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Structural studies on insulin

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STRUCTURAL STUDIES ON INSULIN

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INTRODUCTION

The sequences of the amino acid residues in the peptide chains of the insulin molecule have recently been proposed by Sanger and his co-workers (1, 2). The basis for this tedious, but admirable, piece of work is a reaction first employed by Abderhalden and Stix (3) but later improved upon by Sanger (4). Other investigators have studied portions of the structure of insulin. Some of these studies agree completely with Sanger's results but others provide evidence which disagrees.

Work done in this laboratory by a previous investigator (5) has provided evidence for one or more aminoid (N-) terminal valine residues in addition to the phenylalanine and glycine residues required by Sanger's structure. The method used to study the amino acid sequence in insulin was one developed in these laboratories (6). This method was a modification of the phenylthiohydantoin (PTH) technique of sequence study proposed by Edman (7), which in turn was a modification of the Abderhalden-Brockmann procedure. Since evidence for an N-terminal valine residue was found by this technique, a study of a portion of the amino acid residue sequence at the N-terminal end of the insulin molecule was begun.

HISTORICAL

A review of the literature on insulin published through June, 1953, has been reported by Blaney (5). Only a brief summary of that part of the previously reviewed work which is pertinent to the present investigation will be reported here in addition to a more detailed review of publications which have appeared since.

Sanger's Complete Amino Acid Residue Sequence of Insulin

The most complete investigation of the amino acid residue sequences in insulin has been carried out by Sanger (4, 8, 9) and Sanger and his co-workers (1, 2, 10, 11). Briefly, the native insulin was treated with performic acid to oxidize the disulfide bonds thought to be holding four chains (two pairs of identical chains) of amino acid residues together. Electrophoretic patterns showed that the performic acid oxidation product contained three fractions (8), but four fractions were separated by precipitation methods (9). Two of these four fractions were thought by Sanger to represent the two pairs of identical peptide chains of the intact molecule with the exception that they would contain cysteic

acid residues in place of half-cystine residues. One fraction containing N-terminal glycine residues was called fraction A and the second fraction containing N-terminal phenylalanine residues was called fraction B. Fraction A represented 30 to 40 percent and fraction B approximately 25 percent of the total oxidized insulin. Two other fractions, X and M, represented 20 to 30 percent of the original material (9). The reaction of 2,4-dinitrofluorobenzene (DNFB) with free amino groups to give, on hydrolysis, dinitrophenyl (DNP-) derivatives of the amino acid residues containing the free amino group was utilized to study the peptides resulting from both partial acid hydrolysis and proteolytic hydrolysis (with pepsin, trypsin, and chymotrypsin) of fractions A and B (1, 2, 10, 11). The proposed sequences for the two fractions resulting from these investigations were, for fraction A, Gly.-Iso.-Val.-Glu.-Glu.-Cys.-Cys.-Ala.-Ser.-Val.-Cys.-Ser.-Leu.-Tyr.-Glu.-Leu.-Gly.-Asp.-Tyr.-Cys.-Asp. (2) and for fraction B, Phe.-Val.-Asp.-Glu.-His.-Leu.-Cys.-Gly.-Ser.-His.-Leu.-Val.-Glu.-Ala.-Leu.-Tyr.-Leu.-Val.-Cys.-Gly.-Glu.-Arg.-Gly.-Phe.-Phe.-Tyr.-Thr.-Pro.-Lys.-Ala. (1).

Sanger's work has not gone unchallenged. Bull (12) is reluctant to accept the fact that the peptides obtained by both enzymatic and acid hydrolysis have the amino acid sequence of the native insulin molecule. There is, he points

out, some evidence of rearrangement of amino acid residues and synthesis of new peptides during proteolytic hydrolysis, and there is no conclusive evidence to show that this does not happen during partial acid hydrolysis. Bull also questions the purity of the various fractions obtained as the result of partial hydrolysis and would like to have a better system of records of the different peptides for easier checking of the proposed sequences (12).

Blaney (5) points out that some of the small peptides of fraction B do not fit the proposed sequence for that chain (10) and that the fractions X and M of the oxidized insulin were not investigated for their amino acid residue sequence. There is no assurance that one or more other peptide chains of insulin are not included in these fractions, a fact recognized by Sanger (9).

Investigations of the Carboxoid Terminus of Insulin

Carboxypeptidase Digestion

The enzyme, carboxypeptidase, was used by Lens (13) to remove the carboxoid (C-) terminal amino acid residues of insulin with subsequent isolation and identification of those residues. One to three residues of alanine were detected,

which result disagrees in part with Sanger's finding of two residues each of alanine and aspartic acid. Harris (14) repeated the technique to find that two moles of alanine were released per mole of insulin from Sanger's fraction B (9) and that an undetermined amount of asparagine was released from the C-terminal end of fraction A. These data agree with those of Sanger (1, 2).

Reduction of the Free Carboxyl Groups

A second technique used for determining the C-terminal amino acid residues was to reduce their free carboxyl groups to the corresponding alcoholic group, then to liberate, isolate, and determine the amino alcohol both qualitatively and quantitatively.

Fromageot, Jutisz, Meyer, and Pennasse (15, 16) used lithium aluminum hydride in N-ethyl morpholine to reduce the free carboxyl groups. Sanger's fractions A and B were found to contain C-terminal glycine and alanine residues, respectively. Quantitative determinations showed two moles of each per mole of insulin. Later work by Jolles and Fromageot (17) during an investigation of the aspartic acid residues in insulin indicated that two of the six aspartic acid residues in the molecule had free alpha carboxyl groups. Only these

two carboxyl groups could be reduced by lithium aluminum hydride. All other carboxyl groups were present in either peptide linkage or as amide groups. Thus, there should be two C-terminal asparagine residues and four asparagine residues within the peptide chains.

Chibnall and Rees (18) first esterified the free carboxyl groups, then reduced the ester groups to the corresponding alcoholic group with lithium borohydride. The amino alcohols found on quantitative determinations were two residues of alanine, one of glycine, and one residue of an unidentified amino acid. Later more careful work by the same authors showed that asparagine was a C-terminal amino acid residue of insulin (19) and their claim of a C-terminal glycine residue was, according to Sanger (2), orally withdrawn.

Formation of the Thiohydantoin of the C-Terminal Residue

The reaction in acetic anhydride of the free carboxyl groups of proteins with ammonium cyanate was used by several investigators for the determination of the C-terminal amino acid residues.

Waley and Watson (20) found only C-terminal alanine residues by this method, while Baptist and Bull (21) found

alanine and a small amount of glycine. The latter authors found that aspartic acid, glutamic acid, lysine, and arginine residues in C-terminal positions could not be determined by their modification of this method. Turner and Schmerzler (22) found that C-terminal asparagine but not aspartic acid would form a thiohydantoin. When these authors treated insulin with ammonium thiocyanate in acetic anhydride and the thiohydantoin was selectively hydrolyzed from the residual insulin with barium hydroxide at room temperature, the thiohydantoins of alanine and asparagine were obtained. The latter data agreed with Sanger's structure. Turner and Schmerzler were able to apply the C-terminal thiohydantoin technique in a stepwise fashion to synthetic peptides to obtain the expected sequence, but unfortunately no sequential determination was made on insulin.

Investigations of the Aminoid Terminus of Insulin

DNP-Derivatives of N-Terminal Residues

Fletcher, Lowther, and Reith (23) used a modification of the DNFB method of Sanger (4) to determine the N-terminal amino acid residues of insulin. The insulin used in the determination was treated with DNFB, hydrolyzed, and the

ether-soluble DNP-derivatives of amino acids were extracted by the same procedure used by Sanger (4). The methyl esters of the ether soluble and the water soluble DNP-amino acids were then prepared. Quantitative determinations of the resulting amino acid derivatives were performed by adsorption chromatography on alumina. The calculated results showed the presence of two residues of N-terminal phenylalanine, two or three residues of N-terminal glycine, two residues of lysine with only its epsilon amino group free, and two to three residues of an unidentified amino acid. The authors had prepared the DNP-derivatives of only a few amino acid esters and so were not able to determine what the unidentified residue was. When the authors carried out the N-terminal determination of the same insulin sample by Sanger's technique (4), they obtained only phenylalanine and glycine derivatives.

Fletcher, Lowther, and Reith (23) also carefully standardized the conditions of hydrolysis of the DNP-protein derivative in order to study the decomposition of DNP-amino acids and the recovery of the unchanged products. It was found that recovery of these derivatives varied considerably with the method of estimating the yield and with the hydrolytic conditions and extraction procedures used. Although Porter and Sanger (24) report that a constant proportion of

the DNP-amino acids can be recovered in the presence of globin, Desnuellè, Rovery, and Fabre (25) found that the recovery of DNP-aspartic acid varied according to the protein present.

The significance of the work by Fletcher et al. is far-reaching, for it indicates that not all DNP-amino acid derivatives can be determined by the partition chromatography method of Sanger (4), and that because of such variable results, the calculations of the quantities of DNP-amino acid residues usually based upon the recovery experiments of Porter and Sanger (24) are not reliable. A more comprehensive and critical study of the technique used by Sanger in his study of insulin is now essential.

I^{131}-Pipsyl Derivatives of N-Terminal Residues

Udenfriend and Velick (26) treated insulin with I^{131}-pipsyl chloride. The product then was hydrolyzed and the I^{131}-pipsyl derivatives of the amino acids having free amino groups were isolated from the hydrolysate. Quantitative determinations showed that insulin had glycine and phenylalanine N-terminal residues as required by Sanger's structure but only one residue of each per mole of insulin of molecular weight 12,000. The authors report that the I^{131}-pipsyl

amino acid derivatives are much more stable to the conditions of hydrolysis than the DNP-derivatives of amino acids. An added precaution against error in the quantitative determination of the terminal residues was taken by addition of the indicator S^{35}-labeled pipsyl amino acid to the labeled protein derivative before hydrolysis. The destruction due to hydrolysis should be the same for both the I^{131}-pipsyl derivative of the N-terminal residue and the S^{35}-pipsyl indicator residue.

In view of the results of Fletcher et al. (23), a re-examination of this method of analysis might be appropriate in order to eliminate calculations to compensate for the loss of derivatives on hydrolysis of the derivatized protein.

PTH's of the N-Terminal Residues

The reaction of a free amino group with phenylisothiocyanate (PTC) in alkaline solution with subsequent acid hydrolysis causes formation of the PTH of amino acids having a free alpha amino group. The reaction was used by Edman (7) as a means of determining the sequence of amino acid residues in a peptide or protein.

Edman (7) ran the reaction in 1:1 pyridine-water solution at 40°C. with the addition of alkali as needed to

maintain the pH of the solution at 8.6. After removal of the pyridine and excess reagent with benzene, the aqueous solution was evaporated to dryness. The PTH of the N-terminal amino acid was formed on cleavage of the N-terminal peptide bond with anhydrous nitromethane-hydrogen chloride leaving the intact residual peptide. Repetition of the treatment and cleavage gave the PTH of the penultimate amino acid residue, and so on in a stepwise fashion. After each treatment, the PTH's were extracted. Quantitative determinations of the PTH's were based on their ultra violet absorption at 267-268 millimicrons. The PTH's were then hydrolyzed with barium hydroxide and the regenerated amino acids were determined qualitatively by paper chromatography.

The Fraenkel-Conrats (27) applied a slight modification of this technique to insulin. The hydrolysis of the phenylthioureido insulin derivative was done with 0.6-1.2 N hydrochloric acid at 75°C. (29). Four N-terminal amino acid residues were found by quantitative methods and the qualitative methods showed that phenylalanine, glycine, and some alanine were present.

H. Fraenkel-Conrat (28) again modified the technique for determining the amino acid sequence in insulin and other proteins and peptides. A solution of the substance whose sequence was to be determined was applied to small strips of

Whatman No. 1 filter paper and dried. A 20 percent solution of PTC in dioxane was used to wet the paper and the strip was placed in a pyridine, dioxane, and water saturated atmosphere at 40°C. for two to three hours. The strips were then thoroughly washed with benzene and a 1:1 mixture of peroxide-free ether and absolute alcohol to extract excess reagent. The PTH's of the N-terminal amino acids were formed by placing the strips in a desiccator containing beakers of glacial acetic acid and 5.7 N hydrochloric acid. The desiccator was evacuated to about 100 mm. After four to 16 hours, the strips were removed and the PTH's were extracted with the alcohol-ether mixture. Quantitative determinations were made spectrophotometrically in this solution at 270 millimicrons. Qualitative identification was made by direct paper chromatography of the PTH's and by hydrolysis of the PTH's with barium hydroxide.

Although 14 steps were carried out on insulin by this technique, Fraenkel-Conrat reported the results of only five of the steps because the chromatographic picture became complex and further identification was difficult beyond the fifth step. The results found for the pentapeptides of native insulin and also the insulin A chain and B chain agreed exactly with those of Sanger. Thus, the insulin A chain contained the pentapeptide, Gly.-Ileu,-Val.-Glu.NH_2 ---

and the insulin B chain contained the pentapeptide Phe.-Val.-Asp.NH_2.-Glu.NH_2.-His.

Fraenkel-Conrat (28) noted that other amino acids appeared to approach in quantity the amino acid residues required by Sanger's structure for some positions in the insulin chains. This then made the determination of the correct amino acid in the sequence difficult.

Fraenkel-Conrat (28) also reported that the direct chromatography of the PTH's indicated the N-terminal and the adjacent peptide bonds were split through the first five to seven steps. Little evidence for non-specific splitting was found. It would seem, however, that the splitting of the peptide bond adjacent to the N-terminal bond is a non-specific cleavage. Perhaps too little information is given in the paper for the proper interpretation of the statement.

Christensen (29) applied the PTH technique to insulin to determine the first five positions beginning at the N-terminus. The hydrolysis was accomplished in this case with 0.1 N hydrochloric acid at 75°C. Only glycine and phenylalanine residues were obtained on the first degradation. The amino acid residues expected from Sanger's structure appeared on the subsequent four degradations with, however, other amino acid residues appearing also. Some of the "extra" amino acid residues could be explained as the result

of incomplete reactions during previous degradations. In some cases, however, the amounts isolated were too large to be explained in this manner.

Kaiser, Maxwell, Landmann, and Hubata (30) prepared phenylthiocarbamyl insulin by the same technique used by Edman (7), then hydrolyzed the material with 2 N hydrochloric acid in a sealed tube in a boiling water bath for one hour. Direct paper chromatography of the extracted PTH's showed that phenylalanine and glycine were the N-terminal residues of insulin. It should be pointed out, however, that the conditions for hydrolysis were rather mild and may not be severe enough to cause complete hydrolysis of peptide bonds involving such difficultly hydrolyzable amino acid residues as valine and isoleucine (31). Thus an N-terminal valine residue could have escaped undetected.

