of zinc insulin in 0 M urea the plot consists of two straight line parts at quite different slopes to each other. The acetylation constant k for each acetylation was estimated from the semilogarithmic graphs (Figures 5 and 6) by calculation of the slope of each straight acetylation line. Table 2 lists the slopes calculated in log (4-n) per hour of acetylation for each set of conditions shown. These slopes are comparable because the pH, temperature, and concentration of acetylimidazole are the same in each acetylation. It can be seen from Figure 6 that the acetylation curves for zinc insulin in 0 M urea, at constant pH 7.5, 6.5, and pH 7.0 to 6.5 are of similar shape, meaning that the small variation in acetylation pH only slightly affects the rate of acetylation. These results indicate that acetylation of proteins with acetylimidazole can provide more information when followed kinetically. Table 2 indicates that all four tyrosyl residues of amorphous insulin are equally exposed, and that urea does not increase their availability. Urea, even without exposing the tyrosyl groups to a greater extent, should speed up their acetylation because of the increase in dielectric constant of the reaction mixture, but this is probably counteracted by the viscosity increase. The acetylation of all four tyrosyl residues of amorphous

Table 2. Rate of acetylation of insulin with acetylimidazole.

Conditions[1]	Slope [in log (4-n)/hour]
Amorphous insulin in 0 or 8 M urea	12.0
Amorphous insulin in 2 M sodium chloride	2.7
Zinc insulin in 8 M urea	7.5
Zinc insulin in 0 M urea (fast acetylation step)	7.5
Zinc insulin in 0 M urea (slow acetylation step)	0.8

[1] In all cases acetylation with acetylimidazole was carried out as described in the Experimental part.

insulin is in agreement with the recent studies by Riordan et al. (1965). The increase of ionic strength decreases the acetylation rate of amorphous insulin most probably because it enhances aggregation of insulin (Oncley, 1952). The present data indicate that the acetylation of zinc insulin in 0 M urea consists of an initial fast acetylation at a rate similar to that of zinc insulin in urea, involving two tyrosyl residues, and an acetylation ten times slower than the initial fast acetylation, involving the remaining two tyrosyl residues. It is apparent from these data that the reactivity of tyrosyl residues towards acetylimidazole depends clearly on the protein conformation and aggregation state. The difference between the acetylation course of amorphous and crystalline zinc insulin is due to the higher aggregation state of crystalline zinc insulin,

probably involving the dimer proposed by Lindley and Rollett (1955). The two-phase acetylation of zinc insulin in 0 M urea suggests that two of its tyrosyl residues are reactive or "free", and the other two are non-reactive or "buried." This suggestion is consistent with the results of Laskowski et al. (1960), Springell (1961), Kurihara et al. (1963), DeZoeten and Havinga (1961), and Aoyama et al. (1965).

The possibility that the Ac_2-insulin preparation is a population of molecules containing the two acetyl groups on more than two distinct tyrosyl residues is excluded by the structural studies on the Ac_2-insulin discussed later in this thesis.

Deacetylation Kinetics of Ac_n-insulin Preparations

The deacetylation of Ac_n-insulin preparations was used here not only for the determination of the n values, but it was also followed kinetically in order to obtain more information about the availability of the O-acetyl-tyrosyl residues to the hydroxylamine reagent. The deacetylations were performed at a constant pH of 6.6 in the presence of a 250-fold molar excess of hydroxylamine per O-acetyl-tyrosyl residue (see Experimental part). Such an excess of hydroxylamine makes the deacetylation pseudomonomolecular, so that the first order equation $\log n = kt$ should be applicable, as the number of O-acetyl-tyrosyl residues, n decreases exponentially with deacetylation time, t. The kinetic constant, k,

can be calculated from the slope of the plot of log n versus deacetylation time. If all O-acetyl-tyrosyl residues of the Ac_n-insulin preparation are equally available to hydroxylamine, one straight line curve must be expected, giving one value for k. On the other hand, different availability of one or more O-acetyl-tyrosyl residues of Ac_n-insulin will result in a log n versus t curve composite of two or more straight line portions, each one with its own slope and k. Figures 7 and 8 give the results of the deacetylation kinetic studies of several Ac_n-insulin preparations, N, O-diacetyl tyrosine, and also native amorphous insulin. The deacetylation conditions for all these deacetylations were the same, including constant pH 6.6, temperature, and concentrations of hydroxylamine, so that the deacetylation rates of the above preparations can be compared (Table 3). The shape of the log n versus t graphs was found to be the following: For N, O-diacetyl tyrosine deacetylation it was a straight line (Figure 7), for native insulin "deacetylation", also a straight line (Figure 7), while for the Ac_n-insulin preparations, the semilogarithmic deacetylation plot consisted of an early curved portion followed by a straight line (Figure 7 and 8). Such a graph can be analyzed by extrapolation of the straight line and subtraction of the values found by extrapolation from the values of the curved part of the regular graph. The resulting points were found to lie on a straight line, so that the original deacetylation graph of each

