It might also be possible to turn the method around, and use it to isolate a pure protein Ag.[18] Assuming that there is available a γ-globulin fraction containing Ab reactive to the desired Ag, one might thiolate it, use the thiolated Ab to precipitate the Ag free of contaminating proteins, and then remove the thiolated Ab from the Ag by MMD precipitation at acid *p*H.

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We gratefully acknowledge the interest and support of Professor J. M. Sturtevant, from whose National Science Foundation grant G-2855, the postdoctoral research assistantship held by one of us (J.E.F.) was provided. This research was also supported in part by U. S. Public Health Service grants E-1204 (C2,3) to Professor S. J. Singer.

NEW HAVEN, CONN.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY]

Tyrosyl Hydrogen Bonds in Insulin[1,2]

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RECEIVED JUNE 8, 1959

The ultraviolet absorption spectra of zinc- and zinc-free insulins at *p*H 8 are modified either by acidification or by tryptic digestion. The character of the difference spectra produced by either treatment is very similar and appears to be due to the breaking of tyrosyl hydrogen bonds. Since the acid-induced shift still persists after tryptic digestion and is of undiminished magnitude, it is concluded that there are two tyrosyl residues which are hydrogen bonded as donors to two acceptor groups. The *p*H-dependence of the acid-induced shift suggests that one of the acceptors is a carboxylate ion side chain. In a 6000 mol. wt. insulin model, involving α-helices, the only tyrosyl-carboxylate ion hydrogen bond which can be formed is one between the B13 glutamic acid residue and the B16 tyrosyl residue. For the spectral shift induced by tryptic digestion, the B26 tyrosyl residue is implicated. It has been shown that trypsin splits native insulin at the same two bonds as in isolated B-chain, *viz.*, at the B22-B23 and the B29-B30 peptide bonds. The former splitting releases one of the four tyrosyl residues in insulin as a heptapeptide fragment. The rate of splitting follows the rate of spectral change during tryptic digestion. Removal of the terminal B-30 alanyl residue by carboxypeptidase digestion has no effect on the spectrum. It is concluded that the B26 tyrosyl residue is hydrogen bonded to an acceptor which does not ionize the *p*H range 1.5 to 8.0. The validity of Beer's Law and the effect of dioxane and added salt on the difference spectra have been examined. It is not yet possible to decide whether the two tyrosyl hydrogen bonds are within the 6000 or 12,000 mol. wt. units of insulin.

Introduction

When the amino acid sequence and the positions of the disulfide cross links in a protein are known, it should be possible to determine the folding of the molecule in solution by locating a few specific interactions (*e.g.*, hydrogen or hydrophobic bonds) between side chains. The present work is an attempt to apply this approach to the determination of the configuration of insulin[4] in solution by searching for possible side chain hydrogen bonds involving tyrosyl residues as donors.

Titration data[5] on zinc- and zinc-free insulins have been interpreted to indicate that the tyrosyl groups ionize normally. However, it should be kept in mind that titration curves give only average *pK* values over several groups and, in the case of insulin, it has not thus far been possible to distinguish between the four tyrosyl groups and the single ϵ-amino group[6] from titration data.[5] Furthermore, the detailed interpretation of insulin titration data is considerably complicated by *p*H-dependent changes in the degree of aggregation. Since titration curves provide no evidence for tyrosyl hydrogen bonds in insulin, the more sensitive and group-specific technique of difference spectrophotometry was employed in this investigation.

Difference spectrophotometry has been used to determine the *pK* values of ionizable groups in compounds of tyrosine and tryptophan.[7-10] It has also been used to determine the *pK* values of the acceptor groups involved with tyrosyl groups in hydrogen bonds in insulin[11] and in ribonuclease.[12,13] A number of workers have also observed the difference spectra arising from shifts of the tyrosyl absorption band in proteins, without investigating the nature of the groups responsible for the perturbation.[14-21] In particular it has not always

(1) This investigation was supported by research grant No. E-1473 from the National Institute of Allergy and Infectious Diseases, of the National Institutes of Health, U. S. Public Health Service.

(2) Presented in part before the Division of Biological Chemistry at the 131st meeting of the American Chemical Society, Miami, Florida, April, 1957.

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(14) D. Shugar, *Biochem. J.*, **52**, 142 (1952).

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(16) M. Sela and C. B. Anfinsen, *Biochim. Biophys. Acta*, **24**, 229 (1957).

(17) C. H. Chervenka, *ibid.*, **26**, 222 (1957); **31**, 85 (1959).

(18) A. N. Glazer, H. A. McKenzie and R. G. Wake, *Nature*, **180**, 1286 (1957).

(19) H. Fraenkel-Conrat, *Federation Proc.*, **16**, 810 (1957).

been clear to what extent vicinal and other similar effects could be excluded. The distinctions between these possibilities will be examined later.

The absorption spectrum of tyrosine in the 270–290 mμ region undergoes an enhancement in intensity and a shift toward longer wave lengths if the tyrosyl group becomes hydrogen bonded as a donor. A difference spectrum between the hydrogen-bonded and the non-hydrogen-bonded forms shows two major peaks[11,12] in the vicinity of 280 and 287 mμ. The tyrosyl hydrogen bonds in a protein can be broken in several different ways, *e.g.*, by acidification so that the acceptor group gains a proton, by treatment with urea or by enzymatic hydrolysis of peptide bonds leading to the liberation of one or more peptide fragments containing either the acceptor group or the tyrosyl donor. Variation of the *p*H, in the acidification method, yields a titration curve for the acceptor group in which the *p*H is correlated with the extent of hydrogen bond breakage. In this way the *pK* and identity of the acceptor group may be determined.[11,12,22]

It is important to note that in the technique of difference spectrophotometry the rupture of tyrosyl hydrogen bonds is detected under conditions (*p*H 2–8) where the tyrosyl hydroxyl group does not ionize. This is in contrast to the method of Crammer and Neuberger[23] in which the protein is treated with alkali to rupture the hydrogen bond, this occurring in the *p*H region of tyrosyl ionization.

In the present work the difference spectra produced by acidification and by enzymatic hydrolysis of insulin have been investigated in more detail. In the latter case data have been obtained for the rate of appearance of the difference spectrum, and this has been correlated with the rate of tryptic hydrolysis of the B22-B23 arg–gly bond of the phenylalanyl-(B) chain. Digestion of insulin with carboxypeptidase indicates that the spectral changes are *not* produced by hydrolysis of the B29-B30 lys–ala bond.

As regards the spectral shift produced by acidification, evidence on the nature and location of the acceptor groups has been adduced by studying the spectral shifts at varying *p*H, ionic strength and concentration. In addition, by studying the *p*H-dependence of the spectral changes both before and after tryptic digestion, it has been possible to show that there is more than one tyrosyl residue involved in hydrogen bonding. Attempts have been made to interpret the data in terms of current hypotheses about the configuration of insulin in solution.

In view of its importance to the discussion of the data obtained here, the amino acid sequence deduced by Sanger and co-workers[4] is reproduced in Fig. 1, where the tyrosyl residues and free COOH groups are given special prominence.

(20) E. J. Williams and J. F. Foster, THIS JOURNAL, **81**, 865 (1959).

(21) O. O. Blumenfeld and G. E. Perlmann, *J. Gen. Physiol.*, **42**, 563 (1959).

(22) It should, however, be pointed out that more complex interpretations of this *p*H dependence are also possible. Ionization of a group in a protein may cause some coöperative structural changes leading to breakage of the hydrogen bond even though the ionizing group was not the acceptor.[20] While such a possibility should be borne in mind, it is not possible at present to prove it or rule it out. As will be emphasized in the Discussion section, tyrosyl hydrogen bonding will be assumed to be the origin of the spectral changes reported here.

(23) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

Experimental

Materials.—Crystalline zinc insulin was obtained from the Eli Lilly Co. (Batch No. 535,664). Zinc-free insulin was prepared, according to the directions of Dr. O. K. Behrens, by dissolving 1 g. of zinc insulin in 60 ml. of 0.025 *N* HCl and dialyzing at 0° for 5 days in a rocking dialyzer with frequent changes of 0.025 *N* HCl. The zinc-free insulin was recovered by lyophilization and found to contain less than 0.04% zinc compared to 0.57% (1 atom of zinc per 12,000 mol. wt. dimer) for the dry crystalline starting material.

The trypsin used was a Worthington twice-crystallized, salt-free product (Batch No. TR450SF). The soybean trypsin inhibitor (STI) and carboxypeptidase were also Worthington preparations (Batch Nos. SI5308 and CO557-DFP, the latter being DFP treated). The synthetic substrate *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride (TAMe) was obtained from the H. M. Chemical Co. Ltd., of Santa Monica, California.

Ammonium acetate buffers and other salt solutions were prepared from analytical grade reagents. Ionic strengths were maintained at 0.10 except where otherwise stated. The standard KOH solutions were CO_2-free[24] and HCl solutions were diluted as required from glass-distilled, constant-boiling HCl. De-ionized water was used throughout.

Dioxane was purified by refluxing over solid NaOH for 12 hours, followed by fractionation over fresh solid NaOH.