Landmann, Drake, and Dillaha (32) also reported phenylalanine and glycine as the only N-terminal amino acid residues found in insulin by essentially the method used by Kaiser *et al.* (30). Peptides and proteins treated for end-group determinations were hydrolyzed with 2 N hydrochloric acid at 70°C. for two hours in this work. Such hydrolytic conditions are used for partial hydrolysis and it would seem that incomplete hydrolysis could certainly be expected.

The PTH method as modified by Fox, Hurst, and Itschner (6) and Fox, Hurst, and Warner (33) was applied to insulin by Blaney (5). Blaney studied the sequences of five amino acids in the insulin molecule. Native insulin was first hydrolyzed and the amounts of the five amino acids, phenylalanine, isoleucine, valine, aspartic acid, and glutamic acid were determined in the hydrolysate by microbiological assay. Glycine was not determined because the microbiological assay of this amino acid had not been successful in this laboratory. Table 1 compares Blaney's results with those of Tristram (34), Brand (35), Fromageot (36), Sanger (1, 2), and Harfenist (31). As can be seen, the results are in close agreement with those of Sanger except that three residues of isoleucine were found.

When the subtractive microbiological sequence technique of Fox, Hurst, and Warner (33) was applied, results in general agreed with those of Sanger. The results for the five amino acids studied are given in Table 2. The three residues of isoleucine appeared in both the untreated hydrolysate and in the hydrolysate of the sample given one subtractive treatment. Blaney found 0.6 residues of isoleucine indicated in the hydrolysates of the second and third subtractive treatments. He reports that such an amount of utilization of the PTH of isoleucine might be expected in view of the findings

Table 1. The number of residues of five amino acids in insulin[a]

Amino acid	Tristram (34)	Brand (35)	Fromageot (36)	Sanger[b] (1, 2)	Harfenist[c] (31)	Blaney (5)
phenylalanine	5.9	5.8	6.0	6	6	5.6
isoleucine	2.5	2.7	1.5	2	2	2.9
valine	7.9	9.0	7.6	10	10	10.0
aspartic acid	6.1	6.1	5.0	6	6	5.4
glutamic acid	15.2	16.4	16.0	14	14	13.3

[a]The major portion of this table is taken from Blaney (5).

[b]Numbers of residues as required by Sanger's structures.

[c]Harfenist's results were doubled to conform to a molecular weight of 12,000.

Table 2. Number of residues[a] before and after PTC treatments[b]

Amino acid	Number of reatments				
	0	1	2	3	4
phenylalanine	6(6)[c]	4(4)	4(4)	4(4)	-(4)[d]
isoleucine	3(2)	3(2)	0-1(0)	0-1(0)	-(0)
valine	10(10)	9(10)	8(8)	6(6)	-(6)
aspartic acid	5(6)	4(6)	11(6)	2(4)	-(4)
glutamic acid	13(14)	-(14)	-(14)	-(14)	11(10)

[a]The numbers of residues have been rounded to the nearest whole number.

[b]Table is taken from the unpublished Ph.D. thesis of D. J. Blaney (5).

[c]The number of residues of each amino acid residue required by Sanger's structure (1, 2) is given in parentheses.

[d]Blanks indicate that the amino acid residue was not assayed.

of Serat (37) that this PTH and the one derived from leucine are utilized to some extent by the assay organism.

The disappearance of one residue of valine in the hydrolysate of the first subtractive treatment indicated that one valine residue occurred in an N-terminal position. These results were borne out when the PTH's were extracted with ether from the hydrolysate of another sample given one subtractive treatment. On hydrolysis of the extracted PTH's with barium hydroxide and paper chromatography of the regenerated amino acids, strong spots appeared for phenylalanine, glycine, and valine (5). After his thesis had been completed and submitted, Blaney repeated the extraction and hydrolysis technique. Paper chromatography of the regenerated amino acids again yielded strong spots for phenylalanine, glycine, and valine as well as two faint spots between the glycine and valine spots (38).

The results for aspartic acid are not consistent, probably due to some inconsistency in the assay organism or procedure at the time these assays were run. The microbiological assay of aspartic acid has been somewhat troublesome in this laboratory until recently (39). Therefore, the appearance of one aspartic acid residue as an N-terminal residue may not be significant.

It should be noted that the presence of six N-terminal amino acid residues, two of phenylalanine, two of glycine, one of valine, and one of aspartic acid would, as pointed out by Blaney (5), agree with results reported by Chibnall (40) that 18 free amino groups per molecule of weight 35,500 in addition to the free ϵ-amino groups of lysine were found by the Van Slyke amino nitrogen determination. Crowfoot's (41) X-ray data also indicates there are 18 peptide chains per insulin molecule of molecular weight 36,000. On a basis of an insulin molecule of a minimum molecular weight of 12,000, both Chibnall's and Crowfoot's results would indicate six peptide chains per molecule, thus lending support to Blaney's results of six N-terminal amino acids.

Additional Investigations

Variation in Amino Acid Composition of Insulin from Different Sources

Harfenist (31) has published the results of complete amino acid analyses of beef, pork, and sheep insulins. Previous work by Harfenist and Craig (42, 43) had indicated beef insulin could be separated into two biologically active components, called insulin A and insulin B by the authors.

The amino acid analysis of both beef insulins A and B and of pork and sheep insulins was carried out. The results reported by Harfenist (31) are given in Table 3.

As can be noted from Table 3, beef insulins A and B differed from each other by only one ammonia residue and did not differ from the amino acid composition of Sanger's structure. The differences in insulins from other sources are shown in the numbers of residues of isoleucine, valine, glycine, alanine, threonine, and serine.

Harfenist (31) reports that no differences in immunological properties or physiological activity were shown by any of the insulins from these sources. The differences in amino acid composition must, therefore, have a very small effect on their biological properties.

Fractionation of Performic Acid-Oxidized Insulin

Andersen (44) separated performic acid-oxidized insulin into crude preparations of fractions A and B using ammonium acetate buffer as was done by Sanger (9). The crude preparations were then subjected to partition chromatography on silane-treated Hyflo-Super Cel. The solvent system used was 2-butanol and 0.01 N trichloroacetic acid (1:2.5). The peaks

Table 3. Amino acid compositions of insulins from different sources[a]

Amino acid	Number of residues[b]			
	Beef-A	Beef-B	Pork	Sheep
isoleucine	2	2	4	2
valine	10	10	8	10
glycine	8	8	8	10
alanine	6	6	4	6
threonine	2	2	4	2
serine	6	6	4	2
aspartic acid	6	6	6	6
glutamic acid	14	14	14	14
proline	2	2	2	2
cystine	6	6	6	6
leucine	12	12	12	12
tyrosine	8	8	8	8
phenylalanine	6	6	6	6
histidine	4	4	4	4
lysine	2	2	2	2
arginine	2	2	2	2
ammonia	6	5	6	6

[a]These results were reported by Harfenist (31).

[b]The numbers of residues were converted to a molecular weight of 12,000 for the insulin molecule for comparison with previous analyses.

of peptide material were located by determining the optical density at 277 millimicrons of the fractions collected.

A single sharp peak was obtained for fraction A and two peaks were obtained for crude fraction B. One peak of the latter two represented Sanger's fraction B, although Andersen gives no details on how this was determined. The trichloroacetic acid was removed from the fractions with Dowex-2 and each was lyophilised. Paper electrophoresis of the two fractions indicated that complete separation had been obtained.

Although the author apparently disregards the second peak obtained in the partition chromatography of the crude preparation of fraction B, it would be interesting to know the nature of the material forming this peak. Could it perhaps have been a third peptide chain of insulin?

Fractionation of Insulin by Electrophoresis-Convection

Brown, Shumaker, Timasheff, and Kirkwood (45) investigated the fractionation of insulin by means of electrophoresis-convection. The insulin used in these preliminary experiments was found to be separable into two components. Timasheff, Brown, and Kirkwood (46) found, in an extension of the investigation, that commercial Lilly Zinc-crystalline

Insulin and a specially prepared Lilly amorphous insulin could be fractionated into a major, active, electrophoretically homogeneous component and a minor electrophoretically heterogeneous fraction containing a considerable lower activity. Study of the fractionation data indicated that the major electrophoretically rapid-moving component probably contained all the activity and that the slow highly heterogeneous minor component probably contained none of the insulin activity. The total amount of the slow minor component was estimated to contain as much as 40 percent of the total protein. The similarity in behavior between the two components indicated that their chemical differences were very minute and mobility data indicated differences of three electronic charges per molecule of 36,000 molecular weight.

The two fractions separated by electrophoresis-convection (46) differed from those separated by counter-current distribution by Harfenist and Craig (42, 43) because the latter two fractions did not differ in activity. Harfenist and Craig (42), however, reported that all the insulin investigated by counter-current distribution contained from ten to 50 percent protein other than the major component. There is no data given in either paper which will allow one to decide whether the minor heterogeneous component of Timasheff,

Brown, and Kirkwood (46) is perhaps some of this protein or not. The possibility should be investigated.

Timasheff and Kirkwood (47) studied the fractionation of the complexed insulin-protamine systems by means of electrophoresis-convection. The insulin-protamine complex was very easily separated at pH's very close to the isoelectric point of insulin. This was not possible for the free insulin alone. As shown by the activity of the separated insulin components, the complexed insulin-protamine system could be more completely separated. The data obtained from this work seemed to support the conclusions of the previous work that the slow minor component contained little or no activity.

No attempt was made during these investigations to determine the amino acid analysis or amino acid sequence of the major or minor components of the fractionated insulin. The investigations have probably not progressed to this point. Until such data are available the significance of the fractionations cannot be evaluated or compared with information obtained by other investigators.

Summary

Although there is considerable evidence in the literature to support portions of Sanger's proposed amino acid sequence in insulin, there is mounting evidence to show that his structure may not be entirely correct. Fletcher, Lowther, and Reith (23) have certainly cast doubt on the ability of Sanger's partition chromatography technique to determine all the amino acids having free amino groups. As pointed out in the discussions of various pieces of work, other portions of Sanger's operations used to identify peptides or amino acid derivatives may have to be scrutinized more carefully and perhaps be reinterpreted. Such reinvestigation need not detract from the admiration one rightly holds for such a prodigious task so ably performed by Sanger and his co-workers. The scope of the work and the large number of operations necessitated by the nature of the investigations of portions of the amino acid sequence of insulin, however, make a reinvestigation of the complete structure of insulin a necessity.

METHODS

Paper Chromatography

Apparatus

Chromatography jars equipped with stainless steel racks, Pyrex troughs and antisiphon rods, and covers (Research Equipment Corporation) were used for all the descending chromatograms. Two sizes of jar were used. A 12 x 24 in. jar was equipped to hold four 20 x 57 cm. sheets of paper. Two 6 x 18 in. jars were each equipped to hold two 10 x 45 cm. sheets of paper.

Ascending chromatograms were run in a 10 x 12 in. jar equipped with a desiccator lid for a top. A 46.5 x 28.5 cm. sheet of chromatography paper, when stapled in the form of a cylinder (see p. 30) could easily be fitted in this jar.

Paper

Whatman No. 1, a medium speed and general purpose chromatography paper, was used for all the paper chromatograms described.

Solvents

All solvents used were either reagent grade solvents or were purified by redistillation in an all glass apparatus.

Standard Compounds

All the standard solutions used were made to concentrations of 1 mg. per ml.

The standard solutions of amino acids were prepared from commercially available DL-amino acids except where specifically noted otherwise.

The standard solutions of PTH's of amino acids were prepared from the PTH's synthesized by Serat (37).

The DNP-amino acids used to prepare standard solutions were synthesized by the method of Porter (48).

Location of the Compounds on the Developed Chromatogram

Amino acids were at first detected on paper chromatograms by spraying the paper with a 0.2 percent solution of ninhydrin in water saturated n-butyl alcohol. After heating the sprayed paper at 100°C. for seven minutes, the areas occupied by the amino acids were blue or purple.

The ninhydrin reagent of Levy and Chung (49) was later found to give reproducible differential colors with certain of the amino acids. These colors were: phenylalanine, blue-gray; tyrosine, gray; glycine, red-purple; cystine, brown; histidine, brown-purple; proline, yellow; aspartic acid, blue; leucine, isoleucine, alanine, threonine, glutamic acid, lysine, and valine, blue-purple.

A 0.2 percent solution of ninhydrin in either water-saturated butanol or absolute ethanol was prepared. Two ml. of 2,4,6-collidine and 15 ml. of glacial acetic acid were added to each 50 ml. of this solution.

The reagent was sprayed on the developed chromatogram and the sheet was heated for three minutes at 100°C. The colors were intense for five gamma quantities of each of the amino acids chromatographed. The ninhydrin-acetic acid-collidine reagent appears to be superior in that it is sensitive to very small quantities of amino acid and the colors of the individual amino acids are reproducible in every case.

PTH's were detected by spraying the paper (impregnated with a 5 percent starch solution) with a solution of a 1:1 mixture of 0.01 M iodine in 0.5 M potassium iodide and 0.5 M sodium azide. The areas occupied by the PTH's remained white against a brown background.

The DNP-amino acid derivatives are bright yellow and so can easily be seen on the paper. No reagent is required to detect their presence. Faint spots are easier to see if the paper is held between a light source and the observer.

General Procedure

Descending chromatography. The general procedure followed in preparing and developing a paper chromatogram was the same in all experiments. The paper was cut to the size required for the chromatography jar used. If the paper was to be impregnated with a buffer solution or with a starch solution, this was done and the paper was dried thoroughly. A line 6 cm. from one of the narrow ends of the paper sheet was marked with a pencil. The solutions, standards and unknowns, were spotted at intervals of 2 to 3 cm. on this line. Five lambda aliquots of the solution were applied at a time from a micropipette (Microchemical Specialties Company) until the proper amount had been added. Each five lambda aliquot was dried before another was added.

The solvent systems were always freshly prepared. The paper chromatogram was hung in the chromatography jar in the presence of the lower solvent phase to equilibrate with the

solvent. The length of time of equilibration depended upon the solvent system used.

After equilibration of the paper, the organic phase of the solvent system was placed in the trough. The chromatograms were usually developed until the solvent front had nearly reached the end of the paper. If a longer developing time than this was required, a piece of Kleenex was stapled to the bottom of the sheet and the solvent was allowed to run off the bottom.

After development, the sheets were dried, then treated with the proper reagent for location of the areas occupied by the compounds being chromatographed.