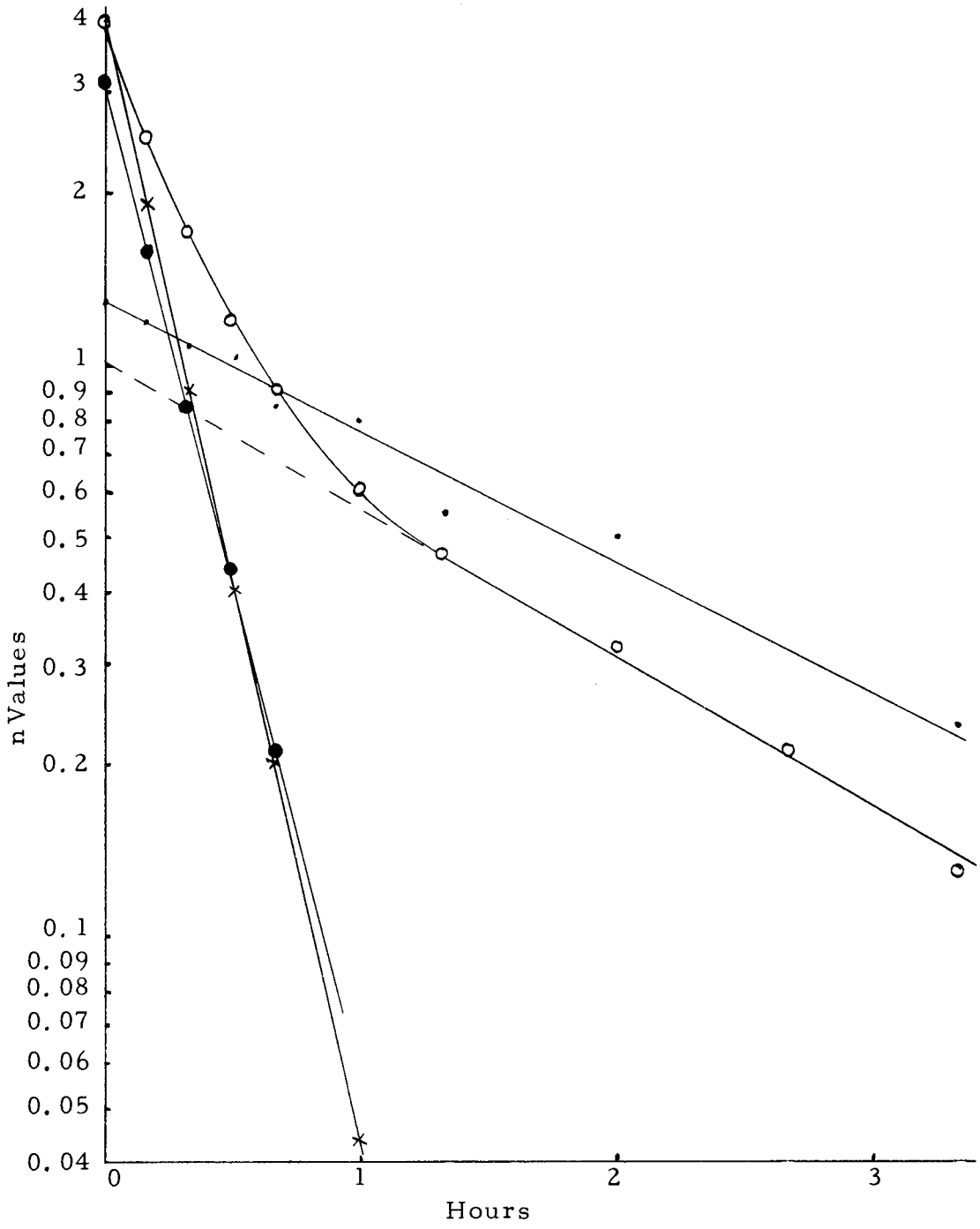

Figure 7. Deacetylation kinetics of N, O-diacetyltyrosine (x), native insulin (·), and Ac_4-insulin (o for the experimental points and ● for the points found by subtraction of the extrapolated straight line from the experimental points of the curved part of the graph) with hydroxylamine, under the conditions described in the Experimental part.

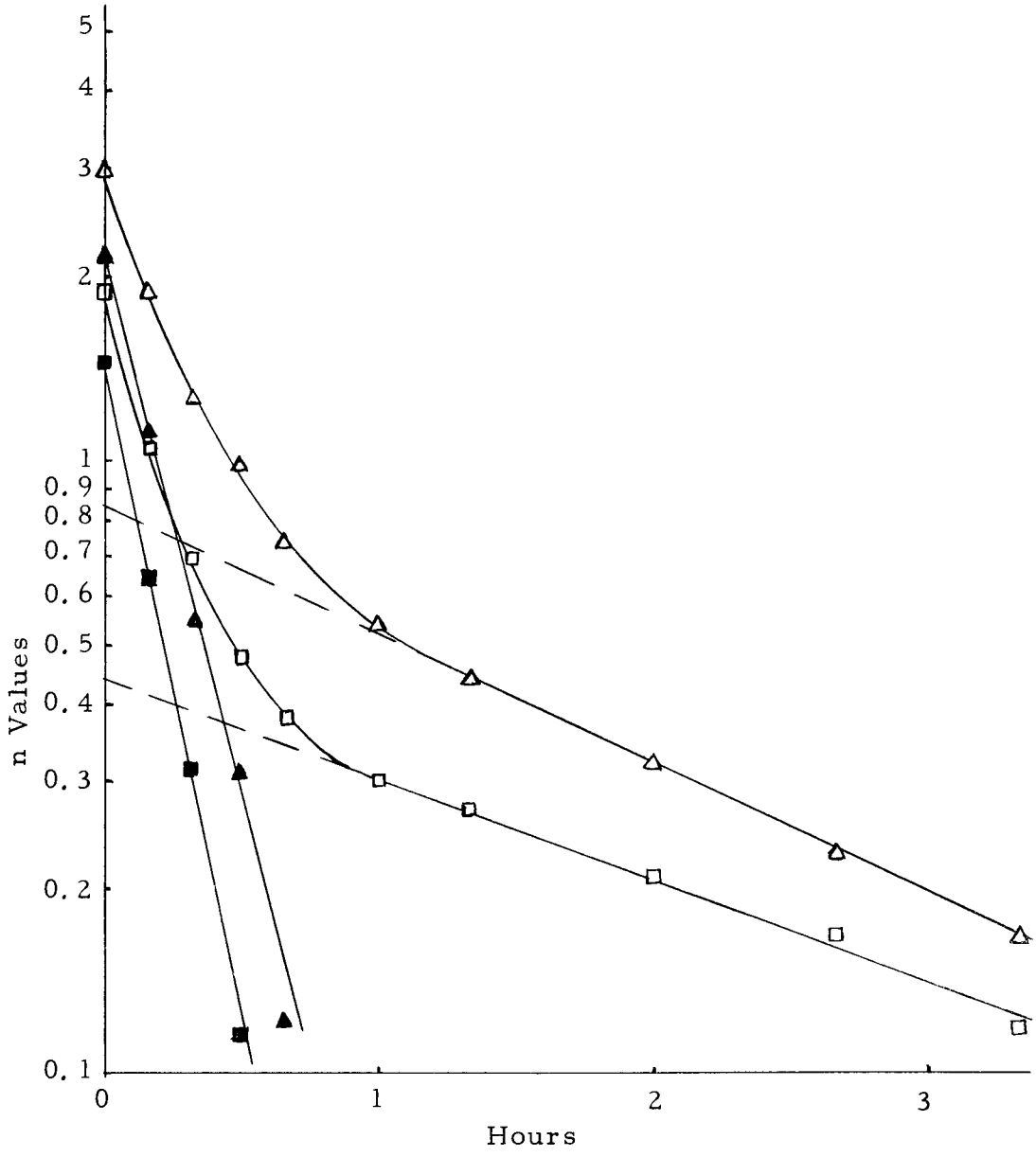

Figure 8. Deacetylation kinetics of Ac_2-insulin (□), and Ac_3-insulin (△) with hydroxylamine, under the conditions described in the Experimental part. Open symbols (□, △) are experimental points, solid symbols (■, ▲) were calculated by subtraction of the extrapolated straight lines from the experimental points of the curved parts of the Ac_2-insulin and Ac_3-insulin graphs respectively.