Insulin Methyl Ester.—Methylated insulin was prepared by Dr. M. L. McFadden according to the procedure of Mommaerts and Neurath.[25] Zinc insulin was suspended in 0.1 *N* HCl in anhydrous methanol (100 mg. per 10 ml.) at 25° for 24 hr. with occasional shaking. Complete solution was not effected in this time. Methanol was removed by dialysis against 0.025 *N* HCl. The lyophilized insulin methyl ester hydrochloride was soluble to the extent of at least 0.1% in 0.075 *M* KCl below *p*H 5.3.

Zinc Analysis of Insulin.—Zinc analyses were carried out by Dr. L. Gruen using the Versene titration method developed by Flaschka.[26] The sample was adjusted to *p*H 8 to 10 with an ammonia–ammonium chloride buffer and titrated with a standard solution of 0.01 *M* Versene using a 0.1-ml. ultramicroburet and Eriochrome Black T indicator. The Versene was standardized against a standard solution of Zn^{++} prepared by dissolving the weighed metal in a small amount of triply glass-distilled HCl and diluting to volume with zinc-free water. The presence of insulin did not interfere with the zinc titration, as was verified by adding known amounts of zinc to solutions of zinc-free insulin and observing that the amounts of zinc found in ashed and unashed samples were the same within the experimental error.

Spectrophotometry.—Spectrophotometric measurements were made manually with the Beckman model DU spectrophotometer using a photomultiplier attachment at full sensitivity. The concentration of the insulin solutions varied from 0.1 to 0.5%, and matched silica cells of 0.5 and 1 cm. light path, respectively, were used. Many of the results, however, have been converted to a standard basis of 0.5% insulin for a 1 cm. cell. Difference spectra were obtained by using reference solutions of identical protein concentration to the solution under examination.[27] As will be discussed below, a difference spectrum arises from changes in the environment of the tyrosyl residues, produced either by *p*H changes or as a result of enzymatic digestion. In either case, the optical density *differences* (ΔD) are relatively small, arising as they do from shifts of only 1–2 mμ in the absorption spectra. It is therefore necessary to use comparatively concentrated insulin solutions of up to 0.5% which, even in 0.5 cm. cells, would have very high optical

(24) I. M. Kolthoff, *Z. anal. Chem.*, **61**, 48 (1922).

(25) W. F. H. M. Mommaerts and H. Neurath, *J. Biol. Chem.*, **185**, 909 (1950).

(26) H. Flaschka, *Mikrochemie ver. Mikrochim. Acta*, **39**, 38 (1952); *Chemist Analyst*, **42**, 84 (1953).

(27) A similar use of the method of difference spectra for the accurate determination of copper was made by R. Bastian, *Anal. Chem.*, **21**, 972 (1949).

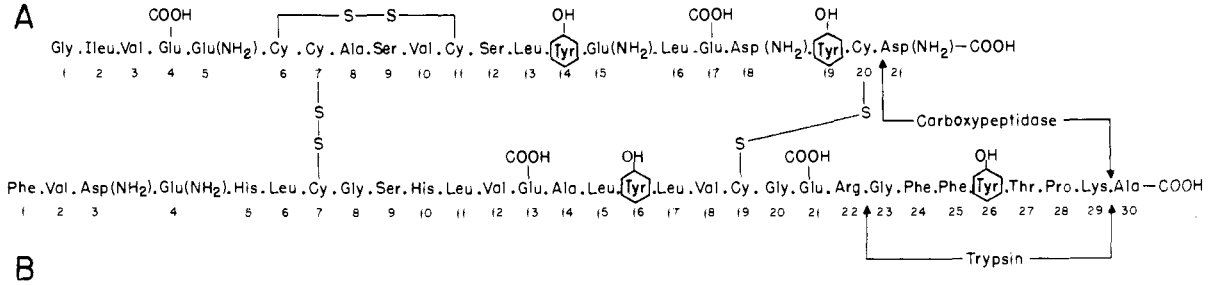

Fig. 1.—The amino acid sequence for beef insulin.[4] The tyrosyl- and carboxyl- side chains are given special prominence, and the points of trypsin and carboxypeptidase attack are shown.

densities of up to 2.5 at 276 mμ. It should be stressed that under these conditions one cannot obtain precise Δ D values (which are in the range 0 to 0.3) by measuring the absorption spectra of the two insulin solutions separately and then subtracting one from the other. The difference spectra must be measured directly as described above. Even then, good precision could not be obtained if the optical density at 276 mμ exceeded that of a 0.5% solution of insulin in a 0.5 cm. cell. With protein solutions of high optical density, and in the regions of low wave length, it is important to minimize errors due to stray radiation and fluorescence of the cells and the protein solution.[28-31] Artifacts due to these sources are minimized by working with narrow slit openings and confining measurements to the wave length range above 240 mμ. Readings used in interpreting difference spectra were all made at about 286 mμ and Beer's Law was obeyed (see later). Whenever applicable, a correction for the difference in optical density between two solvents was made.

pH Measurements.—pH measurements were made with a Beckman model G pH meter using external electrodes. In some cases the glass and calomel electrode assembly was sufficiently small to fit into a 1 cm. silica cell, so that spectral changes could be followed more conveniently as the pH was varied. The pH meter and electrodes were standardized at pH 4 at room temperature and checked at pH 7 and 10 with Beckman standard buffers. For the small electrodes a linear correction calibration curve was obtained and used. For pH measurements in dioxane it was assumed that 0.05 M potassium acid phthalate in 40% dioxane has a pH of 5.50.[32] Although the glass electrode was left for long periods in buffers containing 40% dioxane,[32] it was found that equilibration was rapid for the electrode used.

Trypsin Assay.—Trypsin activity was assayed by following the rate of hydrolysis of 0.02 M TAMe at 25° by the enzyme (1 to 5 × 10^{-5}%), maintaining the pH at 8.0 by addition of 0.05 M KOH.

Tryptic Digestion of Insulin; Rate of Peptide Bond Hydrolysis.—Sanger and Tuppy[33] found that trypsin attacked the B22-B23 arg-gly and the B29-B30 lys-ala bonds of the isolated B-chain of insulin, liberating the B30 alanine residue and the tyrosine-containing heptapeptide B23 to B29 (see Fig. 1). It was necessary to establish first of all whether intact zinc insulin is similarly susceptible to trypsin attack[34] and then whether the appearance of the difference spectrum is due to hydrolysis of the B22-B23 arg-gly bond or to hydrolysis of the B29-B30 lys-ala bond. For this purpose the rates of hydrolysis of the arg-gly and lys-ala bonds were compared with the rate of appearance of the difference spectrum.

A stock solution of zinc insulin was prepared by suspending 100 mg. of the crystalline material in 4 ml. of acetic acid (0.2 N) and adding 6 ml. of ammonium hydroxide (0.2 N) with stirring to bring the pH to 8.8. Addition of 1.1 ml. of acetic acid (0.2 N) and 7 ml. of ammonium acetate buffer (pH 8, ionic strength 0.1) gave a solution of insulin of 0.55% concentration at pH 8.0.

The stock solution of trypsin was 0.2% enzyme dissolved in HCl (0.0025 N) containing $CaCl_2$ (0.01 M). Dilutions were prepared from this stock solution. Stock solutions of soybean trypsin inhibitor (0.4%) were prepared in a similar solvent.

In a typical digestion at 23°, insulin (0.5%) and trypsin (0.01%) were incubated together at pH 8. At intervals, 1-ml. aliquots were removed and added, at room temperature, to 1 ml. of 10% trichloroacetic acid (TCA). After leaving for 20 minutes to ensure complete precipitation of undigested insulin, digested core material and trypsin, the precipitate was centrifuged off. The supernatant solution together with washings of the precipitate (using 5% TCA) were made up to 3 ml.

By measuring the optical density at 276 mμ against a suitable blank solution, it was possible to determine the concentration of the tyrosine-containing heptapeptide. A blank value at zero time was obtained by precipitating intact insulin under the same conditions, and a final value was obtained by allowing the digestion to proceed for 48 hours, when values of D_{276} became constant. Replicate experiments gave good agreement both for the rates and final values.

Attempts were made to measure the rate of tryptic liberation of the C-terminal alanine residue by spot dilution paper chromatography using aliquots of the same digests as used in the rate measurements described above. Before chromatography in butanol–acetic acid–water, ammonium acetate buffer was removed from each 0.1-ml. aliquot by sublimation. These experiments were only semi-quantitative but indicated unexpectedly that the rates of tryptic hydrolysis of the arg–gly and lys–ala bonds were of the same order, with the latter possibly slower. Since no octapeptide was detected in the experiments of Dr. M. L. McFadden, described below, it appears likely that the C-terminal alanine residue is hydrolyzed off much more rapidly from the octapeptide fragment than from the intact insulin molecule.

Since the rate of hydrolysis of the lys–ala bond could not be accurately determined, the relation of this bond cleavage to the occurrence of the spectral shift had to be investigated by digesting with carboxypeptidase (see below).

Preparation and Analysis of the B23-B29 Heptapeptide.—For use in the interpretation of experiments involving tryptic hydrolysis of insulin, the tyrosine-containing heptapeptide B23-B29 (see Fig. 1) was prepared and analyzed by Dr. M. L. McFadden using the following procedure. Approximately 250 mg. of zinc insulin and 8 mg. of trypsin were incubated together at pH 8.0 in 50 ml. of 0.1 N ammonium acetate buffer for 20 hr. at 25°. The protein core[40] was removed by precipitation at pH 5.4 and the supernatant

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(31) A. H. Mehler, B. Bloom, M. E. Ahrendt and D. W. Stetten, Jr., *ibid.*, **126**, 1285 (1957).