Ascending chromatography. A 46.5 x 28.5 cm. sheet of chromatography paper was cut (the original 46.5 x 57 cm. sheet was cut in half). A pencil line was marked 2 cm. away from and parallel to one of the long edges of the sheet. The standard and unknown solutions were applied at 2 to 3 cm. intervals just as was done for descending chromatography. The narrow edges of the sheet were then brought together to form a cylinder of the sheet. Ordinary staples were used to hold the edges about 0.5 cm. apart. The cylinder thus formed was then placed in the 10 x 12 in. jar with that end containing the applied substances on the bottom of the jar. After the proper equilibration period in the presence of a beaker

of the aqueous solvent phase, the organic solvent phase was placed in the bottom of the jar. The chromatogram was allowed to develop until the solvent front had just reached the top of the paper. The chromatogram was then removed, dried, and sprayed with the proper reagent to detect the separated compounds.

Silica Chromatography of DNP-Amino Acids

Apparatus

Silica chromatograms were run in glass columns made from 14 inch lengths of 12 mm. (outside diameter) glass tubing. One end was heated until the edge of the glass formed a slight constriction.

An apparatus for applying a positive air pressure of about 5 inches of mercury to the top of the column was prepared. This was needed to force the solvent system through the tightly packed silica column.

Pipettes for transferring solvent and the solutions of the DNP-amino acids to the surface of the silica columns without disturbing the surface particles were made of 6 mm. glass tubing. A rubber bulb at one end was used to fill the pipette and the other end was drawn out at a slight angle

for applying the solutions to the side of the glass column just above the silica surface.

Filter paper disks having a diameter of exactly 1 cm. were cut with a sharp cork borer from Whatman No. 50 filter paper. These were boiled in 0.1 N nitric acid for a few minutes, then dried. Just before use, the disks were wet with 2 N hydrochloric acid, blotted between pieces of filter paper, and fitted against the constriction in the end of the glass tube of the column. The silica gel was held in the glass column by this disk.

A plunger was prepared by attaching cotton to one end of a piece of 6 mm. glass tubing with a doubled piece of string. The doubled string was passed through the glass tube and tied to a match stick at the other end so that the cotton was held tightly against the tube. The quantity of cotton was such that it just fitted in the chromatography tube but at the same time would slide freely in it. This plunger was used to pack the silica column.

Preparation of the Silica Gel

One pound of sodium silicate (Baker Chemical Company, 40°-42° Be, lot 7595) was placed in a large beaker. One and one quarter volumes of distilled water were added and the

solution was cooled to 2°C. in an ice bath. Concentrated hydrochloric acid was added slowly with stirring. The solution was not allowed to warm above 10°C. during the addition of the acid. The silicate thickened to a gel which was difficult to stir but the addition of more acid caused the gel to liquefy again and finally to become a suspension of particles. The larger lumps were broken up. When the solution was strongly acid, it was left to warm slowly to room temperature. After standing overnight, an equal volume of water was added. The aqueous acid was decanted and the silica gel was washed several times with water. The washings, containing suspended fine particles of silica which would make filtration difficult, were poured off each time and discarded. The remaining particles were filtered and washed again until no more chloride ion was found in the washings. The gel particles were dried for four days at 110°C. with frequent stirring.

Preparation of the Solvents

The chloroform used in the solvent system was redistilled, then washed five times with an equal volume of water to remove ethanol. It was stored in the dark in an amber bottle.

Ethanol was allowed to stand over potassium hydroxide pellets for a few days to polymerize any aldehydes present and to remove water. It was distilled and stored in the dark in amber bottles.

Phosphate buffer of pH 3.7 was prepared by dissolving 13.8 g. of sodium dihydrogen phosphate monohydrate in water and diluting it to the 100 ml. mark in a volumetric flask. This was used as the stationary phase.

Standard Solutions of the DNP-Amino Acids

The standard solutions of DNP-amino acids were prepared as described on p. 27.

General Procedure

Three g. of the dry silica gel were ground with 1.5 ml. of the phosphate buffer (pH 3.7) until a very fine dry powder was obtained. The grinding was done in a beaker. A flat bottomed test tube was used as a pestle.

One of the 1 cm. filter disks was wetted with 2 N hydrochloric acid and blotted between filter papers. The disk was then placed in the large end of the glass column and carefully pushed to the constriction at the other end

of the column. Care had to be taken that the filter disk was perfectly flat on the inside of the column and had no creases to allow the silica suspension to run between the disk and the glass wall.

A 0.25 percent solution of ethanol in wet chloroform was used as the moving phase. The finely ground silica containing the stationary phase was suspended in a little of the moving phase and the suspension was poured into the glass column. Care was taken to let the suspension run slowly along the side of the column in order not to dislodge the filter disk at the bottom.

After the silica gel suspension had all been poured into the column, positive air pressure was applied until the suspension had descended about half-way down the column. The air pressure was removed and the column was rotated rapidly between the palms of the hands to help settle the silica gel particles evenly. Positive air pressure was then again applied until the surface of the solvent had just reached the top surface of the settled silica gel. The plunger was used to put slight pressure on the top of the column to pack the silica particles together. Too much pressure at this point was found to pack the column too tightly and the solvent ran through very slowly even with the air pressure. A small amount of the moving solvent

was placed on the column with the pipette and air pressure was applied until the solvent surface had just reached the top surface of the silica gel again. The column was now ready for the application of the DNP-amino acids. At no time during the use of the column was the solvent surface allowed to fall below the surface of the silica column. When this accidentally occurred, the silica gel cracked and a new column had to be prepared.

Between one and two micromoles of each DNP-amino acid in 0.5 ml. of moving phase were applied at one time. The insulin hydrolysates were diluted so that approximately this amount of the DNP-derivative of the terminal amino acids would be applied in 0.5 ml. of the moving phase. The DNP-amino acid solution was pressed into the column with air pressure and a few milliliters of the moving phase was carefully added to the top of the silica to wash any DNP-amino acid off the side of the glass column. After pressing this into the column, the moving phase was applied to the silica gel and forced through with pressure.

Standard DNP-amino acid solutions were first run through each newly prepared column to measure their R values. The distance of the meniscus of the solvent above the silica gel surface was measured before applying air pressure and again after releasing the pressure. The distance of the lower

edge of the yellow band formed by the DNP-amino acid was also measured before and again after releasing the pressure. The R value was calculated from these values as follows:

$$R = \frac{\text{distance moved by the DNP-amino acid band}}{\text{distance moved by the meniscus of the solvent}}$$

After obtaining R values of the standard DNP-amino acids, the unknown solution was applied and the R values of the bands formed were measured in the same manner. The R values of the unknowns were then compared with the R values of the standards to determine which derivatives were in the unknown. For further checking, the band of one of the unknown amino acids was completely eluted and mixed with some of the standard solution of the DNP-amino acid whose R value was approximately the same. The mixture was chromatographed to see if it ran as a single band. A single band indicated the derivative from the hydrolysate and the standard DNP-amino acid were the same.

It should be noted that the same standard DNP-amino acid solution had a different R value on each new silica column prepared. Preparation of a new batch of the 0.25 percent ethanol in chloroform solvent also required determining the R values of the standards again. Excess solvent

was always prepared so that the R values of the standards did not have to be determined more than once for each run.

Enough silica gel had been prepared in the one preparation reported here for all the experiments done. However, batches of silica are known to vary in their chromatographic properties even when carefully prepared by the same method (48, 50). For this reason, the R values obtained with one silica gel preparation may differ considerably from those obtained with another. Dr. W. R. Middlebrook (50) has also found that the solvent systems may have to be varied from one preparation of silica gel to another. For example, where 0.25 percent ethanol in chloroform gave the best separation of DNP-phenylalanine from DNP-valine with the silica gel preparation used for these experiments, a solution of 0.5 percent ethanol in chloroform gave the best separation of the same two DNP-amino acids on columns packed with silica gel Dr. Middlebrook had prepared by the same method.

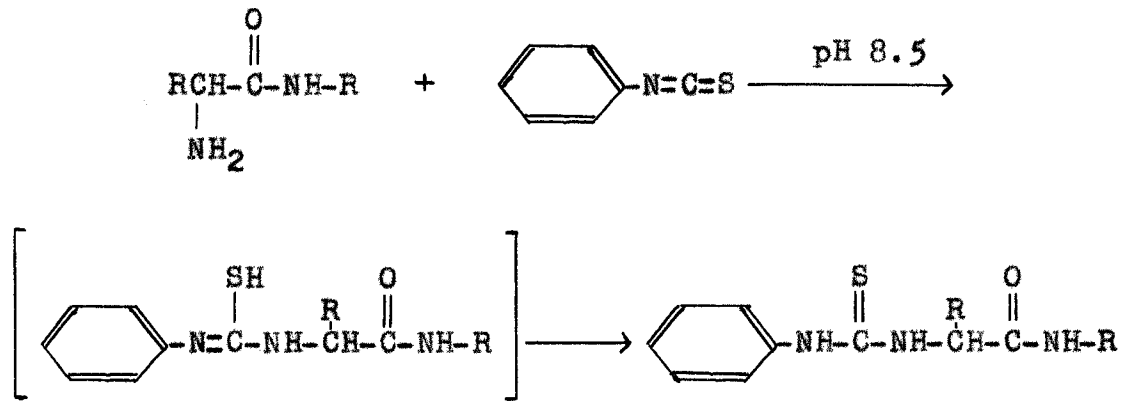
Terminal Residue and Residue Sequence Studies

Treatment with PTC

The procedure followed by Edman (7), Blaney (5), and Fox *et al.* (33) was essentially that used in these experiments.

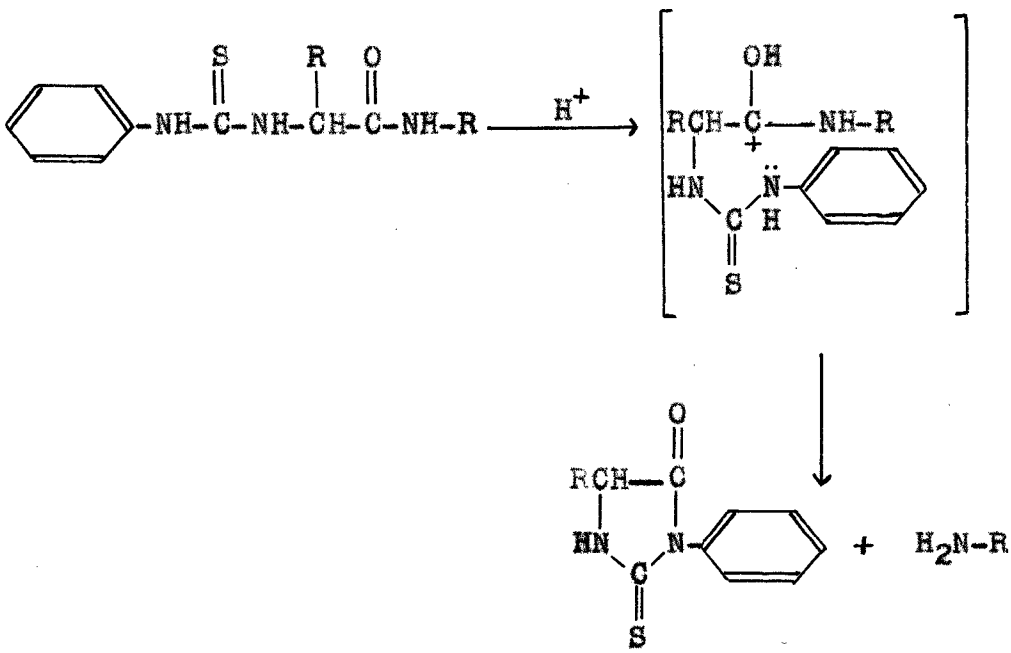
Approximately 5 mg. of insulin was weighed into a weighing bottle. One ml. of water and 1 ml. of pyridine containing 20 moles of PTC per mole of reactive groups in insulin were added to the weighing bottle. A few crystals of brom thymol blue and enough of a mixture of 1:1 pyridine and 0.1 N sodium hydroxide to turn the color of the solution blue-green were added. The blue-green solution indicates a pH of about 8.5. In some cases where more than one sample was being treated at the same time, the brom thymol blue was added to only one sample and the same number of drops of the alkali solution needed to bring that sample to a blue-green color was added to each of the others. The weighing bottle containing the sample was heated at 37°C. with frequent addition of the alkali solution to bring the color back to a blue-green because acidic by-products are released as the reaction proceeds.

When the color of the solution had not changed from blue-green within a two hour period of incubation, the reaction was considered complete. The solution was then evaporated to dryness <u>in vacuo</u> in the presence of sodium hydroxide pellets and concentrated sulfuric acid. At this point the insulin should contain a phenylthioureido group attached to each free reactive group. The groups which are known to react with PTC are free amino, phenolic, thiol, and imidazol groups. The typical reaction with the amino group follows.



Hydrolysis of the Phenylthioureido Derivative

The formation of the PTH of terminal amino acids is catalyzed by acids (5, 7).



If only the PTH of the amino acid residue to which the phenylthioureido group was attached was wanted and no further sequence studies were planned for the sample, the phenylthioureido insulin was completely hydrolyzed in 6 N hydrochloric acid in the autoclave for 16 hours.

If further sequence studies were desired, the phenylthioureido insulin was treated with anhydrous dioxane saturated with hydrogen chloride gas. Only the peptide bond of the terminal amino acid whose alpha amino group has an attached phenylthioureido group is selectively cleaved by the anhydrous dioxane-hydrogen chloride (dioxane-HCl) leaving the interior peptide bonds intact for further treatment.

After PTH formation by either method, the hydrolysate was evaporated to dryness *in vacuo* in the presence of sodium hydroxide pellets and concentrated sulfuric acid.

Subsequent Treatment

If further positions were to be studied, the dried residue of the dioxane-HCl was again treated with PTC and the complete cycle was repeated in the same manner as before.

Extraction of the PTH's

When the PTH's were to be studied, 5 ml. of 0.1 N hydrochloric acid were added to the dry residue of the hydrolysate. The PTH's were then extracted. A glass apparatus was constructed for continuous ether extraction of the PTH's directly from the weighing bottle in which the reaction had been run. Extraction for about two hours produced a colorless ether phase above the aqueous phase of the hydrolysate. The aqueous hydrolysate was on other occasions extracted with from ten to twenty 4 ml. portions of ether and two to five 4 ml. portions of ethyl acetate. The ether and ethyl acetate extracts were combined. The extracts were, in either case, evaporated to dryness.

Hydrolysis of the PTH's

The dried extracted PTH's were dissolved in a small amount of ethyl acetate or ether and transferred to a small Pyrex test tube. After the addition of 0.2 ml. of 0.25 N barium hydroxide to the test tube, it was sealed and suspended for 48 hours in an oil bath kept at 140°C. The test tube was opened and the barium was precipitated with a gentle stream of carbon dioxide or with a slight excess of oxalic

acid. The precipitate was centrifuged to the bottom of the test tube and the supernatant, containing the regenerated amino acids, was used for paper chromatography.