Ac_n-insulin preparations was analyzed as two straight lines, one representing a fast deacetylation, and the second the slow deacetylation. The results from Figures 8 and 9 are that both N, O-diacetyl tyrosine and native insulin deacetylate at a steady rate, while Ac_n-insulin preparations deacetylate in early rapid reaction followed by a later slow deacetylation. Table 3 gives the slopes for each deacetylation graph shown in Figures 7 and 8. These slopes represent the deacetylation kinetic constants under standard deacetylation conditions. Other Ac_n-insulin samples show similar slopes as the ones in Table 3, deacetylated under same conditions.

Table 3. Rate of deacetylation of N, O-diacetyl tyrosine, Ac_n-insulin and native insulin with hydroxylamine[1].

Deacetylated Compound	Slopes [log n/hour]
N, O-diacetyl tyrosine	1.9 --
Ac_2-insulin	2.0 , 0.17
Ac_3-insulin	1.7 , 0.21
Ac_4-insulin	1.7 , 0.25
Native amorphous insulin	-- , 0.22

[1]Compounds were deacetylated at a concentration of about 0.2 mM O-acetyl-tyrosyl residue in 0.1 M sodium phosphate buffer pH 6.6, made 0.07 M with hydroxylamine (at zero time) for four hours at 25°.

It is concluded that the initial rapid deacetylation of Ac_n-insulin preparations is as fast as the deacetylation of N, O-diacetyl tyrosine, and the late slow deacetylation of Ac_n-insulin is as slow as the "deacetylation" of native amorphous insulin.

These results verify the correction of n_t by the $n_{control}$ value because hydroxylamine increases the absorptivity of Ac_n-insulin preparation in a fast deacetylation reaction, and at the same time causes a slow absorptivity increase similar to the one of native insulin.

All four O-acetyl-tyrosyl groups of Ac_4-insulin seem to be equally available to the hydroxylamine (Table 3).

The absorptivity increase on treatment with hydroxylamine corresponds to an $n_{control} = 1.3$ value in the case of native amorphous insulin (Figure 7) with or without previous precipitation by trichloroacetic acid. This phenomenon was reproducible and was not due to evaporation of the sample or to any impurities of hydroxylamine. Furthermore hydroxylamine was shown to be quite specific, because same ionic strength of NaCl or KCl added to insulin solution instead of hydroxylamine did not cause any change of the absorbance. Preliminary experiments on the effect of hydroxylamine on ribonclease, chymotrypsin, and bovine serum albumin showed a small absorptivity increase for ribonuclease ($n_{control}$ about 0.4), no effect for chymotrypsin ($n_{control} = 0$), and a

significant increase in the case of bovine serum albumin ($n_{control}$ about 3).

The remote possibility of an O-tyrosyl-ester bond in native insulin was supported by Gallop et al. (1959), who detected "bound" hydroxamate on collagen, insulin, and bovine serum albumin, but not ribonuclease, after treatment with hydroxylamine. By means of the hydroxamate method by Balls and Wood (1956), modified as described in the Experimental part, hydroxamate bound to insulin treated with hydroxylamine was found to be only 0.1 moles per mole of insulin. Furthermore the methods by Blumenfeld and Gallop (1962) for involvement of α-, β- or γ-carboxyl groups in possible ester bond in insulin were negative.

The above results seem to eliminate the possibility of an ester bond in native insulin so that its absorptivity increase by hydroxylamine must be explained in terms of a change in aggregation of insulin, or in terms of the specific tyrosyl-carboxylate interaction in a hydrophobic environment as has been suggested by Tanford et al. (1955), Hermans and Scheraga (1961), and Riehm and Scheraga (1966). In this interaction a tyrosyl group and a carboxylate anion form a bond stronger than a hydrogen bond, although the two groups might not be close enough to form a hydrogen bond.

Effect of Acetylation and Hydroxylamine Treatment of Insulin on Its Activity

The mouse convulsion test was used as described in the Experimental part.

Each mouse was injected only once, because during the assay at 37° most of the convulsed mice died, and the surviving ones were less sensitive. Only the assays resulting in 40 to 60% convulsions were used to calculate activity. The 50% convulsion dose was calculated from the 40 to 60% convulsion results, and the average 50% convulsion dose for the 30 mice assayed per insulin preparation was used to calculate the activity based on a 100% activity for native insulin. Table 4 presents the results of the convulsion assay. The activity calculated, although no better than 15% accurate, demonstrates that precipitation of insulin by trichloroacetic acid (according to the isolation of Ac_n-insulin from the acetylation reaction mixture) does not impair the activity of insulin. Ac_2-insulin, isolated after ten minutes acetylation of zinc insulin in 0 M urea, was found fully active, while Ac_4-insulin was only 30% active. Furthermore, both native and acetylated insulin lost about 70% of their activity after deacetylation with hydroxylamine.

It is a fact that the amino groups are also acetylated by acetylimidazole (Figure 4), but it has been established

Table 4. Mouse convulsion assay of insulin acetylated with acetylimidazole under conditions described in the Experimental part.

Sample Injected	Treatment with Hydroxylamine[1]	50% Convulsion Dose[2] (μg insulin)	% Activity
Native amorphous insulin	No	0.70	100
Native amorphous insulin	Yes	2.1	33
Trichloroacetic acid precipitated insulin (zero time acetylation control)	No	0.76	92
Trichloroacetic acid precipitated insulin (zero time acetylation control)	Yes	2.3	30
Ac_2-insulin	No	0.67	100
Ac_2-insulin	Yes	2.0	35
Ac_4-insulin	No	2.3	30
Ac_4-insulin	Yes	2.5	28

[1]Samples were deacetylated under the standard conditions described in the Experimental part, then diluted to appropriate concentrations for the assay.

[2]Average of three tests (ten mice per test).

(Fraenkel-Conrat and Fraenkel-Conrat, 1950; Evans and Saroff, 1957) that the two α-amino and the one ε-amino groups of insulin are not essential for activity. This result can be also drawn from Figure 4 for Ac_2-insulin preparation which is fully active although all its amino groups are acetylated.