(32) E. Fredericq, *J. Polymer Sci.*, **12**, 287 (1954).

(33) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).

(34) There is reason to believe that samples of zinc insulin differ in their susceptibility to trypsin digestion, particularly at the B22-B23 arg-gly bond. While Harris and Li[35] and Nicol and Smith[36] have reported positive results, other workers[37,38] have observed no hydrolysis at this bond. In the present study we have also confirmed observations[36,39] that the removal of zinc speeds up the rate of attack.

(35) J. I. Harris and C. H. Li, THIS JOURNAL, **74**, 2945 (1952).

(36) D. S. H. W. Nicol and L. F. Smith, *Biochem. J.*, **64**, 17P (1956).

(37) J. A. V. Butler, E. C. Dodds, D. M. P. Phillips and J. M. L. Stephen, *ibid.*, **44**, 224 (1949).

(38) H. Lindley, private communication.

(39) F. H. Carpenter and J. D. Young, *Federation Proc.*, **18**, 201 (1959).

(40) Recently Carpenter and Young[39] have described the isolation of this protein core and have named it "Desoctapeptide-insulin."

solution lyophilized *in vacuo*. The ammonium acetate buffer was removed during lyophilization.

A portion of the supernatant was chromatographed on Whatman No. 1 paper with butanol–acetic acid–water.[41] When the chromatogram was developed by spraying with 0.1% ninhydrin in 95% ethanol containing 4 volumes of collidine and 30 volumes of glacial acetic acid, the products of digestion appeared to be three in number, *viz.*, alanine, the heptapeptide[35] and a small slow moving spot not previously reported.[35,42] Control experiments showed that the latter spot was not due to trypsin self-digestion or insulin breakdown in the absence of enzyme. It was not zinc insulin, zinc-free insulin, core or trypsin but was undoubtedly a product of hydrolysis of insulin by Worthington trypsin. When washed off the paper and hydrolyzed, it yielded aspartic and glutamic acids, arginine, glycine, alanine and traces of serine and lysine. The heptapeptide spot, when eluted and hydrolyzed, showed the expected amino acids with small amounts of aspartic and glutamic acid impurities. The remainder of the supernatant digest was lyophilized, dissolved in a few ml. of the organic phase of a butanol–acetic acid–water mixture (4:1:5 by volume), transferred to the top of a cellulose column (prepared as described by Blackburn[43] and washed onto the column with several 5-ml. portions of solvent. Using a Technicon fraction collector, 160 drop fractions (4 ml.) were collected at a rate of 800 drops per hour.

The tube contents were examined for their ultraviolet absorption at 276 mμ using a Beckman DU spectrophotometer; three ultraviolet absorbing peaks were obtained. One-ml. aliquots of the effluent fractions were also analyzed with ninhydrin. Four ninhydrin-positive peaks were obtained, three corresponding to the three ultraviolet absorbing peaks plus one large peak which emerged later. Paper chromatography with butanol–acetic acid–water indicated that one peak contained only alanine, two peaks contained mixtures of amino acids (possibly peptides) and one peak contained the heptapeptide. Hydrolysis and paper chromatography of the latter peak material yielded the expected composition for the heptapeptide B23-B29 together with traces of glutamic and aspartic acids. Similar anomalies were reported by Feitelson and Partridge[44] who digested the isolated B-chain of insulin with trypsin.

Since the unidentified fragments occur in small amounts, we shall assume here that trypsin digestion of insulin (both zinc and zinc-free) leads to hydrolysis predominantly of the B22-B23 arg–gly and the B29-B30 lys–ala bonds, in conformity with the results of Sanger and Tuppy[33] on the isolated B-chain, and of Harris and Li[35] on intact insulin.

Carboxypeptidase Digestion of Insulin.—Although the rates of appearance of the difference spectra and of tryptic hydrolysis of the B22-B23 argy–gly bond were determined and could therefore be correlated with one another, accurate data could not be obtained for the tryptic hydrolysis of the B29-B30 lys–ala bond. Carboxypeptidase was therefore used to attack the B29-B30 lys–ala bond to see whether its hydrolysis gave rise to the difference spectrum.

Carboxypeptidase was dissolved by treating 0.1 ml. of a suspension (20 mg./ml.) of the enzyme with 10 ml. of 0.1 *M* NaCl (0.05 *M* in tris buffer) at *p*H 9.5 and stirring at 5° for 2.5 hr. The solution was brought to room temperature and the *p*H to 8.0 by carefully adding about 2.5 ml. of 0.1 *M* HCl.

A stock solution of 0.8% insulin at *p*H 8.0 was prepared in 0.05 *M* tris buffer containing 0.1 *M* NaCl. Four ml. of insulin solution was added to 4 ml. of carboxypeptidase solution. At intervals 2-ml. aliquots were removed and heated at 100° for 3 minutes to stop the hydrolysis. The solutions were then analyzed for alanine (as DNP-alanine) as follows. 3.5 ml. of 5% FDNB in ethanol was added and the solution shaken for 2 hr. at room temperature. Two ml. of water was added and the solution extracted with three 5-ml. portions of peroxide-free ether to remove excess FDNB. The water layer was acidified to *p*H 1 and again extracted with ether until no more color was removed. The second set of ether extractions was combined and evaporated to dryness first at atmospheric pressure and then at reduced pressure using a cold finger, heating at 70° to remove dinitrophenol. The residue was dissolved in acetone and transferred quantitatively to Whatman No. 1 filter paper. An ascending paper chromatogram was run in the toluene–chloroethanol–pyridine–ammonia solvent of Biserte and Osteux[45] for 16 hr. Two spots of approximately equal intensity, and corrsponding to DNP-alanine and DNP-asparagine, were observed. The DNP-alanine spots were cut out and eluted with 4 ml. of 1% $NaHCO_3$. The eluate was diluted 10 times with 1% $NaHCO_3$ and its optical density measured at 360 mμ in a 1 cm. cell, reading against a 1% $NaHCO_3$ extract of a blank portion of the chromatogram diluted in the same way. The optical density reading was converted to alanine concentration with the aid of a calibration curve prepared with known amounts of DNP-alanine.

Spectral Change Due to Tryptic Digestion.—In a typical digestion at *p*H 8, insulin (0.5%) and trypsin (0.01%) were placed in a 0.5 cm. silica cell and the top sealed with parafilm and a glass cap. This solution was used as the spectrophotometric blank for obtaining the zero optical density setting. A solution of insulin of identical concentration but containing no trypsin was read against this blank. As the digestion proceeded at room temperature, the change in optical density was measured at 286 mμ. The reason for choosing this wave length will be apparent from the shape of the difference spectrum (see later). The difference in optical density, ΔD_{286}, was measured over a period of about 5 hours. The value of ΔD_{286} at zero time, being the contribution of the trypsin to the difference in optical density between the two solutions, was obtained by extrapolation to zero time and subtracted from all subsequent readings.

A duplicate tryptic digest of zinc insulin, together with an undigested insulin solution prepared as described above, were sealed up and incubated at 25° for 48 hr. The *p*H change at the end of digestion was usually less than 0.04. A mixture of trypsin and soybean trypsin inhibitor was then added to the undigested solution, and inhibitor alone was added to the digest. By diluting from the same stock solutions and using the same micropipets, it was possible to make negligible any errors due to mismatch. The difference spectrum due to complete trypsin digestion was then measured between 240 and 310 mμ.

***p*H Dependence of the Spectral Change.**—To minimize errors due to concentration mismatch of insulin, it was necessary to produce a matched pair of insulin solutions of low and high *p*H and to produce solutions of intermediate *p*H by titrating one with the other. Thus, to provide the data of Fig. 6 a solution of zinc insulin (1%, ionic strength 0.1) was diluted with solutions of KCl, KOH–KCl or HCl–KCl (all of ionic strength 0.1) to give solutions of 0.1, 0.25, 0.4 or 0.5% insulin concentration. At each concentration this provided a pair of matched insulin solutions, one at *p*H 1.7 to 1.8 and the other at *p*H 8 to 9. Setting the acidic solutions to zero optical density, difference spectra were plotted for solutions varying from *p*H 2 to *p*H 8 at each concentration. For the *p*H range 2 to 4, the acidic solution was titrated with the basic one, while the *p*H range 7 to 8 was covered by titrating the basic solution with the acidic one. Readings in the *p*H range 4 to 7 could not be obtained because of the insolubility of insulin. Using this procedure it was possible to test the reversibility of the data by forward and backward titration with the same two solutions. In some cases, data were checked by titrating solutions right through the insolubility region.

The difference spectra for zinc-free insulin were obtained in a similar manner. In this case, the insulin hydrochloride could be dissolved directly in ammonium acetate buffer at *p*H 8 (or titrated to *p*H 8 in the absence of buffer ions) and adjusted to *p*H 8 with standard alkali. Dilutions were then made as above, and difference spectra arising from the *p*H change obtained in a similar manner.

In reporting difference spectra arising from *p*H changes, the reference solution was the insulin solution of *p*H 1.6 to 1.8. The optical densities of the less acid solutions were then read relative to this standard. Over most of the 250 to 310 mμ wave length range, these differences were positive.