Some paper chromatograms of the barium hydroxide hydrolysates of PTH's streaked badly and contained yellow and orange colored spots which did not correspond to any known amino acids. These often interfered with the interpretation of results. Hydrobromic acid was therefore used to hydrolyze some of the PTH's (22, 51). The extracted PTH's were hydrolyzed with 0.2 ml. of 48 percent hydrobromic acid in a sealed tube at 150°C. for 24 hours. The hydrolysate was evaporated to dryness, dissolved in 10 ml. of water and again evaporated to dryness. The dry residue was dissolved in 10 ml. of water and extracted three times with a 5 percent solution of di-2-ethylhexylamino in chloroform to remove hydrobromic acid (51, 52). The aqueous solution was extracted three times with chloroform to remove excess amine. The aqueous phase was again evaporated to dryness, then dissolved in 0.2 ml. of water and used for paper chromatography.

Preparation of the DNP-Derivatives of the N-Terminal Amino Acids of Insulin

The procedure of Sanger (4) and Porter (48) was followed in the preparation of the DNP-derivative of insulin. The

insulin and an equal weight of sodium bicarbonate were weighed into an Erlenmeyer flask. The insulin and bicarbonate were suspended in ten times the weight of water and twice the volume of a 10 percent solution of DNFB in ethanolwas added. The flask was shaken on a mechanical shaker for two hours at room temperature. The bright yellow DNP-insulin precipitate was centrifuged and washed twice with water, twice with ethanol, and twice with ether, then dried in air.

The DNP-insulin was hydrolyzed in 5.7 N hydrochloric acid for 24 hours to completely split all peptide bonds involving valine residues. After cooling, the hydrolysate was extracted with three portions of ether. The ether extracts were combined and extracted three times with water, the water extracts being added to the aqueous hydrolysate. Both the ether extract and the aqueous hydrolysate were evaporated to dryness. The ether extract should contain the DNP-derivatives of all but the basic amino acids. The DNP-derivatives of the basic amino acids should be found in the aqueous hydrolysate residue.

EXPERIMENTAL AND RESULTS

The sequence of the amino acid residues in insulin was studied by two methods: (1) The formation of PTH's of the N-terminal and subsequent amino acid residues by the technique described in the Methods section, p. 39, and (2) the formation of DNP-derivatives of the N-terminal and subsequent amino acid residues by the technique described on p. 43.

Insulin Sample Used for Experiments

The insulin sample used for the following experiments was obtained through the generosity of Dr. W. F. White of the Armour Laboratories. The insulin sample (No. 473-53AB) was prepared by the fibril purification technique (53) from bovine pancreas in 1952. Its potency was 27 units per mg. when assayed shortly after preparation. The total nitrogen content was 15.4 percent. The sample was a portion of one reported as having been tested by Dr. Lyman C. Craig and found to be "extremely pure" (54).

Investigation of the Amino Acid Residue Sequence of Insulin by the PTH Method

Direct Paper Chromatography of PTH's

Landmann et al. (32) and Sjoquist (55) reported that PTH's could be determined by direct paper chromatography. The time-consuming hydrolysis of the substituted hydantoin could be eliminated in the sequence determinations by direct paper chromatography of PTH's. The method of Sjoquist (55) was chosen because it was sensitive to as little as 0.5 micrograms of PTH and gave better separation of the PTH's of phenylalanine and valine than the technique used by Landmann et al. (32).

Sjoquist's solvent system A (55), a mixture of 70 ml. of n-heptane and 30 ml. pyridine, was used to develop the chromatograms.

A 4.725 mg. sample of insulin was treated with PTC, completely hydrolyzed with 6 N hydrochloric acid in the autoclave, and the PTH's were extracted with ether and ethyl acetate as described in the section on Methods, pp. 38-43.

A 10 x 46 cm. sheet of Whatman No. 1 chromatography paper was impregnated with a 0.5 percent starch solution and dried. Five 5 lambda aliquots of the ether solution of the

extracted PTH's was spotted on the paper. Ten gammas each of the PTH's of phenylalanine, glycine, and valine and a mixture of five gammas of each of these three PTH's were placed at 2 cm. intervals on the sheet. The chromatogram was equilibrated for one hour, developed in the solvent system for three hours, dried at 100°C. for ten minutes, and sprayed with the iodine-azide solution described on p. 28 of the Methods section. Bleached areas for all of the PTH standards appeared immediately but no bleached area appeared for any PTH's in the area traversed by the ether extract of the hydrolysate of PTC treated insulin. The experiments were repeated with larger volumes of the ether extracts of the treated insulin hydrolysate with no better success.

In a similar experiment, 8.51 mg. of glycylphenylalanine was treated with PTC, hydrolyzed, and extracted with ether and ethyl acetate. The combined extracts were evaporated to dryness, then dissolved in 0.2 ml. of ethyl acetate. A 25 lambda portion was chromatographed with five gammas of synthetic PTH of glycine on an adjacent spot as a reference standard. Although the 25 lambdas of the extract of the treated peptide hydrolysate would have yielded 375 gammas of glycine PTH if the theoretical yield had been formed and extracted. However, no visible bleached areas appeared at the expected position. There was a faint bleached area with

an R_f of 0.68 compared to 0.24 for the PTH of glycine. The point of origin was very prominently bleached, indicating that a large amount of sulfur-containing meterial had not moved. Further attempts to determine PTH's by direct paper chromatography were abandoned.

Investigation of Bacterial Contamination in Insulin

Dr. W. R. Middlebrook (50) suggested that a minute amount of bacterial contaminant in the insulin might have provided an enzyme which hydrolyzed certain peptide bonds in the insulin. A sterile loop was used to streak some of the insulin on sterile nutrient agar (Difco Bacto Nutrient Agar, Difco Laboratories) in a Petri dish. The Petri dish was incubated at 37°C. for six days. Only a mold contaminant began to grow at one edge of the dish at a considerable distance from any of the insulin.

Sequence of Amino Acid Residues of Native Insulin

Investigation of the N-terminal amino acid residues.

Two samples of insulin, each weighing 5.07 mg., were treated with PTC in the usual way. One sample was hydrolyzed with 6 N hydrochloric acid in the autoclave. The second was

treated with anhydrous dioxane-HCl, then given a second treatment with PTC and completely hydrolyzed in the autoclave. Each sample was dried and extracted with ether and ethyl acetate. The extracted PTH's were hydrolyzed with barium hydroxide and the barium precipitated with carbon dioxide as described in the section on Methods, pp. 38-43. Paper chromatograms of the regenerated amino acids were developed in a butanol-acetic acid-water (4:1:5) solvent system. The regenerated amino acids from the N-terminal position of insulin given one PTC treatment were phenylalanine, glycine, and valine. All three spots were about as intense as those of their reference standards which contained five gammas of each amino acid. The results of the chromatogram of the amino acids regenerated from the PTH's of the N-terminal and penultimate positions of insulin were somewhat ambiguous. The 4:1:5 butanol-acetic acid-water solvent system does not separate phenylalanine and isoleucine. Aspartic acid and glycine are also listed as having the same R_f values in this system. The spot at the position of isoleucine and phenylalanine had the color of the isoleucine reference standard. The valine spot was nearly twice the size of, and more intense than, the same spot in the hydrolysate of once treated insulin. An elongated colored area occurred at the position opposite the glycine and

aspartic acid reference spots. The upper portion of the elongated area, although it had run ahead of the reference standard, appeared to have the color of the aspartic acid reference standard and the lower part that of the glycine reference standard. These results cannot be interpreted because there was no definite separation.

An experiment to study the release of amino acids as their PTH derivatives in the stepwise sequence determination was begun. Four samples of insulin were weighed into weighing bottles. Sample 1-N weighed 4.5 mg.; Sample 2-N weighed 4.6 mg.; Sample 3-N weighed 5.1 mg.; and Sample 4-N weighed 5.8 mg. All of the samples were given one treatment with PTC and anhydrous dioxane-HCl fission, then dried.

A continuous extraction apparatus (see p. 42 of the Methods section) was used for the direct ether extraction of the PTH's of Samples 1-N and 4-N from the weighing bottles in which the reactions were performed.

The aqueous residues of the two samples containing the residual insulin less the N-terminal amino acid residues were evaporated to dryness. The dry residue of Sample 1-N was used for the study of the penultimate amino acid residues by reaction with DNFB (see p. 99). The dry residue of sample 4-N was used for a second PTC treatment (see p. 62).

The extracted PTH's of Samples 1-N and 4-N were hydrolyzed with barium hydroxide and the regenerated amino acids were determined by paper chromatography of the hydrolysate after precipitation of the barium. The paper chromatography was unsuccessful. A weak glycine spot and a yellow spot opposite the valine standard appeared in the chromatogram of the PTH hydrolysate of Sample 1-N. Weak colored spots were obtained for the five gamma quantities of the standard amino acids, phenylalanine, glycine, and valine, and a glycine spot in the area traversed by the Sample 4-N hydrolysate. Such quantities of phenylalanine, glycine, and valine standards had given intense colored spots in test chromatograms. The chromatograms were repeated but were developed in an air conditioned room. On spraying the chromatogram with the ninhydrin-acetic acid-collidine reagent (49), only phenylalanine and glycine spots were prominent. A weak spot having an R_f near that of alanine appeared above the glycine spot. No evidence of valine was present.

The first treatment of a 10.0 mg. sample (Sample AT_1) of insulin with PTC was repeated. After dioxane-HCl fission, the PTH's were extracted and hydrolyzed with 48 percent hydrobromic acid. Paper chromatography of the hydrolysate in a 4:1:1 n-butyl alcohol-acetic acid-water solvent system again gave exactly the same results as shown in the photograph

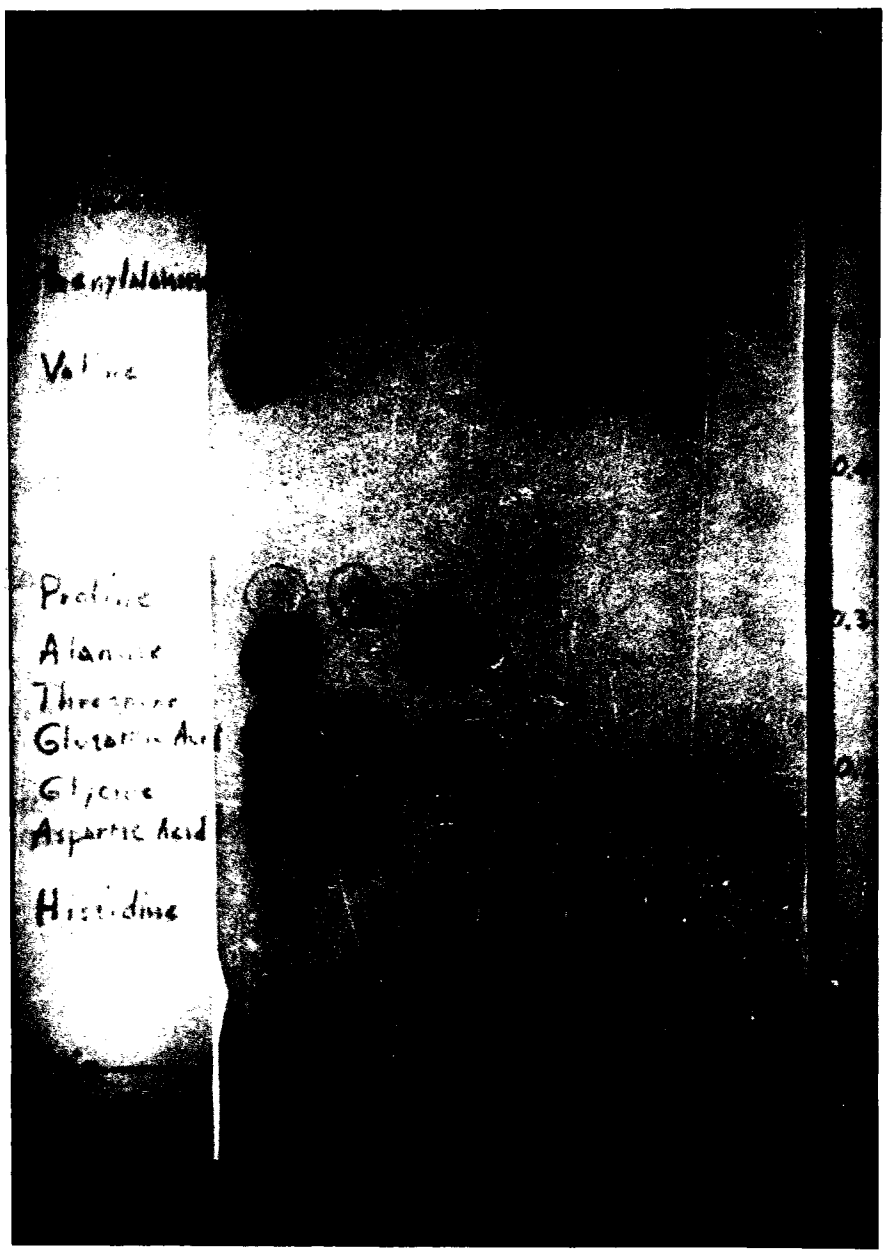
of the paper chromatogram in Figure 1. The aqueous residue remaining after the extraction of the PTH's was evaporated to dryness and used in a second PTC treatment (p. 64).

An 11.1 mg. sample of insulin (Sample 1-R) was treated with PTC and hydrolyzed with 5.7 N hydrochloric acid. The PTH's were extracted with ether and hydrolyzed with 48 percent hydrobromic acid. Paper chromatography of the PTH hydrolysate produced prominent spots for phenylalanine, valine, and glycine plus three other spots. The R_f's of the latter three spots did not match those of the isoleucine, phenylalanine, valine, alanine, tyrosine, glutamic acid, glycine, aspartic acid, or lysine standards chromatographed on the same sheet of Whatman No. 1 chromatography paper.