Acetylimidazole was found (Riordan et al., 1965) not to react with any other groups except tyrosyl hydroxyl, amino, and thiol groups, under the acetylation conditions used, so that any decrease of activity of insulin should be ascribed to acetylation of its tyrosyl residues. The results presented (Table 4) indicate that insulin acetylated only to two of its four tyrosyl residues is fully active, while completely acetylated insulin is only 30% active. At this point the possibility of partial hydrolysis of Ac_n-insulin preparations in the mouse tissues during the two hour assay must be considered. In such a case one would expect even a higher inactivation of the tested preparations.

The effect of complete deacetylation of native, Ac_2-insulin, or Ac_4-insulin with hydroxylamine is to reduce the activity by about 70%.

It is concluded therefore, that the phenyl hydroxyl groups of the two reactive tyrosyl residues of zinc insulin are not essential for activity of insulin, while one or both of the non-reactive tyrosyl residues of zinc insulin are of importance. In the present study no

optical rotatory dispersion studies or other tests designed to detect conformational changes were made on the Ac_n-insulin preparations so that it is not known if Ac_2- or Ac_4-insulin maintain the same conformation as native insulin. It is thus not clear if the hydroxyl groups of the non-reactive tyrosyl residues are a part of the active site of the hormone, or their acetylation simply does not allow the insulin molecule to assume its native conformation.

Of significance is the result that hydroxylamine treated insulin is only 30% active. Hydroxylamine injection alone produces no visible effect on the mice. The inactivation when coupled with the absorptivity increase of native insulin by hydroxylamine treatment, argues in favor of a specific alteration of the hormone structure by hydroxylamine. If there is no O-tyrosyl ester bond in native insulin, it is possible that the hydroxylamine treatment specifically breaks a possible tyrosyl-carboxylate interaction which is essential in holding insulin to its active conformation, increasing the absorptivity of insulin, but altering its native conformation and impairing its activity.

Interestingly enough when hydroxylamine treated insulin was separated by isoelectric precipitation, and dissolved in 0.1 M sodium phosphate buffer pH 6.6, a decrease of absorbance was observed with time. This phenomenon could mean a reforming of the possible tyrosyl-carboxylate interaction as that in native insulin.

Complete acetylation of all four tyrosyl groups of insulin, would also destroy the previously assumed tyrosyl-carboxylate interaction, leading to inactivation of the same extent as with hydroxylamine treatment. This is actually the case from Table 4, where both Ac_4-insulin and the hydroxylamine treated insulin are only 30% active.

Wilson et al. (1962) have supplied convincing evidence that antigenicity of insulin is controlled by its A chain, which makes it possible that Ac_2-insulin might behave immunologically different from the ox insulin injected to diabetic patients. The decrease in chemical activity of injected ox insulin has been attributed (Berson et al., 1956) to the presence of insulin-binding antibody in the serum of diabetics treated with heterologous insulin. The possibility exists that Ac_2-insulin might find therapeutic use because of altered solubility and structure of two tyrosyl residues, possibly in the form of complexes with protamine.

Location of the Two Non-reactive Tyrosyl Residues in Insulin

As shown from the previous kinetic studies Ac_2-insulin seems to contain two acetyl groups on the two reactive tyrosyl residues of zinc insulin. These tyrosyl residues were considered "free," while the remaining two must be non-reactive or "bound." The fact that Ac_2-insulin is completely active while Ac_4-insulin is only 30%

active, would mean that one or both of the two non-reactive tyrosyl groups in zinc insulin must be intact for complete activity of insulin. It is of interest therefore to determine the location of the non-reactive tyrosyl residues on insulin. The four tyrosyl residues are located in insulin at the positions A_{14}, A_{19}, B_{16} and B_{26} as shown in Figure 9. In order to locate the two reactive tyrosyl groups which are acetylated in Ac_2-insulin the following method was used: Ac_2-insulin was oxidized and each chain was isolated and treated with hydroxylamine (see Experimental part). Another portion of the same Ac_2-insulin preparation was digested with trypsin and chymotrypsin separately, and the tryptic and chymotryptic core was isolated and treated with hydroxylamine in order to determine the n_t value for each deacetylated fragment of Ac_2-insulin. Native insulin as a control was also oxidized and digested in the same way as Ac_2-insulin, and each chain and core was treated with hydroxylamine, in order to determine the $n_{control}$ value of each insulin fragment. Table 5 lists the results of the deacetylation experiments, in terms of the $n_{control}$, n_t values found, and the corrected values, n, which show the number of acetyl groups attached to tyrosyl residues in each fragment of Ac_2-insulin. The $n_{control}$ and n_t values shown are averages of two or more determinations with a variation of 15% or less from the mean value. It turns out that all the peptides listed show a small but significant $n_{control}$ value, which justifies the