(41) A. L. Levy and D. Chung, *Anal. Chem.*, **25**, 396 (1953).

(42) Two-dimensional chromatograms were also obtained using the solvent systems described by Harris and Li,[35] *i.e.*, butanol–acetic acid–water in the first direction and phenol–*m*-cresol–*p*H 8.5 borate buffer in the second direction.

(43) S. Blackburn, *Chemistry and Industry*, 294 (1951).

(44) J. Feitelson and S. M. Partridge, *Biochem. J.*, **64**, 607 (1956).

(45) G. Biserte and R. Osteux, *Bull. soc. chim. biol.*, **33**, 50 (1951).

Results

1. Effect of Tryptic Digestion. (a) Rate of Spectral Change.—The data for the typical digestion cited in the experimental section entitled "Rate of peptide bond hydrolysis" were obtained using the spectrophotometric procedure described in the Experimental section entitled "Spectral change due to tryptic digestion," and are shown in Fig. 2. In 273 minutes at *p*H 8, the difference

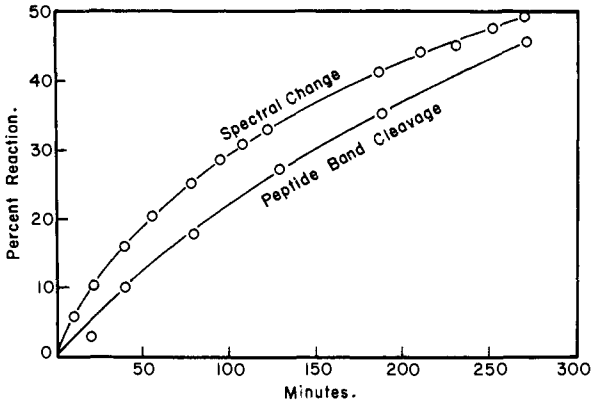

Fig. 2.—The rate of appearance of the difference spectrum and the rate of the B22-B23 arg-gly peptide bond hydrolysis in the digestion of 0.5% beef zinc-insulin at *p*H 8 by 0.01% trypsin at 23°.

in optical density at 286 mμ rose from zero to 0.108. After 48 hr. the readings became constant (see Table I) and the 48 hour final value (0.220 in this experiment) was used for calculating the extent of reaction.

TABLE I

TEST OF COMPLETION OF TRYPTIC HYDROLYSIS IN 48 HOURS AT 25°

(0.5% insulin; *p*H 8)

Trypsin concn., %	Time, hr.	ΔD_{288}
0.01	41.5	0.227
	52	.231
	65	.223
0.02	41.5	.248
	52	.250
	65	.244

(b) Difference Spectrum.—The results for the spectral change due to tryptic digestion are shown in Fig. 3. The data obtained with 0.5% insulin solutions using a 0.5 cm. cell have been recalculated as for a 1 cm. cell. The spectral change produced by tryptic digestion of *zinc-free* insulin was measured in a similar manner and is also shown in Fig. 3. Removal of zinc appears to raise the heights of the two main peaks, with no other alteration in the difference spectrum.

(c) Rate of Peptide Bond Hydrolysis.—In order to correlate the spectral changes on trypsin digestion with the hydrolysis of the B22-B23 arg-gly bond, the rate of hydrolysis of this bond was measured as described in the Experimental section entitled "Rate of peptide bond hydrolysis." The results are shown in Fig. 2 in which the same solution was used for both sets of measurements. The values of D_{276} of the TCA supernatant in-

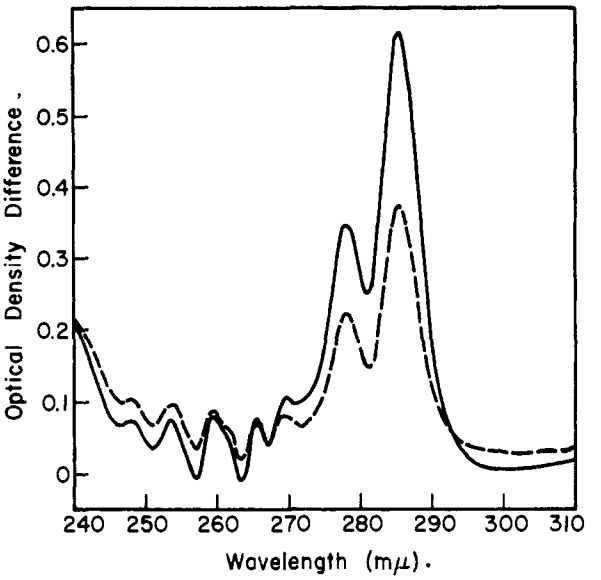

Fig. 3.—Difference spectra of 0.5% (8.72×10^{-4} *M*) beef zinc-insulin (dashed curve) and zinc-free insulin (solid curve) in 1 cm. cells, produced by tryptic digestion. The digest was used as the reference solution of zero optical density. The conditions of digestion are described in the text.

creased from zero to 0.0775 in 273 minutes. The 48 hour value, corresponding to 100% hydrolysis of the arg-gly bond, was 0.170. Correcting this for the 3-fold dilution before measuring, the value of 0.51 is exactly 25% of the D_{276} value for a solution of intact insulin in the same 0.5 cm. cells. This is in accord with the supposition that one of the four tyrosyl residues in the insulin molecule has been split out as a peptide fragment which is soluble in 5% TCA. From Fig. 2 it is apparent that the spectral change and the hydrolysis of the arg-gly bond occur at closely similar rates and are therefore probably related. However, neither phenomenon occurs as a first-order reaction, both reactions slowing down too rapidly.

As already indicated, accurate data for the rate of tryptic hydrolysis of the lys-ala bond could not be obtained and, in order to determine whether this bond had any bearing on the spectral changes, the effect of carboxypeptidase on the insulin spectrum was determined.

2. Effect of Carboxypeptidase Digestion.—The digestion was carried out as described in the Experimental section. As soon as the enzyme was added to the insulin the optical density difference between this solution and a blank containing 1 ml. of insulin and 1 ml. of *p*H 8 buffer was followed for 3 hr. at 285.5 mμ in 0.5 cm. cells. The optical density decreased by about 0.015 in 0.5 hr. and then remained constant. When the carboxypeptidase concentration was halved, the optical density fell by 0.014 in 1 hr. and then remained constant for 3 hours.[46] This change in optical

(46) When the carboxypeptidase action was continued for several hours longer, the optical density began to rise although there was no further yield of alanine. However, this rise could be due to hydrolysis of additional peptide bonds in the B-chain. Although it has been stated[47] that the action of carboxypeptidase is limited to liberation of the C-terminal alanine residue of insulin and its isolated chains, we

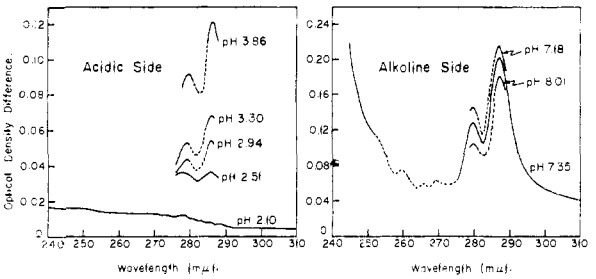

Fig. 4.—Difference spectra produced by varying the *p*H of solutions of native zinc insulin, relative to a solution of *p*H 1.7 set at zero optical density. The insulin concentration was 0.5% and the optical density values are for 0.5 cm. cells.

density is negligible compared to that produced by tryptic digestion (see Fig. 3). During this period the lys–ala bond had been hydrolyzed as shown by the data for DNP-alanine estimation in Table II.

TABLE II

RATE OF HYDROLYSIS OF THE LYS-ALA BOND IN INSULIN BY CARBOXYPEPTIDASE

(0.4% insulin; *p*H 8)

Time	% Alanine liberated
1 min.	10
1.25 hr.	22
3 hr.	22

The percentage of alanine liberated levels off after 1 hr. and then remains constant. We attribute the discrepancy between the final values of 22% and the expected value of 100% partly to losses of alanine during the DNP-ing and subsequent recovery for analysis. Even if the value of 22% were taken literally, it still would mean that the lys–ala bond in insulin can be split without producing the difference spectrum associated with tryptic hydrolysis. The small discrepancy between the two curves of Fig. 2 may be due to a charge effect perturbation upon hydrolysis of the lys–ala bond. It is therefore clear that the main spectral shift is due to the hydrolysis of the B22–B23 arg–gly bond with the liberation of the heptapeptide gly.phe.phe.tyr.thr.pro.lys.

3. Effect of *p*H Changes. (a) Difference Spectrum.—Figure 4 shows the difference spectra obtained at various *p*H values for insulin solutions of ionic strength 0.1 relative to a standard insulin solution of *p*H 1.6 and in the absence of buffer ions. The high ionic strength helped to prevent cloudiness at *p*H 7–8. Similar spectra were obtained in the presence of ammonium acetate buffer (0.1 *M*) over the whole *p*H range. The acetate affected neither the detailed features of the difference spectra nor the height of the main peak which was quite reproducible for a given insulin concentration and cell light-path. Solutions of insulin which had been digested with trypsin produced similar difference spectra when subjected to *p*H change. Figure 5 illustrates the closely similar difference spectra produced by tryptic digestion, by acidification of intact zinc insulin and by acidification of a tryptic digest of insulin. The heights of the major peaks (ΔD) differ slightly due to small differences in *p*H between the three cases.

have found that using Lilly zinc insulin and Worthington DFP-treated carboxypeptidase, prolonged digestion produces small but easily detectable quantities of the amino acids further along the B-chain and beyond the proline residue. This could be due to inadequate DFP-treatment of the enzyme.