The PTC treatment and subsequent aqueous acid hydrolysis of insulin was repeated on a 10.0 mg. sample (Sample AT_1Ba) to determine whether the appearance of spots other than phenylalanine, glycine, and valine was due to the hydrolysis of the PTH's with hydrobromic acid. The PTH's were extracted with ether and ethyl acetate and the combined ether-ethyl acetate extracts were washed three times with water. After evaporation of the ether and ethyl acetate, the PTH's were hydrolyzed with barium hydroxide. Barium ion was precipitated with oxalic acid and the hydrolysate was spotted on a paper chromatogram. The chromatogram was developed with

Figure 1. A photograph of the paper chromatogram of the amino acids regenerated from the PTH's of the N-terminal amino acids of Sample AT_1

Chromatogram No.	Material applied to the chromatogram
1	5 gammas each of all the amino acid standards
2	5 gammas each of aspartic acid, threonine, and proline
3	5 gammas each of glutamic acid and alanine
4	5 gammas of isoleucine
5	5 gammas each of histidine, glycine, valine, and phenylalanine
6	5 gammas each of glycine, valine, and phenylalanine and 20 lambdas of Sample AT_1
7	40 lambdas of Sample AT_1


Valine
Proline
Alanine
Glycine
Aspartic Acid
Histidine

the 4:1:1 n-butyl alcohol-acetic acid-water solvent system and sprayed with ninhydrin-acetic acid-collidine reagent (49). A large blue sword shaped streak was found in the area traversed by the sample. Very prominent spots of phenylalanine, valine, and glycine were found within the sword shaped streak as determined by their positions and colors.

The PTC treatment of a 25.7 mg. sample of insulin (Sample AT_1BL) was repeated in the usual way (pp. 38-43) with two exceptions. First, the solution, after the initial reaction with PTC, was extracted ten times with about 5 ml. portions of ether in order to remove pyridine and excess PTC. The aqueous solution remaining was then evaporated to dryness. The second exception was that after the ether-ethyl acetate extraction of the PTH's, the combined extracts were washed three times with small quantities of water in order to remove any free amino acids which had been carried over. The aqueous washings were dried and used in later paper chromatography (see p. 89).

After barium hydroxide hydrolysis in the usual manner, the regenerated amino acids were chromatographed on Whatman No. 1 paper in the 4:1:1 n-butyl alcohol-acetic acid-water system. Very prominent spots appeared for phenylalanine, glycine, and valine, and weaker spots were easily detected for alanine, and an unknown ninhydrin reaction substance

having an R_f higher than that of any amino acid known to be present in insulin.

Three additional samples of insulin, one weighing 10.0 mg., one weighing 10.1 mg., and one weighing 8.8 mg., were treated with PTC in the usual manner. Pyridine and excess PTC were extracted with ether as was done with Sample AT_1BL. Aqueous acid hydrolysis and extraction of the PTH's by 20 portions of ether and five portions of ethyl acetate was done as usual. The combined extracts of the 10.0 mg. sample were washed five times with small quantities of distilled water. The PTH extracts of the 10.1 mg. sample were washed five times with small quantities of 0.1 N hydrochloric acid. The PTH extracts of the 8.8 mg. sample were not washed at all. The PTH's were hydrolyzed with barium hydroxide as usual. The aqueous and 0.1 N hydrochloric acid washings of the ether-ethyl acetate extracts of the 10.0 mg. and 10.1 mg. samples, respectively, were evaporated to dryness and used for paper chromatography (see p. 92).

Paper chromatograms of the regenerated amino acids were run on Whatman No. 1 paper. The chromatogram was developed in the 4:1:1 n-butyl alcohol-acetic acid-water solvent system. A photograph of the paper chromatograms of the 10.0 mg. and the 10.1 mg. samples is shown in Figure 2. Figure 3 is a

Figure 2. A photograph of the paper chromatograms of amino acids regenerated from the PTH's of the N-terminal amino acids of two insulin samples, a 10.0 mg. sample (sheet on the left) and a 10.1 mg. sample (sheet on the right)

Chromatogram No.	Material applied to the chromatogram
1	5 gammas each of glycine, alanine, valine and phenylalanine standards
2	5 gammas each of the four amino acid standards plus 75 lambdas of the hydrolysate of the PTH's of the 10.0 mg. insulin sample
3, 4, 5, and 6	100, 75, 50, and 25 lambdas, respectively, of the hydrolysate of the PTH's of the 10.0 mg. insulin sample
7	Same as spot no. 1
8	5 gammas each of the amino acid standards plus 75 lambdas of the hydrolysate of the PTH's of the 10.1 mg. insulin sample
9, 10, 11, and 12	100, 75, 50, and 25 lambdas of the hydrolysate of the PTH's of the 10.1 mg. insulin sample

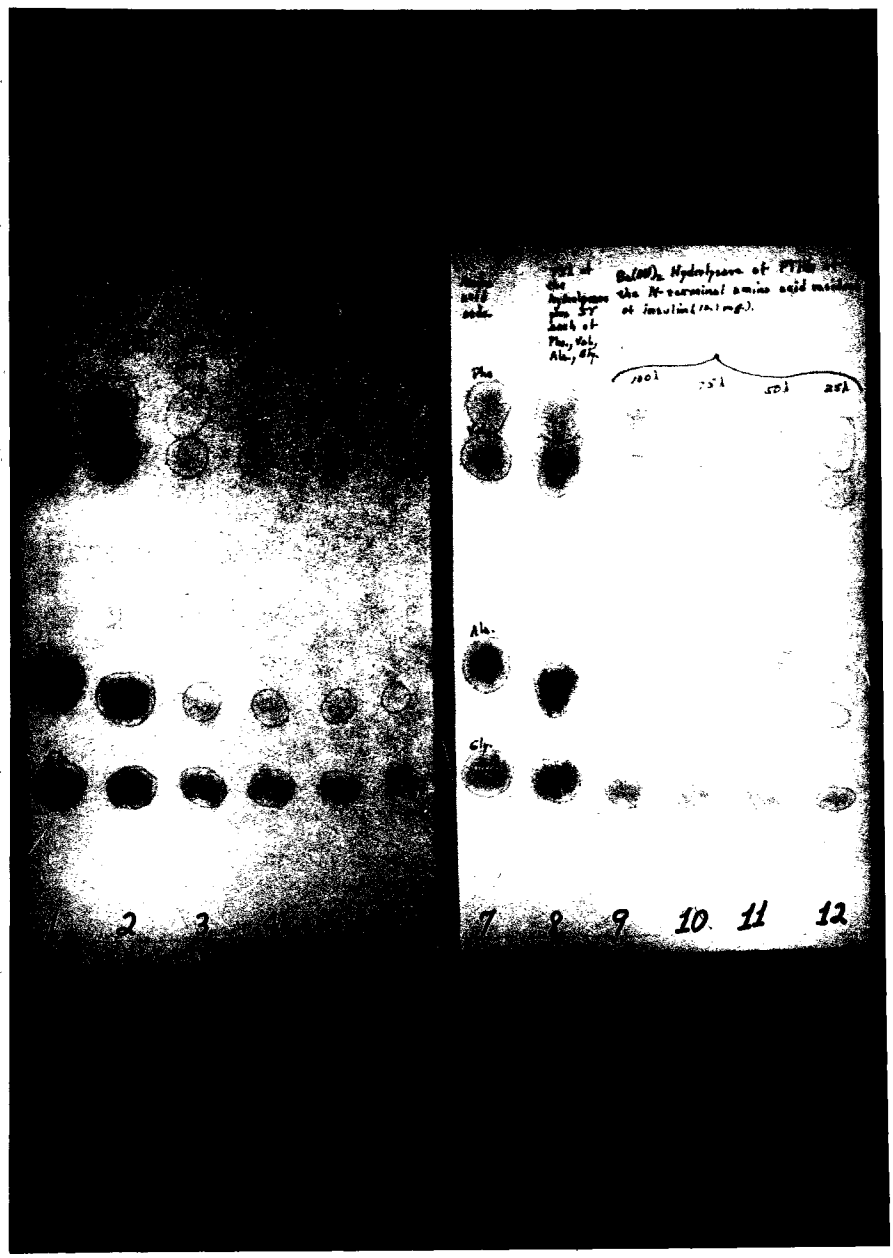
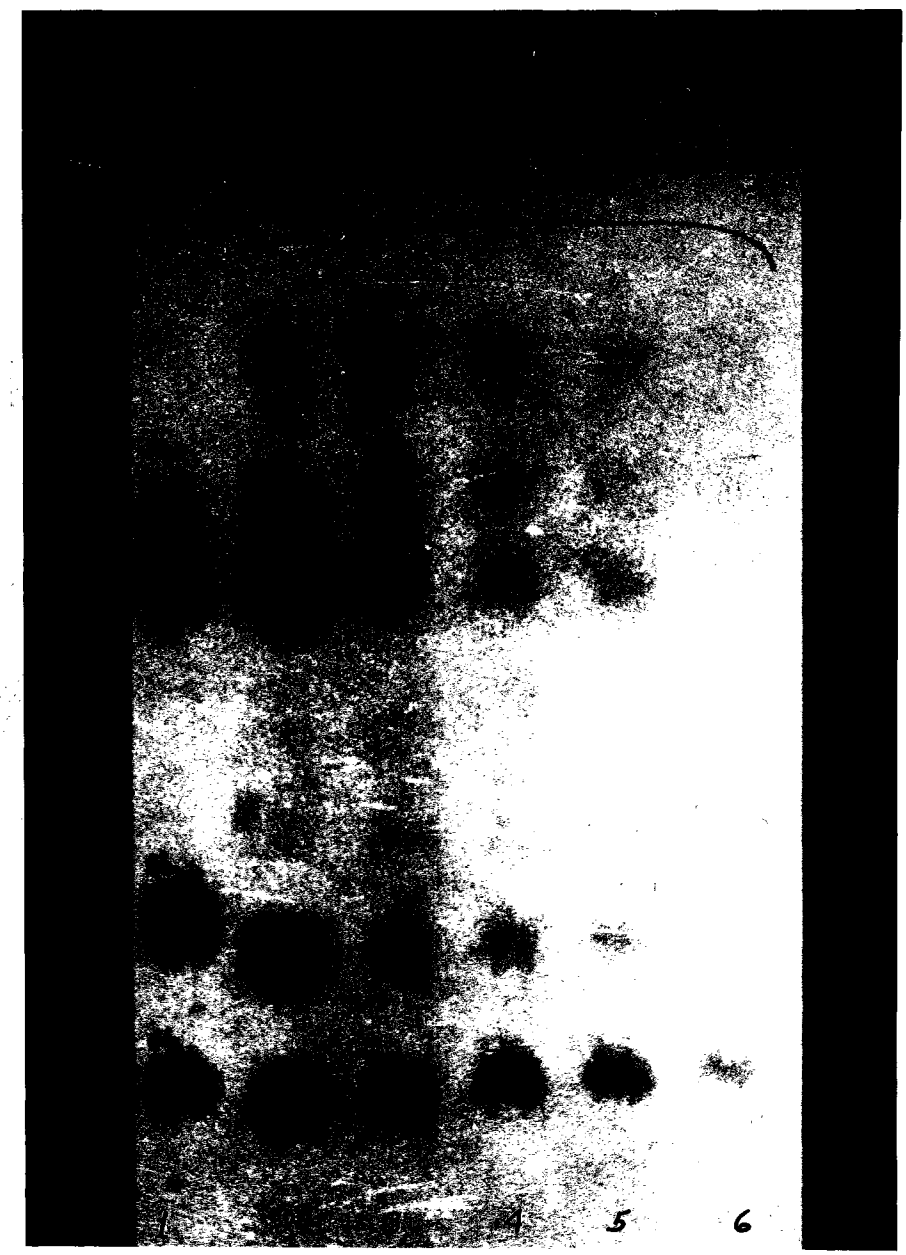

Phe
Ala.
Cly.
2
3
7
8
9
10
11
12

Figure 3. A photograph of the paper chromatogram of the hydrolysate of the PTH's of the N-terminal amino acids of an 8.8 mg. sample of insulin (see p. 56)

Chromatogram No.	Material applied to the chromatogram
1	5 gammas each of glycine, alanine, valine, and phenylalanine standards
2	5 gammas of the amino acid standards plus 75 lambdas of the hydrolysate of the PTH's of the 8.8 mg. insulin sample
3,4,5,6	100, 75, 50, and 25 lambdas, respectively, of the hydrolysate of the PTH's of the 8.8 mg. insulin sample


4
5
6

photograph of the paper chromatogram of the 8.8 mg. insulin sample.

The amino acid residues found to be present in the PTH hydrolysate of the 10.0 mg. sample of insulin were glycine, alanine, valine, and phenylalanine. The alanine spot appeared to be less intense than the phenylalanine, valine, and glycine spots when the spots of these amino acids in the four levels of hydrolysate used were compared with the spots of the five gamma standards. There appeared to be no difference in the intensity of the phenylalanine, valine and glycine spots.

Glycine, alanine, valine, and phenylalanine were also present on the paper chromatogram of the hydrolysate of the PTH's of the N-terminal amino acid residues of the 10.1 mg. sample of insulin. When the relative intensities of the spots made by the regenerated amino acids were visually compared with the relative intensities of the spots produced by the amino acid standards, the regenerated valine and alanine spots appeared to have lower intensities and thus to be present in lower amounts.

Glycine, alanine, valine, phenylalanine, and two unknown residues were regenerated from the PTH's of the N-terminal amino acid residues of the 8.8 mg. sample of insulin. One of the unknown spots had an R_f between that of alanine and

valine but did not have the color produced by tyrosine. The second unknown spot had an R_f greater than that of any amino acid known to be present in insulin.

Investigation of the penultimate amino acid residues. The dry residue remaining after extraction of the PTH's of the N-terminal amino acid residues of Sample 4-N and the dry residues of Samples 2-N and 3-N were given a second complete PTC treatment and anhydrous dioxane-HCl fission in order to produce the PTH's of the penultimate amino acid residues of these insulin samples. The PTH's of Samples 2-N and 4-N were extracted with ether by means of the continuous extraction apparatus. The aqueous residues of Samples 2-N and 4-N were evaporated to dryness. The dry residue of Sample 2-N was used for the study of the amino acid residues in the third positions on the insulin chains by reaction with DNFB (p. 99). The dry residue of Sample 4-N was used for a third PTC treatment (see p. 64).

The extracted PTH's of Samples 2-N and 4-N were hydrolyzed with barium hydroxide. The barium was precipitated with oxalic acid. The hydrolysate was used for paper chromatography on Whatman No. 1 paper in a 1:1 n-amyl alcohol-water solvent system.

All of the hydrolysate of the PTH's extracted from Sample 4-N was spotted on a chromatogram. After development

and spraying with the ninhydrin-acetic acid-collidine reagent, a large blue sword shaped streak was obtained. No interpretation of results was possible.

Thirty lambdas of the Sample 2-N PTH hydrolysate were spotted on a sheet of Whatman No. 1 chromatography paper. The chromatogram was developed in the 4:1:1 n-butanol-acetic acid-water solvent system. Treatment with the 0.2 percent ninhydrin-acetic acid-collidine reagent produced ambiguous results. No isoleucine was present. Valine and glycine may have been present although the colors of the spots were different than those of the standards. A third amino acid was indicated but the position of the spot did not correspond with that of any of the histidine, aspartic acid, glycine, glutamic acid, threonine, alanine, proline, valine, phenylalanine, and isoleucine standards.