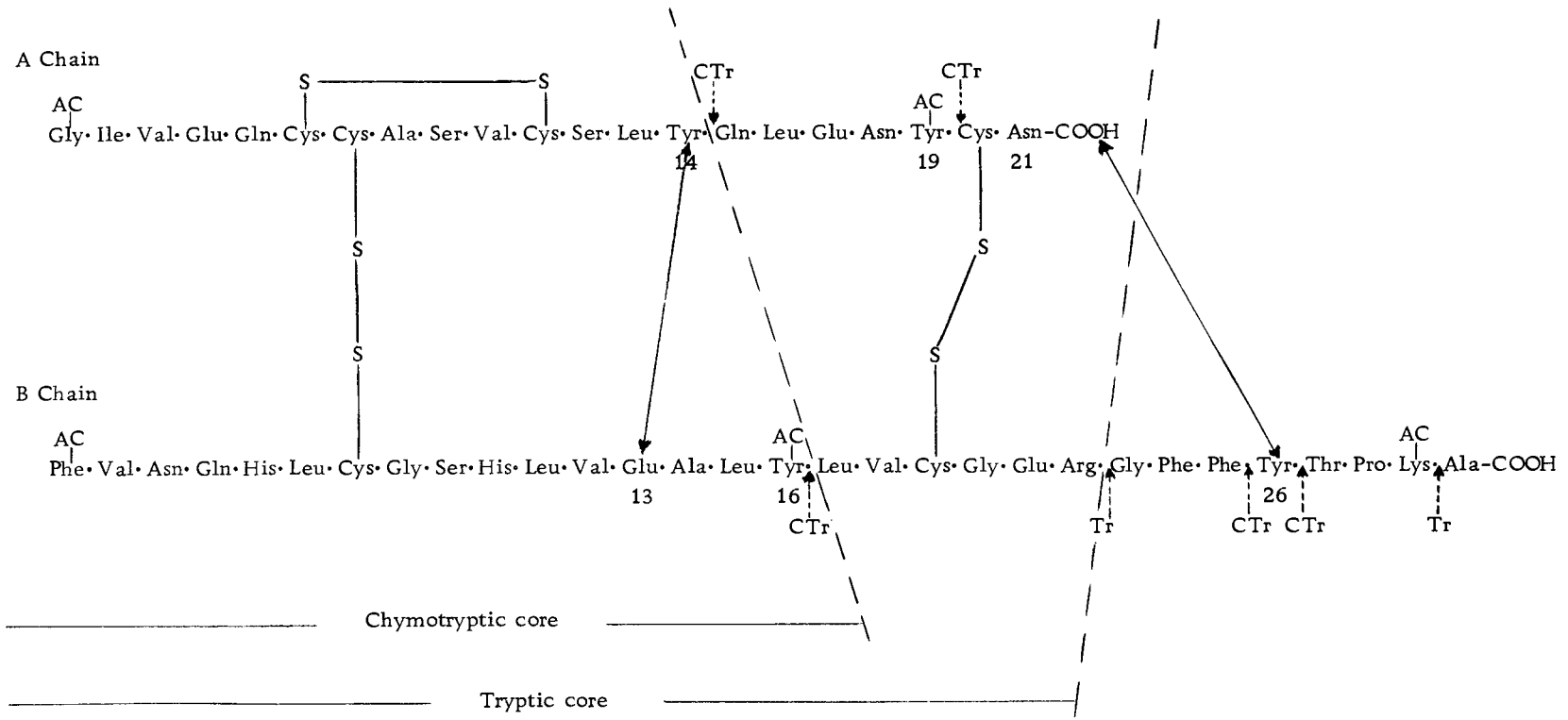

Figure 9. Primary structure of Ac_2-insulin molecule, indicating the peptide bonds hydrolyzed by trypsin (Tr) and α-chymotrypsin (CTr), as well as the tryptic and chymotryptic cores of the molecule. Tyrosyl residues A19 and B16 are acetylated. Solid arrows indicate the two interchain specific tyrosyl-carboxylate interactions of the non-reactive tyrosyl residues.

Table 5. The n values of Ac_2-insulin and its degradation products. The n_{theor} indicates the theoretical value of n expected from the structure of Ac_2-insulin shown in Figure 9.

	$n_{control}$	n_t	n	n_{theor}
Ac_2-insulin	1.3	3.4	2.1	2.0
A and B chains from Ac_2-insulin	0.6	2.5	1.9	2.0
A chain from Ac_2-insulin	-0.4	0.6	1.0	1.0
B chain from Ac_2-insulin	0.1	1.0	0.9	1.0
Tryptic core from Ac_2-insulin	0.8	2.6	1.8	2.0
Chymotryptic core from Ac_2-insulin	0.8	1.7	0.9	1.0

correction of n_t values. This small increase in absorptivity of the peptides on treatment with hydroxylamine could be partially explained by means of the increase of ionic strength, which diminishes electrostatic interactions, unfolds the peptides, and exposes all tyrosyl residues to a greater extent. The A chain is an exception here because it contains seven negative charges and only one positive charge. The repulsion of the negative charges keep the A chain expanded, but on increase of ionic strength, this repulsion is lessened, the peptide gets a more compact form (Craig et al., 1965) and the tyrosyl residues become less exposed. The $n_{control}$ values of the tryptic and chymotryptic cores, however, as well as that of native insulin are too high to be explained by the increase of ionic strength due to the addition of hydroxylamine, and specific action

on possible tyrosyl-carboxylate bonds cannot be excluded.

Sufficient control experiments were performed (as in the Experimental part) to ascertain that none of the degradation and digestion conditions of Ac_2-insulin or native insulin, or the isolation conditions of their fragments, hydrolyze the O-acetyl-tyrosyl ester bond, or oxidize the phenyl hydroxyl group of tyrosyl residues. Digestion of Ac_2-insulin and isolation of the tryptic and chymotryptic cores by trichloroacetic acid precipitation was favored over the digestion of the A and B chain of Ac_2-insulin and separation of the fragments by ion-exchange chromatography, because O-acetyl-tyrosyl esters were found to be labile in resin beds. Furthermore, chymotrypsin is nonspecific enough to hydrolyze the peptide bond of an acetylated tyrosyl residue, as was shown by the amino acid analysis of the chymotryptic core of native and Ac_2-insulin (Table 6). In isolating each chain from the oxidized insulin it was necessary to determine the pH of minimum solubility of the B chain.

The solubility of B and Ac_2-B chains for different pH values is shown in Figure 10. Both B and Ac_2-B chains are least soluble at pH 4.5. The solubility curve of the B chain is quite similar to that of the S-sulphonate of the B chain given by Du et al. (1961).

The excess of trypsin or chymotrypsin used was necessary to shorten the digestion period, after the end of which the enzymes

Table 6. Amino acid composition of the degradation products of insulin and Ac_2-insulin[1]

	Ac_2-insulin		A chain from			B chain from			Tryptic core from			Chymotryptic core from		
Amino Acid	Found	Theory	Insulin	Ac_2-insulin	Theory	Insulin	Ac_2-insulin	Theory	Insulin	Ac_2-insulin	Theory	Insulin	Ac_2-insulin	Theory
Cysteic acid	0.07	0	3.82	3.80	4	2.04	1.80	2	0.24	0.57	0	0.39	0.45	0
Aspartic acid	3.00	3	2.00	2.00	2	1.00	1.00	1	3.00	3.00	3	0.85	1.14	1
Threonine	1.05	1	--	--	0	0.87	0.83	1	0.04	--	0	0.39	0.14	0
Serine	3.25	3	2.06	2.07	2	1.04	1.00	1	3.14	3.69	3	3.19	3.40	3
Glutamic acid	6.75	7	3.70	3.82	4	3.04	2.64	3	7.42	6.71	7	4.00	4.00	4
Proline	1.04	1	--	--	0	0.85	0.81	1	--	--	0	0.22	--	0
Glycine	3.90	4	1.05	0.95	1	2.68	2.52	3	3.02	2.91	3	2.65	2.65	2
Alanine	2.74	3	1.09	0.99	1	1.70	1.66	2	2.03	1.89	2	2.34	1.86	2
Half-cystine	5.92	6	--	--	0	--	--	0	6.50	6.17	6	4.26	4.19	4
Valine	4.98	5	1.80	1.71	2	2.95	2.68	3	5.00	5.00	5	3.70	3.06	4
Methionine	--	0	--	--	0	--	--	0	--	--	0	--	--	0
Isoleucine	0.82	1	0.98	0.68	1	0.09	--	0	0.65	0.85	1	1.11	0.83	1
Leucine	6.06	6	2.00	1.87	2	3.72	3.60	4	6.80	6.51	6	3.93	3.92	4
Tyrosine	3.92	4	2.02	1.96	2	1.88	1.73	2	3.60	3.62	3	2.18	1.94	2
Phenylalanine	3.13	3	--	--	0	2.60	3.06	3	1.50	1.13	1	0.87	0.91	1
Lysine	1.00	1	--	--	0	1.00	1.00	1	0.24	0.36	0	0.32	0.27	0
Histidine	1.92	2	--	--	0	1.98	1.93	2	2.00	1.94	2	2.00	2.00	2
Arginine	1.07	1	--	--	0	1.14	1.09	1	0.67	1.00	1	0.22	0.36	0

[1] Moles of amino acid/mole of protein.