(47) F. Sanger and E. O. P. Thompson, *Biochem. J.*, **53**, 366 (1953).

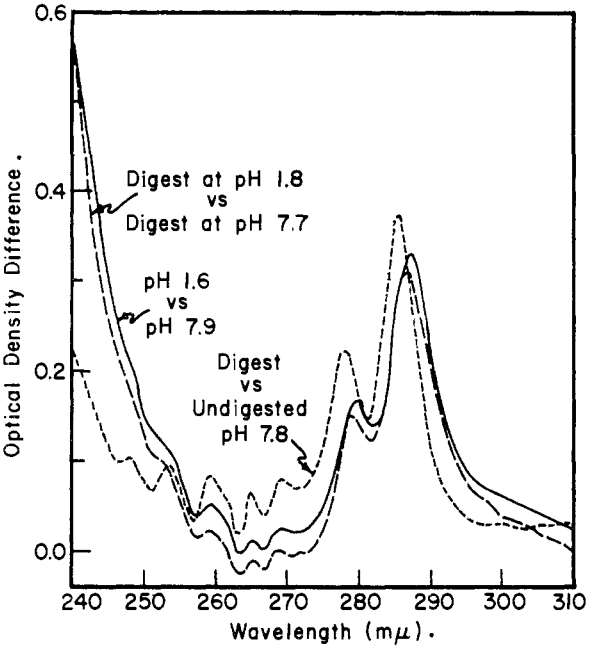

Fig. 5.—Comparison of the difference spectra obtained by tryptic digestion, by acidification of native zinc insulin and by acidification of the trypsin digest of zinc insulin. Optical density differences were measured in 0.5 cm. cells using approximately 0.5% solutions of insulin. In the figure, all readings are adjusted to 1 cm. cells for 0.5% insulin.

(b) *p*H Dependence of the Spectral Shift.—The character of the difference spectra obtained by *p*H change is not identical throughout the whole *p*H range. Below *p*H 2.5 the spectra are not so well-defined (see Fig. 4) and first appear at this *p*H with small double peaks of equal height. As the *p*H increases the peaks become much more pronounced and the peak at 286 mμ becomes the major one. On the basic side of the isoelectric point there is a small displacement (about 1 mμ) toward longer wave length for both peaks until the spectra of Fig. 4 are obtained. The increase in height of the major peak (ΔD_{286}) with increasing *p*H is shown in Fig. 6. The peaks are maximal at *p*H 7 and then decrease in height from *p*H 7 to 8. The *p*H dependence of the sepctral shift is shown for three different concentrations of insulin and the trend is seen to be similar in each case. Similar data are shown for four concentrations of zinc-free insulin in Fig. 7; under comparable conditions of *p*H and concentration the peak values are somewhat higher on the acid side of the isoelectric point but lower on the basic side. The general shape of the curves with respect to *p*H dependence, however, is very similar, and for both insulins the titration curves were reversible. Difference spectra obtained above *p*H 8 showed, in addition to the usual features, slight maxima at

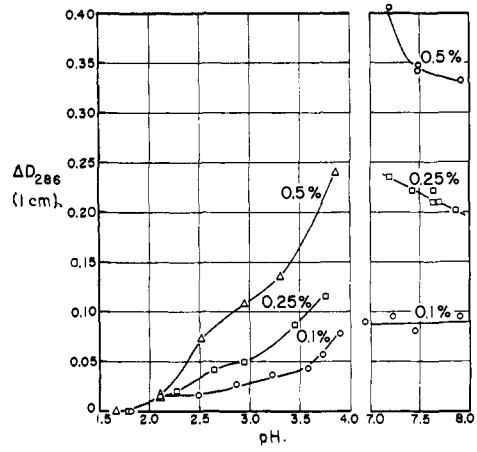

Fig. 6.—The *p*H dependence of the difference spectrum of native zinc insulin (ionic strength 0.1, HCl-KCl), at several concentrations of insulin. The reference insulin solution, set at zero optical density, varied between *p*H 1.6 and 1.8. The readings are all converted to those for 1 cm. cells. The data for 0.4% are omitted for the sake of clarity.

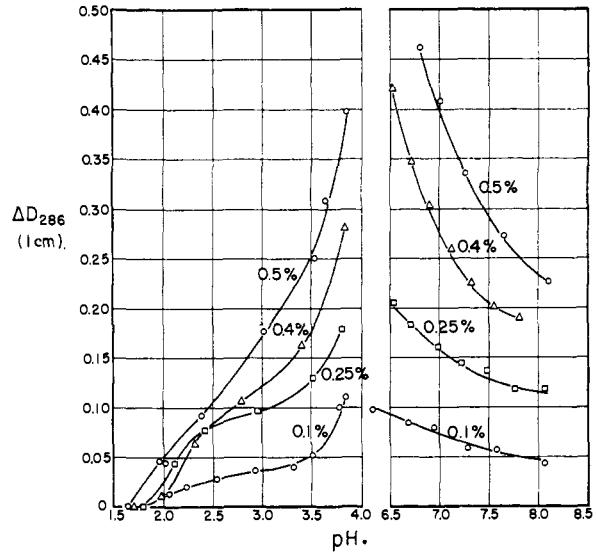

Fig. 7.—The *p*H dependence of the difference spectrum of zinc-free insulin (ionic strength 0.10, HCl-KCl) at several concentrations of insulin. The reference insulin solution, set at zero density, varied between *p*H 1.6 and 1.9. These data are corrected for 1 cm. cells.

295 mμ and enhanced optical densities in the 270–290 mμ region. This is due to the onset of the tyrosyl ionization, and data in this higher *p*H range are therefore of no use in evaluating spectral changes in terms of side-chain interactions.

(c) **Effect of Dioxane on the Difference Spectra.**—By using insulin solutions made up in 40% dioxane and of ionic strength 0.1, it was possible to plot difference spectra almost throughout the whole *p*H range from 2 to 8. The solutions were cloudy only in a small region around *p*H 6.5. The two main peaks at 279 and 286 mμ were absent in the presence of dioxane. While the optical density of the insulin solutions increased in passing from the *p*H 2 standard to the solutions of higher *p*H, the increases were very small, particularly from *p*H 2 to 5, and the difference spectra were almost featureless showing only a low broad maximum around 270–271 mμ. The small optical density differences (ΔD) under these conditions showed a complex *p*H dependence.[48] These small spectral changes due to the *p*H effect in the presence of dioxane are therefore a slight enhancement in optical density with increasing *p*H, occurring over the whole wave length range without any shift to longer wave lengths of the kind observed in the absence of dioxane and normally associated with hydrogen bonding of the tyrosyl residue.

(d) **Effect of Ionic Strength on the Difference Spectra.**—The *p*H dependence of the spectral change has been studied at three ionic strengths (0.1, 0.2 and 0.3). The effect of increasing ionic strength (shown in Fig. 8) is to enhance ΔD_{286} as the *p*H increases from 2.7 to 4.0. This increase in ΔD_{286} parallels an increase in molecular weight at higher ionic strengths[49-51] and a decrease in solubility. Thus it was not possible to obtain data for solutions of *p*H greater than 3 at an ionic strength of 0.3 owing to the onset of insolubility. However, on the basic side of the isoelectric point it will be seen from Fig. 8 that the effect of increasing ionic strength is to decrease the values of ΔD_{286}.

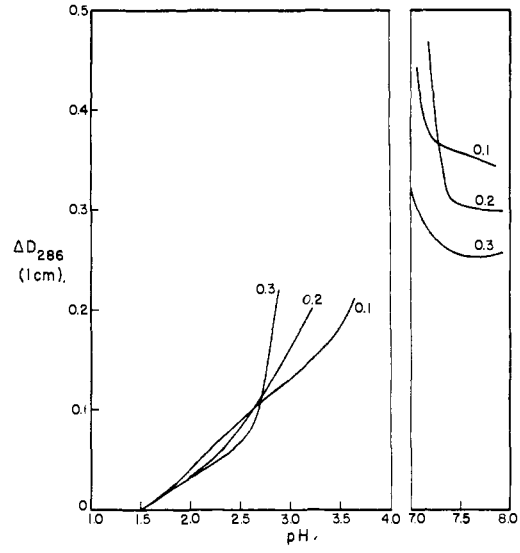

Fig. 8.—The effect of ionic strength on the *p*H-dependence of 0.5% native zinc insulin in 1 cm. cells. Experiments were performed at 0.166% but recalculated to 0.5%.

Discussion

Character of the Difference Spectra.—We would expect that perturbation of the tyrosine ring, and the spectral changes which accompany it, would occur *either via* the $-CH_2-$ group by ionization of a vicinal group (or by a change in *pK* of such a

(48) Observations of the *p*H dependence were complicated by changes in the ultraviolet absorption of the dioxane solvent itself with *p*H. In the presence of chloride ions, KOH produced ultraviolet absorbing material in the solvent. This effect could have been due to impurities in the dioxane which were not removed in the purification process.