The paper chromatography of the PTH hydrolysate of Sample 2-N was repeated with 30 and 60 lambda quantities of the hydrolysate in the same solvent system. Spots comparing with aspartic acid, glycine or glutamic acid, alanine, and valine were observed. An additional blue "V" appeared opposite the yellow spot of the proline standard. Again, no isoleucine appeared.

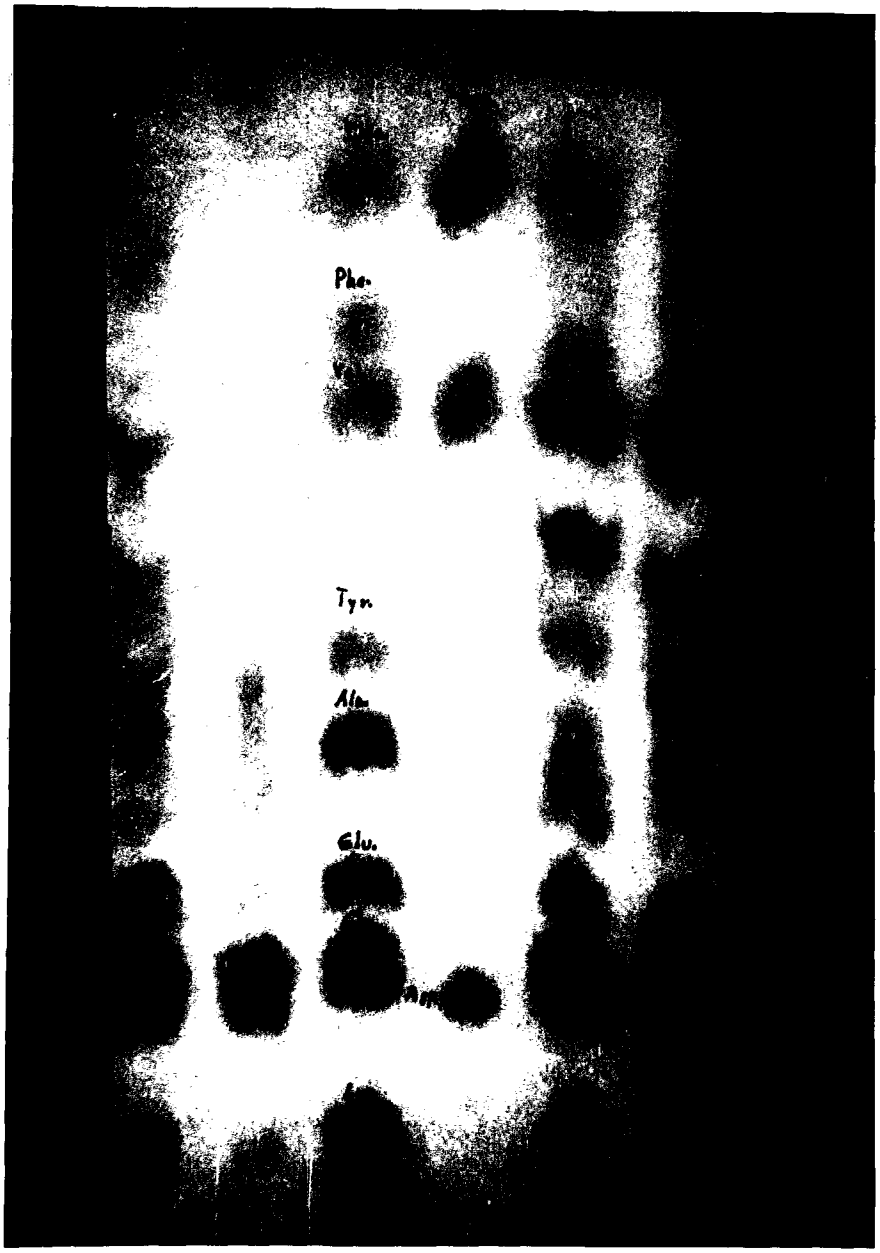
The determination of the amino acid residues in the penultimate positions in the insulin chains was repeated.

The dried residue of Sample AT_1 after extraction of the PTH's of the N-terminal amino acids (p. 52) was given a second treatment with PTC and anhydrous dioxane-HCl. The PTH's were extracted by continuous ether extraction and hydrolyzed with 48 percent hydrobromic acid. Paper chromatography of the hydrolysate in the 4:1:1 n-butyl alcohol-acetic acid-water solvent system resulted in intense spots for isoleucine, valine, phenylalanine, glutamic acid, and glycine. Weaker spots for alanine, aspartic acid, and lysine were also present as was a spot having an R_f which did not compare with that of any of the amino acids known to be present in insulin. Figure 4 is a photograph of the paper chromatogram.

Investigation of the amino acid residues in the third and fourth positions. Samples 3-N and 4-N were given a third treatment with PTC and subsequent dioxane-HCl fission. The PTH's were extracted with ether and hydrolyzed with 48 percent hydrobromic acid. The aqueous residue of Sample 3-N was evaporated to dryness and used in DNFB studies (p. 99). The hydrolysate of Sample 4-N (third PTC treatment) was chromatographed on Whatman No. 1 chromatography paper in the 4:1:1 n-butyl alcohol-acetic acid-water solvent system. Valine and glutamic acid were present in large quantities. A very large, intense spot was present in the area occupied by glycine and aspartic acid. The greater intensity of this

Figure 4. A photograph of the paper chromatograms of the amino acids regenerated from the PTH's of the amino acid residue in the second position in the peptide chains of Samples AT_2 and AT_2Ox and in the third position of Samples 4-N and 4-Ox

Chromatogram No.	Material applied to the chromatogram
1	75 lambdas of the hydrolysate of the PTH's of Sample 4-Ox
2	75 lambdas of the hydrolysate of the PTH's of Sample 4-N
3	5 gammas each of cystine, lysine, aspartic acid, glycine, glutamic acid, alanine, tyrosine, valine, phenylalanine, and isoleucine
4	5 gammas each of aspartic acid, valine, and isoleucine
5	75 lambdas of the hydrolysate of the PTH's of Sample AT_2Ox
6	75 lambdas of the hydrolysate of the PTH's of Sample AT_2


Phe.
Tyr.
Ala.
Glu.

spot was undoubtedly due to a poor separation of some glycine and a large amount of aspartic acid. Weak spots and streaks occurred between the glutamic acid and valine spots, indicating that other amino acids and/or peptides were also present. As a result of the numerous spots which appeared, it was not possible to state definitely which amino acid residues occupied the third positions in the insulin chains. The hydrolysate of Sample 3-N was not used for paper chromatography because the addition of the N-terminal and penultimate amino acid residues would have served no good purpose. The paper chromatogram is shown in Figure 4.

The residue of Sample 4-N, after the extraction of the PTH's of the amino acid residues occupying the third position in the insulin chains, was given a fourth treatment with PTC and anhydrous dioxane-HCl. The PTH's of the amino acid residues in the fourth positions in the insulin chains were extracted with ether and hydrolyzed with 48 percent hydrobromic acid. The residue remaining after the extraction of the PTH's was evaporated to dryness and used for DNFB studies of the amino acid residues in the fifth positions of the insulin chains (see p. 99). The hydrolysate of the PTH's of the amino acid residues in the fourth positions was chromatographed on Whatman No. 1 filter paper with the 4:1:1 n-butyl alcohol-acetic acid-water solvent system. The chromatogram

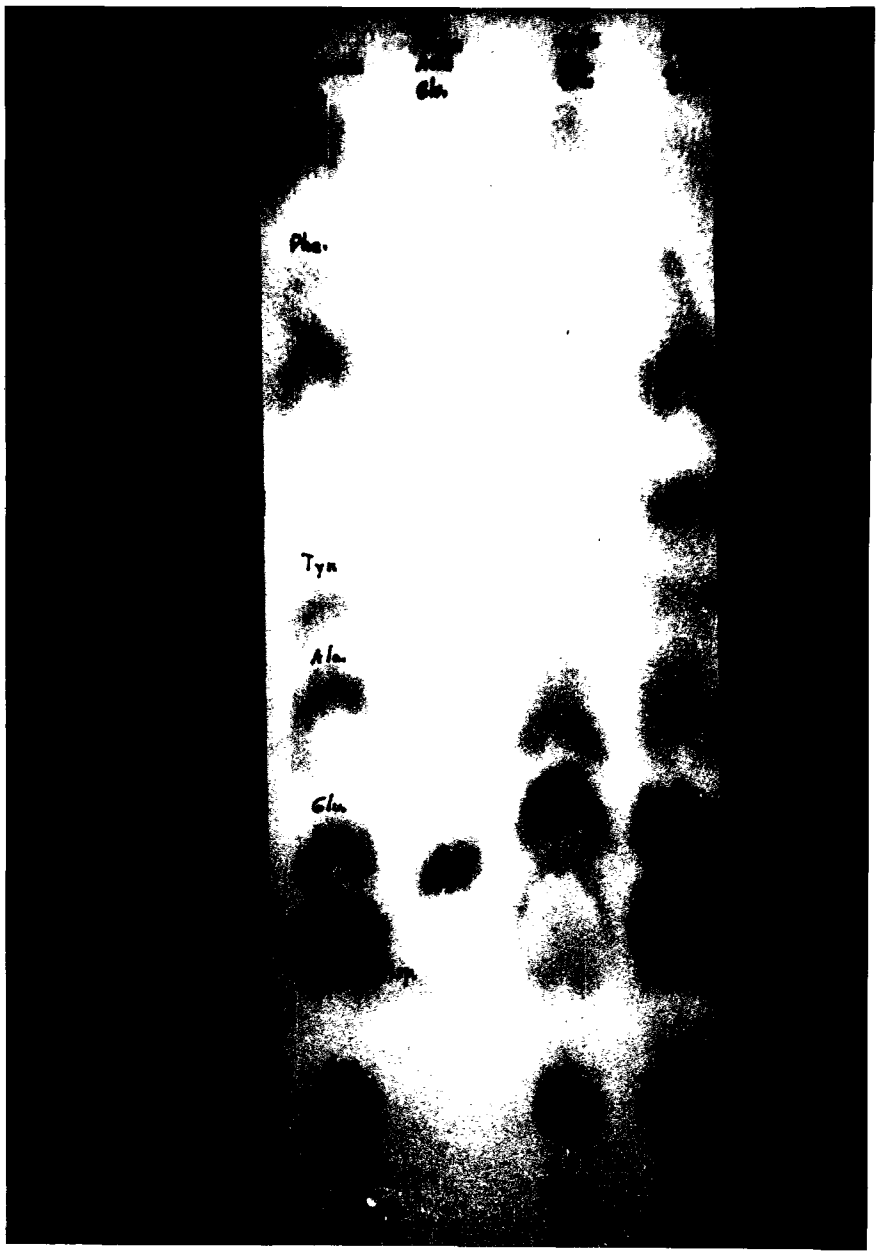
is shown in Figure 5. Numerous spots occurred. The most prominent spots were those for glutamic acid, glycine and-or aspartic acid, and lysine and/or histidine. Weaker spots of valine, alanine, and one having the R_f value but not the color of tyrosine, and faint spots for isoleucine or leucine, phenylalanine, tyrosine and cystine were present. Spots having the R_f's of none of the amino acids known to be in insulin were also visible.

Sequence of Amino Acid Residues of Oxidized Insulin

A portion of the purified insulin was oxidized by dissolving 50.1 mg. in 1.8 ml. of 88-90 percent formic acid, then adding 0.2 ml. of hydrogen peroxide. The solution was thoroughly mixed and allowed to react for 15 minutes at room temperature. The reaction mixture was concentrated to a small volume and a large volume of acetone was added. The white precipitate which formed was centrifuged and the clear supernatant was decanted. The precipitate was washed four times with large amounts of acetone, then air dried (8, 9). The yield was 50.9 mg., or 97 percent of the theoretical yield. A portion of this oxidized insulin was used for sequence studies by the PTH method. Four samples were weighed out as follows. Sample 1-Ox weighed 5.3 mg.,

Figure 5. A photograph of the paper chromatograms of the amino acids regenerated from the PTH's of the amino acid residues in the fourth position in the peptide chains of Samples 4-Ox and 4-N

Chromatogram No.	Material applied to the chromatogram
1	5 gammas each of cystine, lysine, aspartic acid, glycine, glutamic acid, alanine, tyrosine, valine, phenylalanine, and isoleucine standards
2	5 gammas of glutamic acid standard
3	75 lambdas of the hydrolysate of the PTH's of Sample 4-Ox
4	75 lambdas of the hydrolysate of the PTH's of Sample 4-N


Glu
Phe.
Tyr
Ala
Glu

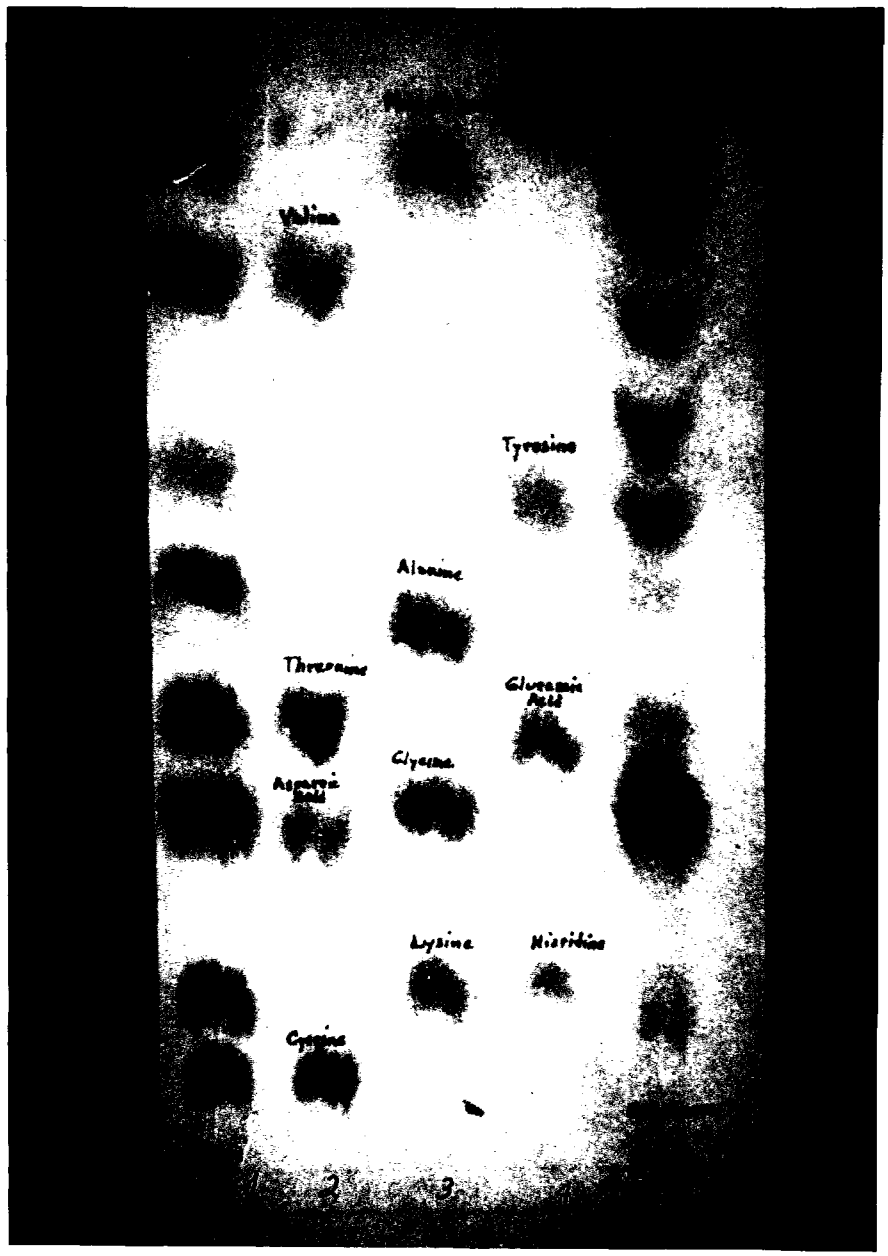
Sample 2-Ox weighed 5.5 mg., Sample 3-Ox weighed 5.6 mg., and Sample 4-Ox weighed 5.8 mg. Each sample of the oxidized insulin was treated in exactly the same way and at the same time as the corresponding numbered sample of native insulin. It will therefore not be necessary to repeat the details of the PTC and dioxane-HCl treatments, the PTH extraction and hydrolysis, or the conditions of the paper chromatography of the PTH hydrolysate. These details can be obtained by referring to the section on the treatment of the native insulin sample having the same Arabic numeral.