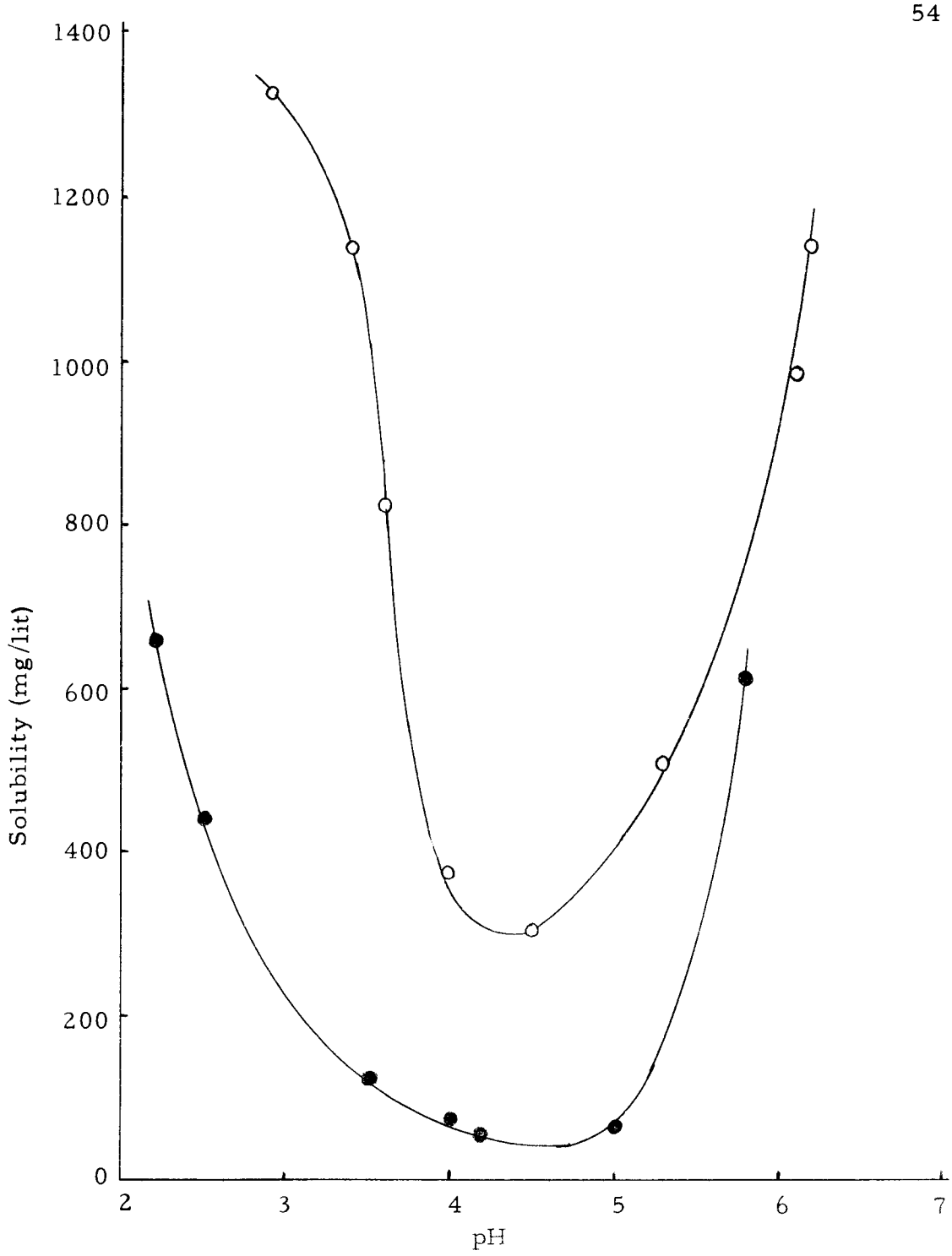

Figure 10. The effect of pH at 25° on the solubility of B chain (○), and Ac_2-B chain (●) in mixtures of 0.1 M sodium phosphate buffer and N HCl at 0°.

were separated from the cores on the basis that the trichloroacetic acid precipitated enzymes do not dissolve in 0.1 M sodium phosphate buffer pH 6.8 to 7.0. The digestion conditions used were the best in obtaining the insulin cores, as judged by their amino acid analysis (Table 6).

Figure 9 shows the tryptic and chymotryptic cores of insulin. From this figure and the results in Table 5 the position of the two acetyl groups of Ac_2-insulin can be deduced. Thus, it is clear that each of the chains from Ac_2-insulin contains one acetylated tyrosyl residue. The fact that the tryptic core of Ac_2-insulin contains two (actually found 1.8) acetyl groups means that the B26 tyrosyl residue is not acetylated, i.e. it is non-reactive or "bound." By the same argument and the fact that each chain of Ac_2-insulin contains only one acetylated tyrosyl residue, it follows that B16 tyrosyl residue is acetylated. The chymotryptic core of Ac_2-insulin contains only one acetyl group, which must be that previously located on B16 tyrosyl residue, leaving the A14 residue not acetylated, i.e. non-reactive or "bound." Since the A chain of the Ac_2-insulin contains one acetyl group, this must be on the A19 tyrosyl residue, which is a reactive one. In conclusion, A19 and B16 are the reactive tyrosyl residues of zinc insulin, while A14 and B26 are the non-reactive or "bound" ones. These findings agree with the results of Aoyama et al. (1965) from his studies employing cyanuric fluoride to react with the

tyrosyl groups of insulin. It is of importance that the two methods give the same results although the cyanuric fluoride reaction takes place at pH 9.7. It is concluded that zinc insulin does not appreciably change its conformation between pH values 6.6 and 9.7. Iodination studies by DeZoeten and Havinga (1961) agree with the present work as to the number of bound tyrosyl residues in zinc insulin, but they propose B16 and B26 as the non-reactive residues.