(49) E. Fredericq and H. Neurath, THIS JOURNAL, **72**, 2684 (1950).

(50) P. Doty, M. Gellert and B. Rabinovitch, *ibid.*, **74**, 2065 (1952).

(51) J. L. Oncley, E. Ellenbogen, D. Gitlin and F. R. N. Gurd, *J. Phys. Chem.*, **56**, 85 (1952).

group), *or* more directly with the chromophore by hydrogen bonding of the phenolic-OH group to a nearby acceptor. Qualitatively the effects should be the same since they merely lead to a displacement of electrons and both effects probably produce the same kind of difference spectrum. However, the magnitude of the vincinal effect depends markedly on the distance between the tyrosine chromophore and the ionizing group responsible for the perturbation. Studies on model compounds of tyrosine and tryptophan have shown[9,10] that the inductive effect diminishes very rapidly when the ionizing group is removed by one residue.

Since none of the four tyrosyl residues in insulin is adjacent to a dicarboxylic acid residue, this makes the foregoing data on insulin difficult to interpret in terms of vicinal effects. A vicinal effect seems to be ruled out also for the spectral shift produced by tryptic digestion of insulin. The breaking of one peptide bond (3 residues removed from the nearest tyrosyl residue) produces a difference spectrum which is qualitatively and quantitatively almost identical with that produced by titrating from neutrality to *p*H 1.5. Furthermore, breaking of the B29-B30 bond (also 3 residues away) causes little change in the spectrum.

It has been shown[9] recently that tyrosine model compounds can exhibit difference spectra with acetate or urea even when the phenolic-OH groups are blocked and the possibility of hydrogen bonding is excluded. This finding raises some question about the interpretation of the protein difference spectra as being due solely to hydrogen bonding. However, the difference spectra of tyrosyl-OH are considerably greater than those of tyrosyl-OMe, thus suggesting that hydrogen bonding is an important although perhaps not the only reason for spectral changes. Furthermore, the ratio of the difference spectra for the OH and OMe compounds is greater in sodium acetate than in urea, an expected result if hydrogen bonding played an important role since sodium acetate produces greater shifts than urea at equimolar concentrations. It is at present difficult to definitely assign the reasons for a spectral change to any single cause.[52] We feel, however, that strong circumstantial evidence has been presented in favor of the assumption that these spectra are due, at least in large part, to hydrogen bonding of the tyrosyl residues. In the remainder of this paper the tyrosyl difference spectra will be assumed to arise *solely* from hydrogen bonding. This may be an approximation, and should be borne in mind in reading the rest of this discussion.

The character of the difference spectra produced by tryptic digestion and by acidification (Fig. 5) are remarkably similar: They consist of two major peaks at 278–279.5 mμ and 285.5–287 mμ (the lower limits, *i.e.*, 278 and 285.5 mμ, pertaining to the digestion experiments) and a series of five minor peaks in the 247–270 mμ region. The major peaks are tentatively assigned to tyrosyl hydrogen bonding[11,12] as discussed below. Donovan, *et al.*,[10] have shown that a difference spectrum can be obtained with peaks at 247, 253, 259, 265 and 268 mμ if there is a change in the charge on the carboxyl group of phenylalanine. The five minor peaks in the insulin difference spectra are at 248, 253.5, 259.5, 265.5 and 269 mμ and are therefore attributable to perturbation of the phenylalanyl residues. When the difference spectrum is produced by tryptic digestion at *p*H 8, the acquisition of a positive charge by the amino group of the B23 glycine residue probably affects the B24 and B25 phenylalanyl residues.[10] When the difference spectrum is produced by acidification, these same phenylalanyl residues are probably affected by changes in charge on a carboxyl group, *e.g.* the B21 glutamic acid residue. Even though there are two chromophores involved, a group this far removed from them would not be expected to exert much perturbing effect and indeed the ΔD values are very small when produced by *p*H change.

The two major peaks arise from a lateral shift of the spectrum toward shorter wave lengths together with a decrease in optical density, produced either by tryptic digestion or acidification. That is, throughout the wave length range examined, a solution of intact insulin at *p*H 8 has a higher optical density than either the digest or the acidified solution. Since no negative differences were observed, this suggests that when the tyrosyl interactions are formed, there is a broadening of the tyrosyl absorption band coupled with a general increase in optical density.

Action of Trypsin.—The rate of appearance of the major peak in the difference spectrum is very similar to the rate at which TCA-soluble tyrosine fragments appear in solution on tryptic digestion (Fig. 2). Sanger and Tuppy[33] and also Harris and Li[35] showed that the action of trypsin on the isolated B-chain of insulin is to split off the C-terminal alanine and the tyrosine-containing peptide which precedes it. Although it has been suggested[37,38] that the second split does not occur with intact zinc insulin, we have found that trypsin still attacks the same two bonds, *viz.*, the B22-B23 arg–gly and the B29-B30 lys–ala bonds. Liberation of the heptapeptide fragment was confirmed by paper and column chromatography, and it was also shown here that the main action of trypsin ceased when one of the four tyrosines in the insulin

(52) In the case of the OMe compound, Wetlaufer, *et al.*,[9] suggested dipole–dipole or ion–dipole interactions. Such an effect could reach the chromophore through an ionizable group or, when no such group is present, it might occur directly between the chromophore and the added solute. The first mechanism would have no relevance to the case of insulin for the same reasons that we can exclude the vicinal effect. While the possibility of direct dipole–dipole effects may have to be considered for the model systems,[9] its relevance to protein difference spectra may be questionable.[20,53,54] The reasons for this are discussed in more detail elsewhere,[53,54] and are based on two kinds of evidence: (1) A consideration of the comparative steric requirements of bonding and non-bonding interactions as well as the comparative magnitude and form of the spectral changes in models and proteins.[53] The conclusions are supported by the observation that the addition of ammonium acetate (0.1 *M*) in 25 to 100-fold excess over the tyrosine residues had no effect on the difference spectra of insulin (0.1 to 0.5%) over the whole experimental *p*H range of 2 to 8 and, therefore, appeared to have no influence on the interactions normally present in the insulin molecule. (2) Recent preliminary results[54] indicate that the change in the spectra of the OMe compound is due to the higher refractive index of the urea or sodium acetate solutions, while the spectra of the OH compound are due *mainly* to hydrogen bonding and partly to the higher refractive index.

(53) S. J. Leach and H. A. Scheraga, in preparation.

(54) R. H. Cramer and M. Laskowski, Jr., in preparation .

molecule had been released as a fragment which was soluble in 5% TCA. This, together with the results of the carboxypeptidase digestions, suggests that at *p*H 8 the B26 tyrosyl residue in the insulin molecule is involved in some kind of interaction and that this interaction is broken when the C-terminal heptapeptide is removed by tryptic digestion. The shift of the tyrosyl absorption band to shorter wave lengths, *i.e.*, in the direction opposite to ionization of the –OH group, suggests that in intact insulin, the B26 tyrosyl residue is acting as a hydrogen bond donor. An alternative explanation, which seems less probable,[55] is that the heptapeptide contains the acceptor group for a tyrosyl donor other than the B26 residue. Possible hydrogen bond acceptors would be the B27 threonyl-OH side chain or the –CO– group of a peptide bond.

If the rate of spectral change is indeed measuring the rate of hydrolysis of a single peptide bond in the insulin molecule, we might expect the upper curve of Fig. 2 to show first-order character. However a logarithmic plot shows that this is not so; nor is the *initial* rate of spectral change first order with respect to the trypsin concentration. This is partly accounted for by a loss in tryptic activity of up to 65% during the first 2 hours of digestion, with a consequent slowing down in the rate of reaction and then remaining constant for 20 hr. The activity, as judged by TAMe assay, fell more rapidly during insulin digestions than in control solutions under exactly the same conditions of *p*H and temperature, and the presence of Ca^{++} ions did not prevent this.

However, the failure to observe first-order kinetics in Fig. 2 cannot be accounted for solely by loss in activity of the trypsin; if the data are corrected for the changing tryptic activity (determined on separate aliquots), they still do not show a first-order behavior. The remaining discrepancy could be due to the binding of trypsin to inactive insulin or to the digestion products. In this case, the fall in activity might be even more pronounced than that shown by the TAMe assays, since the digests were diluted 80 times in the TAMe solutions which would encourage some dissociation of such complexes.

The deviation from first-order character could *not* be due to the attainment of an equilibrium in the digestion process which interferes with the attainment of an end-point, since we have already seen that under the same experimental conditions, the liberation of the terminal peptide, as judged by the assay of TCA-soluble tyrosyl fragments, proceeds to completion.

The deviation from first order character might possibly be due to simultaneous hydrolysis of the lys–ala bond leading to vicinal charge effects.