Investigation of the N-terminal amino acid residues.

The solution of the hydrolysate of the PTH's of the N-terminal amino acid residues of Sample 1-Ox was spilled. The paper chromatogram of the hydrolysate of the PTH's of the N-terminal amino acid residues of Sample 4-Ox indicated that phenylalanine and glycine were present. A weak spot appeared for alanine. No valine was found. The hydrolysate of the PTH's of the N-terminal amino acids of a second sample of oxidized insulin, AT_1Ox (see p. 52 for details of treatment of Sample AT_1), was chromatographed. Numerous spots appeared indicating that a large number of amino acids and/or peptides were present. Spots corresponding to phenylalanine and glycine were the most prominent. A photograph of the chromatogram is presented in Figure 6.

Figure 6. A photograph of the paper chromatograms of the amino acids regenerated from the PTH's of the amino acid residues in the N-terminal position of Sample AT_1Ox

Chromatogram No.	Material applied to the chromatogram
1	A mixture of 5 gammas of each amino acid standard applied to spots 2, 3, and 4
2	5 gammas of cystine, aspartic acid, threonine, and valine
3	5 gammas each of lysine, glycine, alanine, and phenylalanine
4	5 gammas each of histidine, glutamic acid, tyrosine, and isoleucine
5	70 lambdas of the hydrolysate of the PTH's of Sample AT_1Ox


Valine
Tyrosine
Alanine
Threonine
Glutamic Acid
Glycine
Aspartic Acid
Lysine
Histidine
Cystine

Investigation of the penultimate amino acid residues. The hydrolysate of the PTH's of Sample 4-Ox after two treatments with PTC and dioxane-HCl was found to give a large blue sword-shaped streak.

The chromatography of the hydrolysate of the PTH's of the penultimate amino acid residues of Sample AT_2Ox (residue of Sample AT_1Ox given a second complete PTC and dioxane-HCl treatment) produced prominent spots for isoleucine, valine, and glycine. Some aspartic acid was evident at the base of the glycine spot. Weaker spots were present for cystine, histidine and/or lysine, glutamic acid, alanine, and phenylalanine. At least two other weak spots were present having R_f's different than those of the amino acid standards used. Figure 4 is a photograph of the paper chromatogram.

Investigation of the amino acid residues in the third and fourth positions. The hydrolysate of the PTH's of Sample 4-Ox extracted after three PTC and dioxane-HCl treatments gave 12 ninhydrin reactive spots. Aspartic acid, glycine, and valine were very prominent, while isoleucine, phenylalanine, glutamic acid, and histidine and/or lysine spots were also visible as well as other spots which could not be assigned to any of the other amino acid standards used. See Figure 4 for a photograph of this chromatogram.

The paper chromatogram of the hydrolysate of the PTH's of Sample 4-Ox after four complete PTC and dioxane-HCl treatments also contained numerous spots. Glutamic acid was most prominent. A spot corresponding to histidine and/or lysine was quite intense. Alanine and valine were present, while weaker spots for isoleucine, phenylalanine, and at least three other spots were visible. See Figure 5.

Investigation of the Barium Hydroxide Hydrolysis of PTH's

Hydrolysis of the PTH of phenylalanine. The theoretical weight of the PTH of phenylalanine which could be formed from 6.0 mg. of insulin (molecular weight 12,000) was calculated to be 282 gammas. Therefore, 0.3 mg. of the PTH of phenylalanine (synthesized by Serat, 37) was placed in a small Pyrex test tube, 0.2 ml. of 0.25 N barium hydroxide was added and the test tube was sealed. After heating the solution for 48 hours in an oil bath at 145°C., the test tube was opened and the barium was precipitated with a gentle stream of carbon dioxide from dry ice. (This same procedure was used for all the hydrolysis of all the PTH's in this portion of the investigation.) Whatman No. 1 paper chromatography of the hydrolysate in the 4:1:1 n-butyl alcohol-acitic acid-water solvent system produced a very intense, gray to greenish blue spot for phenylalanine, an easily

visible glycine spot, a weak alanine spot, a weak supra-phenylalanine spot whose R_f was too high to be leucine or isoleucine, a weak blue spot between the alanine and phenylalanine spots, and a blue area at the base of the large phenylalanine spot which may have been due to valine.

The melting point of the synthetic PTH of phenylalanine was found to be 184.5-186°C. Edman (61) reported the melting point to be 187°C. The tan colored needles were recrystallized from glacial acetic acid (61). The snow-white crystals obtained had a melting point of 186-187°C.

A sample of the PTH of phenylalanine before recrystallization and one of the recrystallized PTH were hydrolyzed with barium hydroxide. The amino acids found by paper chromatography of the hydrolysates (Figures 7 and 8) were exactly the same for both samples. A very intense blue-green spot of phenylalanine and prominent spots for glycine and a ninhydrin reactive substance whose R_f was greater than that of isoleucine and leucine appeared on the chromatograms.

Phenylalanine (Dow Chemical Co.,), which had been recrystallized from water and found to be pure by paper chromatography, was used to synthesize the PTH of phenylalanine by the method of Edman (61). No attempt was made to recrystallize the synthetic PTH of phenylalanine because the PTH's of the N-terminal amino acids of insulin were not

Figure 7. A photograph of the paper chromatograms of the barium hydroxide hydrolysates of the PTH of phenylalanine before recrystallization (sheet on the left) and after recrystallization (sheet on the right)

Chromatogram No.	Material applied to the chromatogram
1 and 7	5 gammas each of glycine, alanine, valine, and phenylalanine standards
2 and 8	5 gammas of the four amino acid standards and 20 lambdas of the hydrolysates of the PTH of phenylalanine before and after recrystallization, respectively
3 and 9	40 lambdas of the hydrolysates of the PTH of phenylalanine before and after recrystallization, respectively
4 and 10	30 lambdas of the hydrolysates of the PTH of phenylalanine before and after recrystallization, respectively
5 and 11	20 lambdas of the hydrolysates of the PTH of phenylalanine before and after recrystallization, respectively
6 and 12	10 lambdas of the hydrolysates of the PTH of phenylalanine before and after recrystallization, respectively

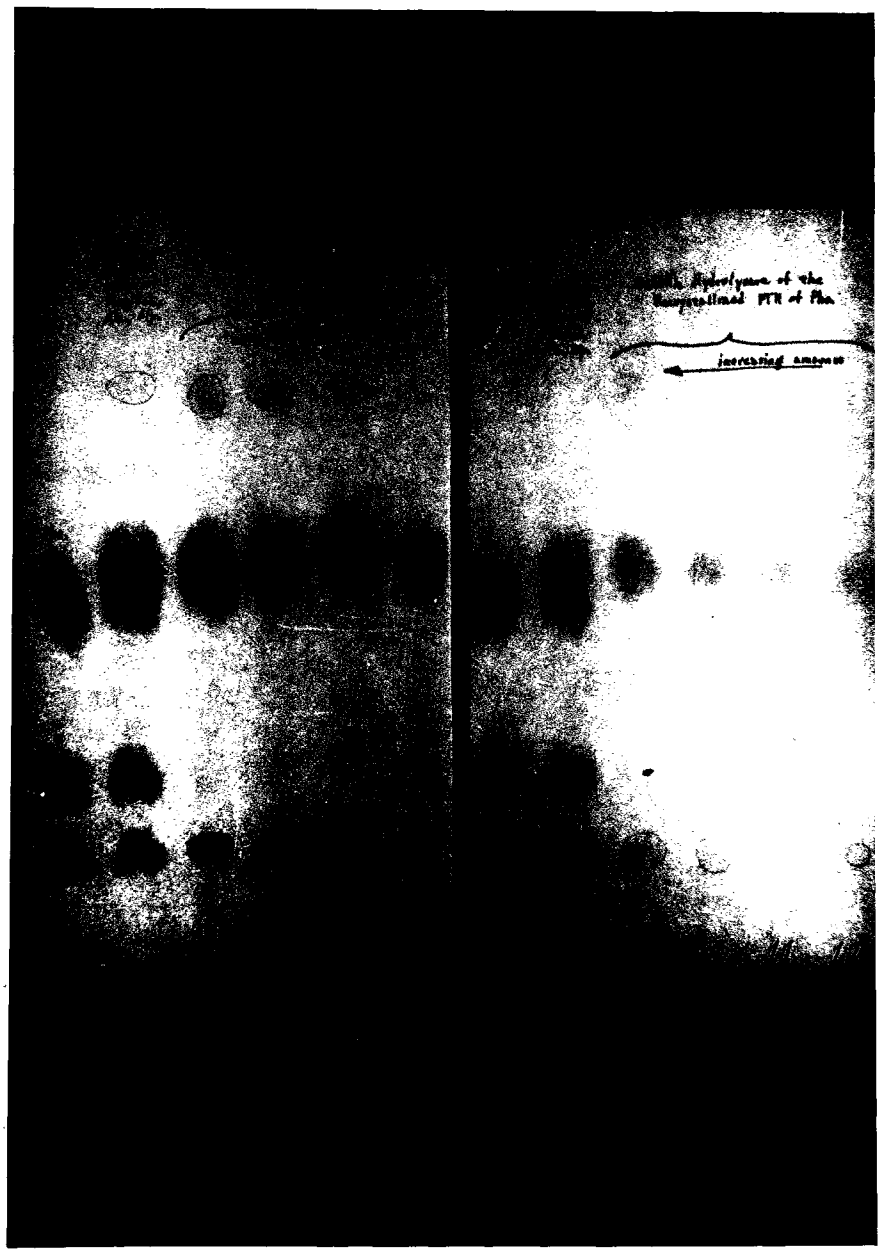
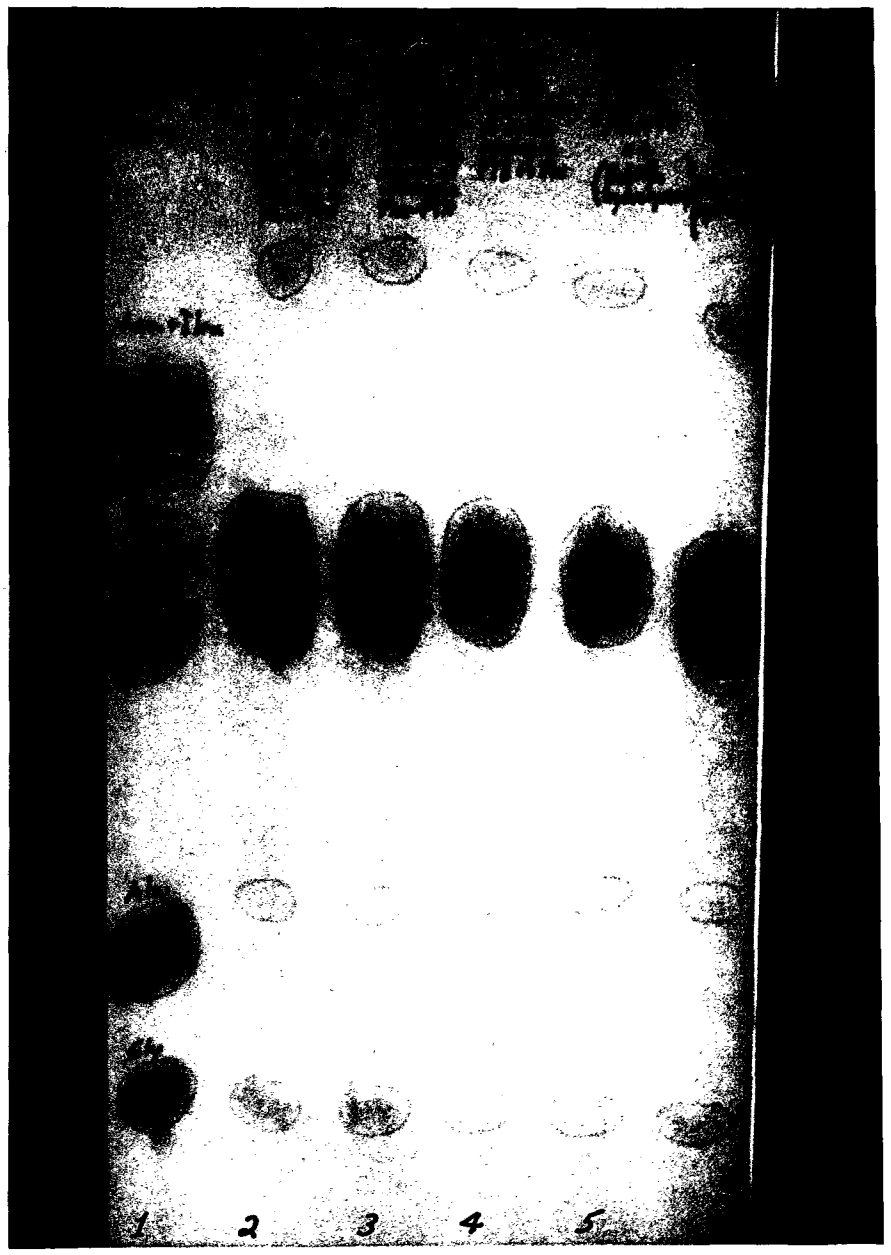

Hydrolysis of the
Unpolished PTR of Pha
increasing amount

Figure 8. A photograph of the paper chromatograms of the hydrolysate of the product of the first synthesis of the PTH of phenylalanine and the hydrolysate of two additional samples of the recrystallized PTH of phenylalanine chromatographed in Figure 7

Chromatogram No.	Material applied to the chromatogram
1	5 gammas each of glycine, alanine, valine, phenylalanine, isoleucine, and leucine standards
2	60 lambdas of the hydrolysate of a 4.5 mg. sample of the product of the first synthesis of the PTH of phenylalanine
3	60 lambdas of the hydrolysate of a 3.0 mg. sample of the product of the first synthesis of the PTH of phenylalanine
4	60 lambdas of the hydrolysate of a 1.5 mg. sample of the product of the first synthesis of the PTH of phenylalanine
5	60 lambdas of hydrolysate no. 2 of recrystallized phenylalanine PTH
6	60 lambdas of hydrolysate no. 1 of recrystallized phenylalanine PTH


1
2
3
4
5

recrystallized before hydrolysis. The residue obtained after evaporating the reaction mixture to dryness was suspended in 0.1 N hydrochloric acid and extracted three times with ether and once with ethyl acetate. The combined extracts were evaporated to dryness. The melting point of the light brown material obtained in this manner was 178-184°C.