Finally under the controlled iodination conditions of Springell (1961), only the two tyrosyl residues of the A chain were found reactive.

The acetylation reaction is carried out under more mild conditions than those used in the treatment with cyanuric fluoride by Aoyama et al. (1965). Furthermore, the acetyl group is a "physiological" one (Hofmann, 1963) and the results from the activity assays (Table 4) argue in favor of one or both of the "bound" A14 and B26 tyrosyl residues, as necessary for hormone action, or for maintaining the active conformation of insulin molecule.

The fact that the trypsin core of insulin is inactive (Young and Carpenter 1961; Carpenter and Baun, 1962) emphasizes the importance of the octapeptide at the C-terminal end of the B chain for the activity of the hormone. Tyrosyl residue B26 is contained in this octapeptide, and it has been concluded (Laskowski et al. 1956; Lakowski et al., 1960) from a blue shift of the insulin spectrum after

trypsin digestion, and on further acidifying the tryptic core, that there are two bound tyrosyl residues in insulin, one of them being B26. From the discussion above it seems that B26 tyrosyl residue is well established as a non-reactive one, but there is a disagreement on the location of the second non-reactive tyrosyl residue in zinc insulin. An additional evidence for the A14 as the second non-reactive tyrosyl residue comes from analysis of the insulin molecule according to Guzzo (1965), who used other proteins of known amino acid sequence and three dimensional structures as models. The presence in the peptide of four critical residues; proline, aspartic acid, glutamic acid, or histidine appears to be necessary for a helical disruption to occur. Any sequence of six or more amino acids without any of the critical ones can be considered helical, or in a hydropholic region. On the basis of this analysis, insulin contains only three possible amino acid sequences with six or more non-critical amino acids. These sequences are A5 to A16, B14 to B20, and B22 to B27, and according to Guzzo (1965) should be α-helices, pleated sheet structures, or in general compact and inaccessible hydrophobic regions with many hydrogen bonds. It is of importance that both A14 and B26 tyrosyl residues are in such regions while A19 is not. The problem remains as to which are the residues with which the bound tyrosyl residues A14 and B26 interact. Although no direct experimental evidence has been worked out in the

literature or in this investigation, some indirect evidence favors certain amino acid residues more than others. Nichol (1961) showed that insulin deprived of its C-terminal asparagine residue was only 20% active. This value is very close to the 30% activity of Ac_4-insulin or the hydroxylamine treated native or acetylated insulin. Furthermore, Slobin and Carpenter (1966), showed that carboxypeptidase cleaves the C-terminal alanine of the B chain ten times faster than the C-terminal asparagine of the A chain. From their O.R.D. studies, they conclude that desalanine-insulin is in the native conformation, while desalanine-desasparagine-insulin is unfolded. It is evident therefore that the C-terminal asparagine of the A chain is "bound" and necessary for maintaining the native conformation and the activity for insulin. The above circumstantial evidence suggests that there might exist a specific tyrosyl-carboxylate interaction between the C-terminal carboxyl group of the A21 asparagine and the B26 tyrosyl residue which is important in maintaining the native conformation of the insulin molecule. Such an interaction would explain the inaccessibility of the A21 asparagine to carboxypeptidase, its importance for maintaining the native conformation and the activity of the hormone molecule, as well as the inaccessibility of B26 tyrosyl residue to acetylimidazole, and its importance for activity. Removal of A21 asparagine from insulin, acetylation of the B26 tyrosyl residue, or treatment of native insulin with

hydroxylamine, seems to destroy this important interaction between A21 and B26, leading in all cases to inactivation of insulin by 20 to 30%. The inactivation of insulin on acetylation of its carboxyl groups by Fraenkel-Conrat and Fraenkel-Conrat (1950) argues also in favor of the essentiality of one or more carboxyl groups in insulin. Specific tyrosyl-aspartate interactions have been shown to be present in ribonuclease, by more direct experimental evidence (Broomfield et al., 1965; Riehm and Scheraga, 1966). Aoyama et al. (1965) indicate that the B22 arginine residue is involved in an interaction with tyrosyl B26, while the evidence presented above seems to favor the A21-B26 interaction. Furthermore, according to Guzzo (1965), the charged guanidyl group of arginine is outside the hydrophobic environment of α-helices, a fact that contradicts the arginine B22-tyrosine B26 interaction proposed by Aoyama et al. (1965).

As to the residue to which the "bound" tyrosyl residue A14 interacts, Aoyama et al. (1965) propose an interaction between A14 tyrosyl and B13 glutamyl residue, on the basis of their proximity.

It is of importance that on breaking each of the tyrosyl-carboxylate interactions proposed above, with hydroxylamine there appears a measurable increase of absorptivity shown by the $n_{control}$ values of native insulin, the tryptic or chymotryptic core. One could assume that hydroxylamine breaks both tyrosyl-carboxylate interactions of native insulin, exposing both tyrosyl groups involved

which results in an increase of absorptivity measured by $n_{control}$ = 1.3. On breaking one of the two specific interactions, however, by enzymatic digestion, followed by isolation and hydroxylamine treatment of the tryptic or chymotryptic core, each core gives an $n_{control}$ value of only 0.8, due to the exposure of one tyrosyl group, instead of two tyrosyl groups in the case of native insulin.

Evaluation of the Acetylation Method Used in This Investigation as a General Method of Studying Protein Structure and Function

Riordan et al. (1965) showed that the number of free tyrosyl residues measured by spectrophotometric titration (Crammer and Neuberger, 1943) correlates well with the number of acetylimidazole-reactive tyrosyl residues in native proteins. In the case of spectrophotometric titration the results as to the availability of tyrosyl residues obtained seem more quantitative because a pK value can be assigned to each tyrosyl residue. The results presented in this investigation, however, suggest that the use of acetylimidazole with careful acetylation kinetic studies under standard conditions, might prove a more sensitive method than spectrophotometric titration, for evaluating the degree of availability of tyrosyl groups in proteins, especially if the slope of the semilogarithmic acetylation graph (see Figures 5 and 6, and Table 2) is assigned to each group of tyrosyl residues being acetylated at similar rates. Thus in the case

of zinc insulin, the acetylation kinetics (see Figure 3) provide more detailed information about the availability of tyrosyl groups than the results by the spectrophotometric titration method of Crammer and Neuberger (1943), who assigned the pK value of 11.0 to all four tyrosyl residues of zinc insulin. Using the improved spectrophotometric method of Inada (1961), the pK value of 11.4 was given to one "bound" tyrosyl residue of zinc insulin, while the other three were characterized as "free" with pK 10.4. In this investigation, the acetylation course of zinc insulin gives an even more detailed picture of the states of the four tyrosyl residues, distinguishing the two reactive or normal, a third which reacts slower than the two reactive ones, and a fourth that reacts even slower (after addition of more acetylimidazole reagent).