Effect of *p*H Changes.—The *p*H dependence of the spectral shift is shown for zinc insulin (Fig. 6) and zinc-free insulin (Fig. 7). Qualitatively, the presence of the zinc atom appears to have no bearing on the shape of the curves. Between *p*H 1.5 and 3.0 the curves are sigmoid in shape and consistent with the idea that the hydrogen-bond acceptor group is one which ionizes with a pK_a of about 2.4 and therefore a carboxylate anion. That is, as the *p*H is raised from 1.5 a COOH group commences to ionize and provides the acceptor for a tyrosyl-carboxylate ion hydrogen bond. The fact that the tyrosyl residue is acting as a donor rather than an acceptor is indicated by the nature of the spectral shift, which is in the direction of ionization of the OH group. At the same time it should be pointed out that there is also an enhancement in the height of the tyrosyl absorption peak and a slight broadening which is sufficient to maintain the same sign for all values of ΔD over the wave length range 250 to 310 mμ. If the change were a simple lateral shift to longer wave lengths, the two spectra would cross each other and the difference spectrum would change sign at about 251 and 277 mμ. It is possible, of course, that the un-ionized COOH group may act as a hydrogen bond acceptor. In this event, some degree of tyrosyl hydrogen bonding exists even at *p*H 1.5, and the *p*H 1.5 insulin solutions are not ideal reference solutions. This possibility is indicated from difference spectra obtained with the model system: acetyltyrosine ethyl ester–potassium acetate.[57] However, as in the present case, the increase in ΔD_{286} with increase in *p*H indicates that any such tyrosyl–carboxyl bonds must be considerably weaker than those involving the carboxylate anion.

The rapid increase in ΔD_{286} above *p*H 3 could arise in several different ways:

(a) It has been suggested on theoretical grounds[58] and shown experimentally with synthetic amphoteric polyelectrolytes[59] that the extent of helical folding in proteins should vary with *p*H and that the folding would be maximal around the isoelectric region.[60] If the tyrosyl hydrogen bond(s) in insulin are between pairs of side chains in the same polypeptide chain, it is possible to visualize a situation in which the bonds are sterically possible only when the intervening residues exist in a helical configuration, *i.e.*, around the isoelectric region of *p*H. At higher or lower *p*H, electrostatic repulsion along the chain would open up the helix, unwinding and extending it so that pairs of hydrogen-bonding groups were separated. In such a case, the *p*H-dependence of the spectral change would be expected

(55) For example, preliminary experiments on the isolated B-chain of insulin indicate that both acceptor and donor groups are within this chain. Since the isolated B-chain exists largely in a folded form,[56] steric considerations make it difficult to couple the B-16 tyrosine with an acceptor near the C-terminal end. Further, in intact insulin, neither tyrosyl residue of the A-chain could reach the heptapeptide in a 6000 MW unit.

(56) S. J. Leach and H. A. Scheraga, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **30**, 271 (1958).

(57) M. Laskowski, Jr., Abstracts of 131st A.C.S. meeting, p. 47C, Miami, April, 1957.

(58) J. Schellman, *Compt. rend. trav. Lab. Carlsberg, Sér. Chim.*, **29**, 230 (1955).

(59) P. Doty, K. Imahori and E. Klemperer, *Proc. Natl. Acad. Sci., U. S.*, **44**, 424 (1958).

(60) An alternative theory[61,62] is that the helical folding acquires additional stability from the presence of side-chain hydrogen bonds. If such interactions were more important than electrostatic effects, the folding would be maximal in a *p*H region intermediate between the pK's of the donor and acceptor groups and would not necessarily bear any relationship to the isoelectric point. Current theories on the factors affecting helical stability have recently been reviewed.[32,63]

(61) H. A. Scheraga, Abstracts of 134th A.C.S. meeting, p. 51C, Chicago, September, 1958.

(62) H. A. Scheraga, *Ann. Rev. of Phys. Chem.*, **10**, 191 (1959).

(63) S. J. Leach, *Revs. Pure and Appl. Chem. (Australia)*, **9**, No. 1 (1959).

to follow the titration curve of insulin, including the sigmoid COOH ionization curve, *even if the acceptor were a non-ionizing group* such as serine, threonine or a –CO– group of a peptide link.

This possibility seems unlikely for two reasons. First, the curves of Figs. 6 and 7 are not those of a simple titration curve of insulin. In particular, the pK of 2.4 seems an abnormally low mean value if it is to apply to the β- and γ-COOH groups of insulin (of which there are four). It is more in keeping with the value for one carboxyl group which is hydrogen bonded as an acceptor. Under these conditions a low pK value is to be expected.[64] The situation may be compared with that found in carbon monoxyhemoglobin; the curve relating sedimentation constants to pH values is unsymmetrical with respect to the isoelectric point, and it appears likely that histidines in the acid region, and lysines and/or tyrosines in the alkaline region, are critically involved in stabilizing the molecule.[65] Secondly, there is reason to believe that insulin shows little change in molecular configuration over a wide range of pH. Yang and Doty[66] have shown that the specific optical rotation of insulin is unchanged from pH 4 to 10 and that throughout this range the value is the same as that found in dimethylformamide solution, where the extent of helical folding is the maximum possible for insulin. The unusual stability of the insulin configuration is reflected also in its resistance to denaturation by heat or by organic solvents. The steep rise in ΔD values, as we approach the isoelectric point from either side, is therefore not compatible with the idea of a molecular unfolding.

(b) The rapid fall in ΔD values as the pH is increased from 6.5 to 8 could be explained by the appearance of a new acceptor group, *e.g.*, un-ionized histidine, which competes with the carboxylate ion. As the histidine group loses its charge, it destroys the tyrosyl–carboxylate hydrogen bonds in favor of tyrosyl–histidine bonds. Hydrogen bonds of this type have been suggested as occurring in the polymerization of monomeric fibrin.[67,68] A zero value of ΔD cannot be expected, owing to the onset of tyrosine ionization at pH 8 with a concomitant increase in the optical density at 286 mμ. However this explanation would require two assumptions; that the new tyrosyl-histidine bonds (say) produce a much smaller spectral shift than the original tyrosyl–carboxylate bonds and that the histidine is a "better" acceptor than the carboxylate group. Neither assumption is very convincing and indeed, the two requirements are to some extent mutually incompatible.

(c) Since the changes in ΔD with pH follow the changes in molecular weight,[51] it is possible that the pH-sensitive tyrosyl hydrogen bonds are intermolecular and that the spectral changes are a reflection of aggregation and disaggregation. Bonds of this type might well be important in stabilizing molecular aggregates.[65,67,68] However, in the present instance, they would be required to exist between units of molecular weight 12,000, this being the minimum for the experimental pH range. The fact that Beer's Law is obeyed (see later) seems to exclude the possibility of hydrogen bonding between the 12,000 MW units. We must still explain the correlation between ΔD and the extent of aggregation.

(d) The rapid increase in ΔD above pH 3 and the decrease above pH 6.5 could be due to general light scattering from large molecular aggregates. These must be present prior to precipitation on both the acid and alkaline sides of the insolubility region, and sedimentation data on insulin[49–51] indicate rapid aggregation in this region. It is possible that if the contribution to ΔD_{286} due to light scattering were subtracted out, the curves of Figs. 6 and 7 would flatten off at and above pH 3. The effect of ionic strength on values of ΔD (Fig. 8) supports the idea that scattering from aggregates is responsible for the behavior of ΔD close to the insolubility region, since the effects follow the changes in molecular weight.[51]

It is impossible to decide conclusively between the above explanations without further data, but at present the last seems the most plausible.

Effect of pH Changes after Trypsin Digestion.—The acid-sensitive hydrogen bonds indicated by the data of Fig. 6 are distinct from the hydrogen bonds which are broken by trypsin digestion. This is clear from the effect of acidifying the trypsin digest. Thus, although digestion has gone to completion, a difference spectrum is obtained which is identical with that obtained before digestion (see Fig. 5). In addition, the change in ΔD_{286} with pH between 1.6 and 3.8 is the same for trypsin-digested insulin, as for the intact material.

It therefore appears that there are at least two tyrosyl residues in the insulin molecule which are involved in hydrogen-bonding interactions with other groups. One residue is the B26 tyrosine, which is hydrogen bonded to an acceptor which either is non-ionizing, or at least does not ionize in the pH range 1.5 to 8.0. In addition, both native insulin as well as the trypsin digest have at least one hydrogen bond between a carboxylate ion acceptor and at least one of the three remaining tyrosyl residues (A14, A19 or B16). The close agreement between the maximal ΔD values for trypsin digestion and acidification suggest that only one of the three remaining tyrosines is in fact so bonded.

Other Observations.—Preliminary experiments on the effect of 4 and 6 M urea on native zinc insulin, the protein being soluble over the whole pH range in these solvents, gave difference spectra which suggest that tyrosyl hydrogen bonds in the insulin molecule are broken by urea. Also, the insulin methyl ester (a derivative in which all the carboxyl groups were esterified) still gave some evidence of a pH-dependent spectrum in the region of carboxyl ionization. However, these results are preliminary and require further investigation.

Role of Aggregation.—In an attempt to determine whether the carboxylate acceptor was in the

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same or in a different insulin molecule, experiments were carried out to see whether the ΔD_{286} values produced by acidification obeyed Beer's Law. If D_1 and D_2 are the optical densities (against water) at a given wave length for the reference solution (pH 1.6) and for the solution at any higher pH, respectively, then if Beer's Law holds

$$D_1 = k_1 c$$

and

$$D_2 = k_2 c$$

Hence

$$\Delta D = (k_2 - k_1)c$$

where $(k_2 - k_1)$ varies with pH at a given wave length. If there is a contribution from light scattering, this contribution would be

$$\Delta D = Hc\Delta M$$

where $H = 32\ \pi^3 n_0^2 (dn/dc)^2 / 3N\lambda^4$, and ΔM is the difference in molecular weights in the two solutions, provided that the largest dimension is less than $\lambda/20$.

Since ΔM is a function of c we should then observe departures from Beer's Law as c increases (depending on whether ΔM is very concentration-dependent in the range of concentrations studied). In addition, as stated above, departures from Beer's Law could also arise if the hydrogen bond responsible for the difference spectrum is an intermolecular one. Since the molecular weight never falls below 12,000 in the pH range studied[50] the intermolecular bonds could be between 12,000 MW units.

Experimental data on zinc insulin are shown in Fig. 9. Similar data were obtained for zinc-free insulin. Within the precision of the data, ΔD is linear with respect to c with a slope which depends on pH. Since Beer's Law holds, we conclude that the tyrosyl–carboxylate hydrogen-bond resides within the 12,000 MW unit and that ΔM for the aggregation process above pH 3 is not very concentration-dependent.

Location of Interactions.—The precise identity of the carboxylate acceptor cannot be determined at this stage. If it is assumed that both donor and acceptor are in the same 6000 molecular weight unit and that the intervening residues are in the form of an α-helix, only one interaction is possible. This is between the B16 tyrosine and the B13 glutamic acid residues. If, as has been assumed,[5] the zinc atom in zinc insulin performs the function of holding together the 12,000 MW dimer, we should then expect zinc insulin to show a different MW–pH relationship and therefore a different ΔD–pH relationship than zinc-free insulin. However, we have already seen that zinc-free insulin behaves in a very similar fashion to zinc insulin with regard to difference spectra, and this would support the idea that the pH-dependent hydrogen-bond is within the 6,000 molecular weight unit. On the other hand, we have also seen that in the presence of 40% dioxane the difference spectrum due to pH change is abolished. The effect of dioxane is to dissociate insulin into its monomer units,[69] and this evidence would therefore support the idea of an intermolecular hydrogen bond. It will be necessary to examine the effect of other disaggregating agents which have

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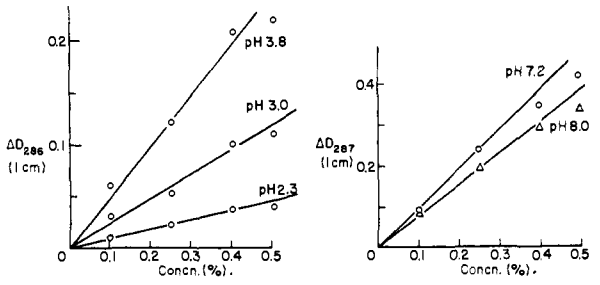

Fig. 9.—Test of Beer's Law for the pH-dependent difference spectrum of native zinc-insulin at an ionic strength of 0.1 (HCl–KCl).

less effect on the dielectric constant of the medium, before this can be taken as conclusive evidence that the disappearance of the hydrogen bonding is due entirely to the disaggregating effect.

In choosing an acceptor for the B26 tyrosine, similar difficulties arise. If we restrict ourselves to a non-ionizing acceptor in the same 6000 MW monomer unit, only the adjacent B27 threonine residue is possible, and this only if the four terminal peptide bonds are not in the form of an α-helix. Owing to the presence of a proline residue at the B26 position, an α-helix would not be possible and a B26 tyr- B27 thr hydrogen bond is, therefore, feasible.[70] On the other hand, if we assume that the acceptor is in another monomer unit, it is possible to make a 12,000 MW dimer model in which the two monomer units run in an antiparallel sense and in which the B26 tyrosine residue from each B chain is hydrogen bonded to say the B3 asparaginyl residue of the other B chain. Such a model would bear some resemblance to that of Lindley and Rollett[71] but differs in detail with their Fig. 4. Another possible interaction involving two B-chains would be a hydrogen bond between the B26 tyr of one chain and the B27 thr of another chain.[72] This type of dimer model is compatible with the data obtained in 40% dioxane. If it is assumed, moreover, that the zinc atom is not necessary to hold the 12,000 MW unit intact,[74] this would explain the similarity in spectral behavior between zinc and zinc-free insulins over the whole pH range.

On the other hand, it is quite possible that the insulin molecule may not be entirely helical in character.[63] In such a case, it would not be surprising to find that essentially all of the tyrosyl residues of insulin could function as hydrogen bond donors to acceptors in various parts of the molecule. In this connection, the location of hydrogen bonds in *fragments* of the insulin molecule (*e.g.*, isolated A and B chains) may shed some light on the insulin structure.[73]

(70) If such a hydrogen bond existed, it could be ruptured during tryptic hydrolysis of the B22-B23 arg–gly bond since the configuration of the free heptapeptide would differ from its configuration when it is bonded to the insulin core.

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Acknowledgments.—We are indebted to Dr. O. K. Behrens of the Eli Lilly Co. for generous gifts of crystalline beef zinc insulin, to Dr. M. L. McFadden for the preparation of insulin methyl ester and for the identification of the heptapeptide produced by tryptic hydrolysis of insulin and to Dr. L. Gruen for carrying out the zinc analyses.

ITHACA, NEW YORK

[CONTRIBUTION FROM THE PALO ALTO MEDICAL RESEARCH FOUNDATION]

The Urea Denaturation of Chymotrypsinogen as Determined by Ultraviolet Spectral Changes. The Influence of *p*H and Salts[1]

BY CHARLES H. CHERVENKA

RECEIVED AUGUST 7, 1959

The rate of change of the spectrum of chymotrypsinogen in urea solutions is strongly influenced by the ionic environment. At low *p*H the rate is proportional to the third power of the hydrogen ion concentration, and this dependence varies with increasing *p*H in a manner which can be correlated with changes in the ionic form of the protein. In general, salts increase the rate of change below *p*H 4.8 and decrease it above this *p*H, and these alterations are proportional to the ionic strength. Calcium and strontium chlorides have an additional and more specific effect which is associated with binding of the metal ion in a 1:1 ratio with the protein molecule, probably to an imidazolyl side chain or the α-amino terminal group.

Several recent reports describe changes in the ultraviolet absorption spectra of proteins during urea treatment.[2-5] These spectral shifts are related to changes in bonding or other factors in the environment of the side chains of certain of the amino acid residues,[6,7] so that they are inherently a reflection of the configurational changes occurring during the general process of denaturation of proteins. Thus a new means of studying this process was suggested.

Previous work had indicated that such a method could be applied to the pancreatic protein, α-chymotrypsinogen.[4] When treated with a concentrated solution of urea, this protein shows a large shift in its ultraviolet spectrum, with a maximum change at 293 mμ. Calcium chloride was found to inhibit the spectral change at neutral *p*H. In the present study this effect was investigated more fully, particularly because the stabilizing action of certain divalent metal ions, notably calcium, upon the enzyme chymotrypsin[8,9] suggested a possible common mechanism. A study of the influence of *p*H upon the rate of urea denaturation was also undertaken so that this factor could be separated from the salt effect; also very little such data are available in the literature.[10] The changes of optical rotation of chymotrypsinogen in urea have been investigated previously,[11] but the effects of variation in *p*H upon the rate were not reported.

Experimental

α-Chymotrypsinogen was purchased from the Worthington Biochemical Laboratory, Freehold, N. J. as the once crystallized filter cake. The material was recrystallized five times from ammonium sulfate,[12] dialyzed thoroughly against 0.001 *M* hydrochloric acid and lyophilized. Fresh solutions in 0.001 *M* hydrochloric acid were prepared as needed.

Both of two brands of commercially available reagent grade urea were found to contain colloidal material which interfered with their use in the spectrophotometer. Before use, a warm, concentrated solution of urea in 30% ethanol was filtered through a Seitz bacterial filter and allowed to crystallize in the cold. The crystals were collected and dried at room temperature. Fresh stock solutions of this material were made up daily. Other chemicals were either C.P. or reagent grade and were used with no further purification.

Ultraviolet absorption measurements were made in the Beckman model DU Spectrophotometer. Temperature control was obtained by circulating water of constant temperature through the thermospacers. Although the cuvette compartment of the instrument could be maintained at 25 ± 0.1° easily, the temperature variation of samples in cuvettes during reaction times was greater, ±0.2°.

The urea solution and the required amount of hydrochloric acid or sodium hydroxide and salt were equilibrated at 25°. In some instances the salt was dissolved in the chymotrypsinogen solution. At zero time the protein sample was added and mixed thoroughly. The final total volume in the cuvette was 3.0 ml. Readings of the difference in absorbance at 293 mμ between the test cuvette and a reference cuvette containing an equal concentration of protein in 0.001 *M* hydrochloric acid were made with time; the initial reading was made at 30 sec. and subsequent readings were made at 15 sec. intervals or longer. The final absorbance value was the same for all reactions which went to completion.

The first-order rate constants were calculated from the change in absorbance by the usual graphical method and are expressed in reciprocal seconds. The molar extinction coefficient of the spectral change at 293 mμ is 7.6×10^3, using a molecular weight of 25,000 for chymotrypsinogen.[13]

Measurements of *p*H were made using the Radiometer model 22 *p*H meter. In the presence of urea the measurements were recorded as taken, and no attempt was made to correct for the effect of urea on the system.

(1) This investigation was supported by research grant numbers C-2289(C4) and A-2800 from the National Institutes of Health, Public Health Service. A report of the preliminary results of this investigation was made at the annual meeting of the Pacific Slope Biochemical Conference at Los Angeles in December, 1958.

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