The big advantage of the acetylation method is the simple, rapid, mild, and relatively specific reaction, by which the acetylation of only the free tyrosyl groups and the free amino groups of a protein can be achieved. Further degradation studies of this acetylated protein can provide a means of pinpointing the free tyrosyl residues in each protein. No spectrophotometric titration method can possibly achieve this.

Furthermore, because of the fact that the introduced acetyl groups are "physiological" (Hofmann, 1963), the acetylation method is a powerful tool for structure-and-function work with proteins.

The methods used on insulin in this investigation could be easily extended to other proteins in order to determine the number, location, and function of their free tyrosyl groups. Moreover, because the free tyrosyl groups lie on the surface of the protein molecules, the above methods could give an insight on the tertiary structure of proteins.

Acetylation of proteins in the presence of 8 M urea results in complete acetylation of all tyrosyl residues. This was shown in the case of zinc insulin in this investigation and for other proteins by Riordan et al. (1965). The possibility exists that a completely acetylated protein could be renaturated, as shown by O. R. D. measurements, and then assayed for activity. If active, it would mean that none of its tyrosyl hydroxyl groups are in the active site, or are important for maintaining the native conformation of the protein. On the other hand, if the renatured molecule is inactive, or if the completely acetylated protein cannot be refolded to its native conformation, one or more of the tyrosyl groups acetylated must be of importance for the right conformation and activity of the protein. By these means one would be able to study the significance of the free, or the bound tyrosyl groups in each protein from the structure-and-function point of view.

Studying the kinetics of the acetylation of a protein, or the deacetylation of the acetylated protein, in the presence or absence of

necessary ions and cofactors, or inhibitors, one could obtain even more selective acetylation of tyrosyl groups and acquire more detailed information for the relation between structure and activity in proteins, and the role of the tyrosyl hydroxyl groups in it.

There is also a possibility of acetylating a protein only to its buried tyrosyl residues, if after complete acetylation in the presence of 8 M urea, and renaturation, one could stop the deacetylation reaction (with hydroxylamine) at the right time so that only the reactive tyrosyl groups would be deacetylated. Such a method would be also useful in detecting the counterparts of the specific tyrosyl-carboxylate interactions (Tanford et al., 1955; Hermans and Scheraga, 1961; Riehm and Scheraga, 1966).

SUMMARY AND CONCLUSIONS

Amorphous and crystalline zinc insulin were acetylated with acetylimidazole under a set of different conditions, and the number of tyrosyl and amino groups acetylated was determined as a function of the reaction time. Amorphous insulin, or zinc insulin in 8 M urea were acetylated at all four tyrosyl residues at a similar rate, while zinc insulin in the absence of urea showed a two-step acetylation. The initial fast acetylation step involved two tyrosyl residues, and the remaining two were acetylated about ten times slower than the first two groups.

Insulin acetylated at all three of its amino groups and at both (Ac_2-insulin) or all four of its tyrosyl groups (Ac_4-insulin) were isolated. These preparations as well as native insulin, with or without hydroxylamine treatment were studied for biological activity. Ac_2-insulin was found to be completely active, Ac_4-insulin was only 30% active, while treatment of native insulin, Ac_2- and Ac_4-insulin with hydroxylamine resulted in a 30% active preparation.

The location of the two reactive O-acetyl-tyrosyl groups which are not essential for activity in Ac_2-insulin were determined by oxidative splitting of Ac_2-insulin and isolation of the A and B chains, as well as digestion of Ac_2-insulin by typsin, and

chymotrypsin, followed by determination of the number of O-acetyl-tyrosyl groups in each chain and the tryptic, and chymotryptic core of Ac_2-insulin. It was concluded that the two reactive tyrosyl residues of zinc insulin are those at positions A19 and B16, while the unreactive residues are those at positions A14 and B26. One or both of the latter are important for complete activity of insulin. The acetylation of all three amino groups of insulin did not impair its biological activity.

The kinetic studies of deacetylation with hydroxylamine showed that all preparations of acetylated insulin showed two distinct steps. The initial fast deacetylation step was essentially identical to the deacetylation of N, O-diacetyl tyrosine, while the terminal rate was about 20 times slower, and of similar rate as the "deacetylation" of native amorphous insulin. Both N, O-diacetyl tyrosine and native amorphous insulin on treatment with hydroxylamine resulted in straight line deacetylation kinetics.

The absorptivity increase of native insulin, and its tryptic and chymotryptic cores, on treatment with hydroxylamine is significant and was not observed when hydroxylamine was replaced by NaCl or KCl solutions. Assays for possible O-tyrosyl ester bond(s) in native insulin were essentially negative.

The specific tyrosyl-carboxylate interaction proposed by Tanford et al. (1955), Hermans and Scheraga (1961) and Riehm and

Scheraga (1966), was tentatively proposed in order to provide an explanation for the absorptivity increase of native insulin and its cores, as well as for the deactivation of native and acetylated insulin, on treatment with hydroxylamine. Complete acetylation of insulin, and treatment with hydroxylamine resulted in the same extent of inactivation, so that it was assumed that both treatments break the specific tyrosyl-carboxylate interaction of one or both of the non-reactive tyrosyl residues A14 and B26, in zinc insulin. The fact that the absorptivity increase on hydroxylamine treatment of native amorphous insulin is about twice as much as that of the tryptic or chymotryptic core would be in favor of both non-reactive tyrosyl residues being bound in tyrosyl-carboxylate interactions in the zinc insulin molecule. Both non-reactive tyrosyl residues were found in amino-acid sequences of the insulin molecule, which according to Guzzo (1965), could be characterized as hydrophobic regions.

As to the general usefulness of the acetylation of other proteins with acetylimidazole, when followed kinetically according to the methods used in this investigation, it is concluded that these methods can supplement the spectrophotometric titration method for detection of the "free" and "bound" tyrosyl residues in proteins. Moreover, the acetylation methods are superior in finding the location of "free" and "bound" tyrosyl residues, and for structure and function work on proteins.

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