Samples containing 1.5 mg., 3.0 mg., and 4.5 mg. of this synthetic PTH of phenylalanine were placed in test tubes. One ml. of 0.25 N barium hydroxide was added to each. The test tubes were sealed and suspended in an oil bath at 145°C. for 48 hours. The test tubes were then cooled and opened. The barium was precipitated with carbon dioxide and 60 lambdas of each of the resulting solutions was used for paper chromatography. The phenylalanine spot of each hydrolysate was intense blue-green. In addition, glycine, alanine, and a ninhydrin reactive substance having an R_f greater than that of the leucine-isoleucine standard were easily visible. The chromatogram is shown in Figure 8.

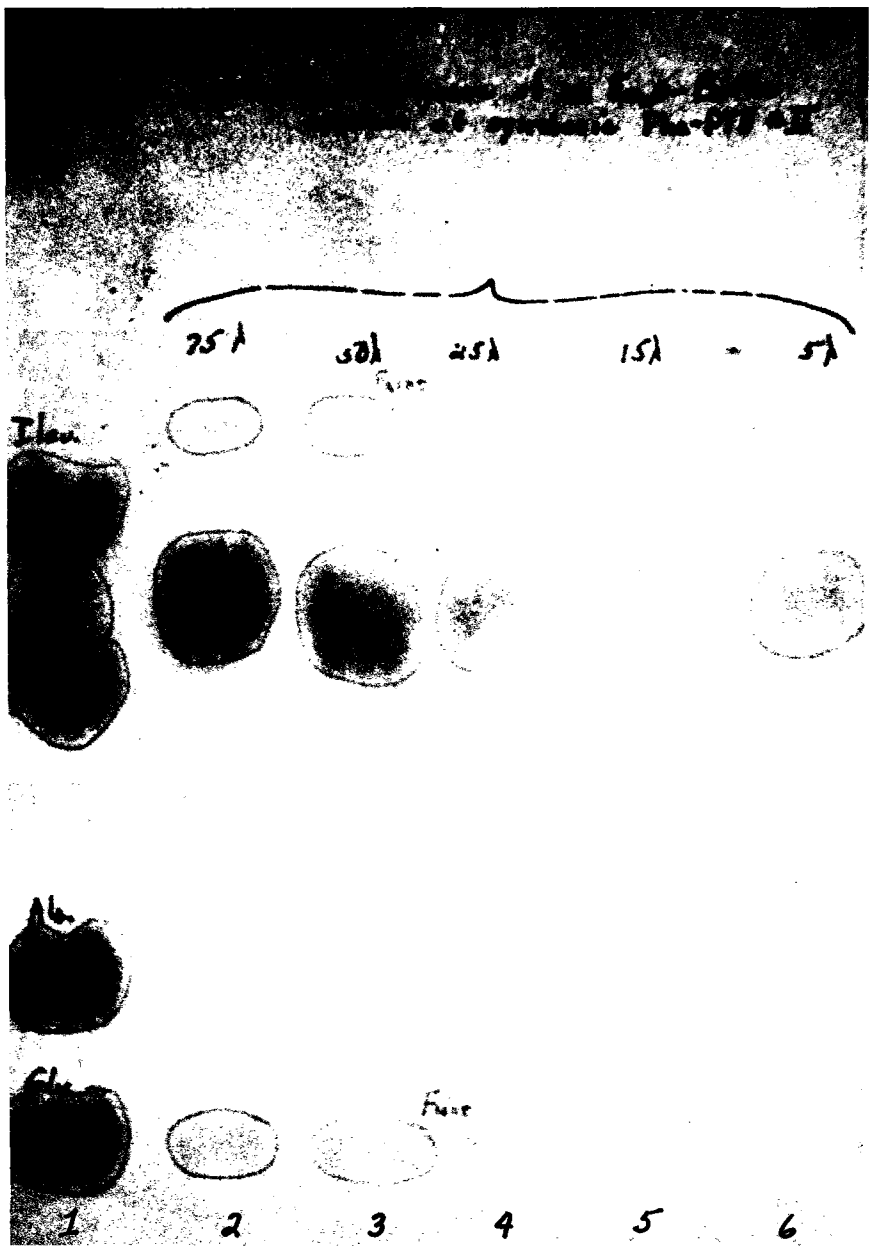
The barium hydroxide hydrolysates of two 1.5 mg. samples of the recrystallized PTH of phenylalanine (see p. 76) were chromatographed on the same sheet of Whatman No. 1 paper as the above synthetic PTH to determine whether there were variations between results of two identical hydrolysis procedures. The results were identical when 60 lambdas of each

of the two hydrolysates were chromatographed. Phenylalanine was present as an intense blue-green spot. Glycine, alanine, and a third ninhydrin reactive spot with an R_f above that of leucine and isoleucine were also easily visible.

A 1.65 g. sample of the Dow Chemical Co. recrystallized phenylalanine was used for a second synthesis of the PTH of phenylalanine by the method of Edman (61). A 1.8 mg. sample of the tan colored needles was suspended in 0.1 N hydrochloric acid and extracted ten times with ether and twice with ethyl acetate. The combined extracts were evaporated to dryness. The PTH was hydrolyzed with 1 ml. of 0.25 N barium hydroxide for 48 hours at 145°C. The hydrolysate, after precipitation of barium with carbon dioxide, was used for paper chromatography. The solution was spotted on the paper in 5 lambda, 15 lambda, 25 lambda, 50 lambda, and 75 lambda quantities. The results showed increasing intensities in the phenylalanine spots with increasing quantities of the applied hydrolysate (see Figure 9). Only the phenylalanine spot was visible in the chromatograms to which less than 50 lambdas of hydrolysate had been applied. In the 50 lambda chromatogram, however, a weak glycine spot and a weak spot with an R_f greater than that of isoleucine or leucine were plainly visible. These same spots appeared with greater intensity in the 75 lambda chromatogram.

Figure 9. A photograph of the paper chromatograms of the barium hydroxide hydrolysate of the material extracted with ether and ethyl acetate from the reaction mixture of the second synthesis of the PTH of phenylalanine (p. 82)

Chromatogram No.	Material applied to the chromatogram
1	5 gammas each of glycine, alanine, valine, phenylalanine and isoleucine standards
2 through 6	75, 50, 25, 15, and 5 lambdas, respectively, of the hydrolysate of the PTH of phenylalanine


25 λ
10 λ
5 λ
1 λ
Fluor
Fluor
1
2
3
4
5
6

Hydrolysis of the PTH of glycine. A sample of the synthetic PTH of glycine prepared by Serat (37) was used for studies of the products of its barium hydroxide hydrolysis. The theoretical weight of the PTH of glycine produced when 6.0 mg. of insulin (molecular weight 12,000) was treated with PTC and dioxane-HCl was calculated to be 171 gammas.

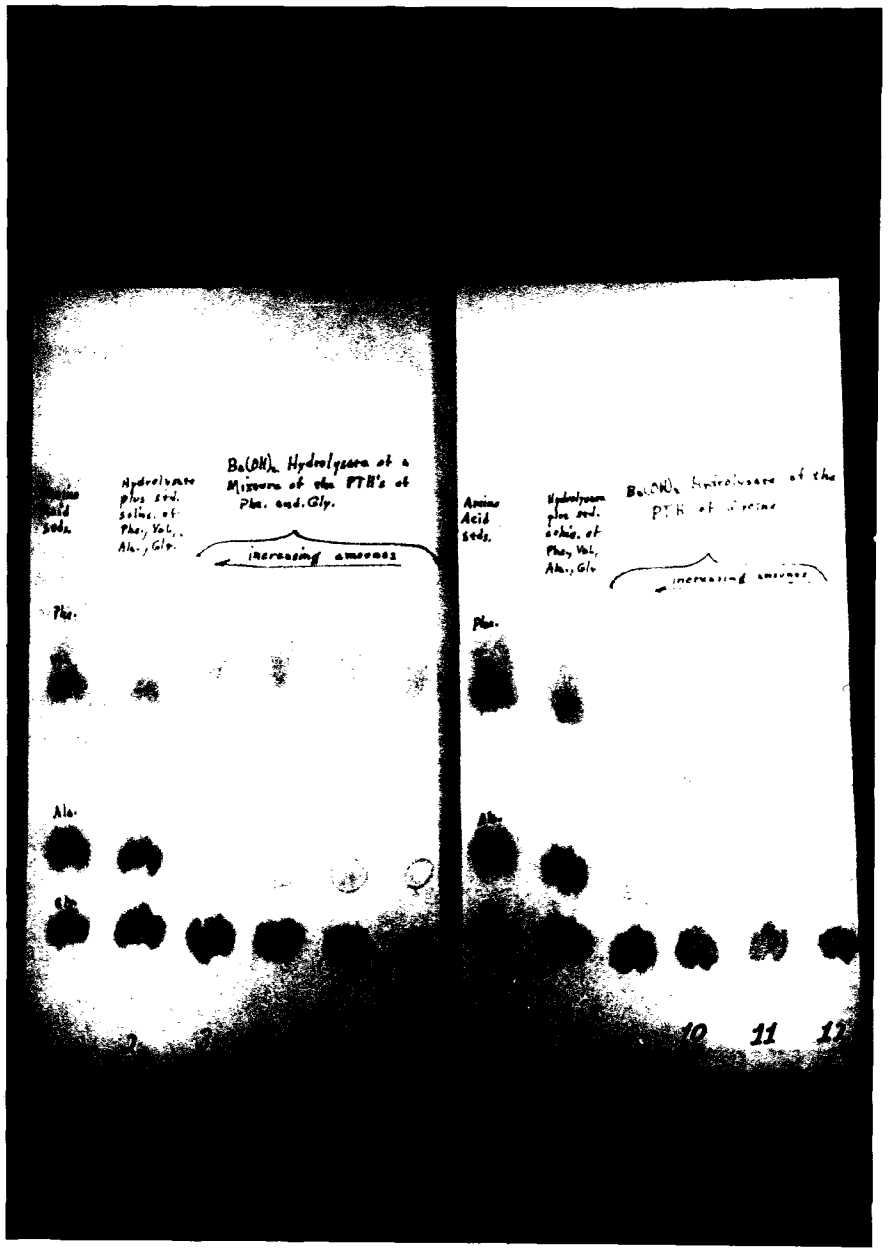
A 0.2 mg. sample of the PTH of glycine was hydrolyzed with 0.2 ml. of 1.25 N barium hydroxide and the resulting hydrolysate was chromatographed in the usual way. A very intense glycine spot and a less intense but prominent alanine spot were obtained.

The barium hydroxide hydrolysis of another 0.2 mg. sample of the PTH of glycine was repeated to determine whether the results were reproducible. Ten, 20, 30, and 40 lambdas of the hydrolysate were applied to adjacent chromatograms on the sheet of chromatography paper. An intense glycine and a prominent alanine spot were obtained from each application. The spots increased in intensity as the amount of hydrolysate applied increased as shown in the photograph in Figure 10.

Hydrolysis of mixtures of the PTH's of phenylalanine and glycine. A mixture of 0.3 mg. of the PTH of phenylalanine (m.p. 184.5-186°C.) and 0.2 mg. of the PTH of glycine was hydrolyzed with 0.2 ml. of 1.25 N barium hydroxide and

Figure 10. A photograph of the paper chromatograms of the hydrolysate of a mixture of 0.3 g. of phenylalanine PTH and 0.2 mg. of glycine PTH (sheet on the left) and of the hydrolysate of glycine PTH (sheet on the right)

Chromatogram No.	Material applied to the chromatogram
1 and 7	5 gammas each of glycine, alanine, valine, and phenylalanine standards
2 and 8	5 gammas each of the four amino acid standards plus 20 lambdas of the hydrolysates of the mixed PTH's and of the PTH of glycine, respectively
3 and 9	40 lambdas of the hydrolysates of the mixed PTH's and of the PTH of glycine, respectively
4 and 10	30 lambdas of the hydrolysates of the mixed PTH's and of PTH of glycine, respectively
5 and 11	20 lambdas of the hydrolysates of the mixed PTH's and of PTH of glycine, respectively
6 and 12	10 lambdas of the hydrolysates of the mixed PTH's and of PTH of glycine, respectively


Hydrolysate plus std. solns. of Phe, Val, Ala., Gly.
Ba(OH)2 Hydrolysate of a Mixture of the PTH's of Phe. and Gly.
increasing amounts
Phe.
Ala.
Hydrolysate plus std. solns. of Phe, Val, Ala., Gly
increasing amounts
Phe.
10
11
12

chromatographed in the usual way. Very intense phenylalanine and glycine spots were obtained in addition to prominent spots for alanine and the ninhydrin reactive spot with a greater R_f than leucine and isoleucine.

The experiment was repeated with 0.3 mg. of the recrystallized PTH of phenylalanine and 0.2 mg. of the PTH of glycine. Again, very intense phenylalanine and glycine spots and a prominent alanine spot were obtained. The fast blue spot was absent from this chromatogram. Figure 10 contains a photograph of these paper chromatograms.

Extraction or Carrying-Over of Amino Acids by the Water-Saturated Ether-Ethyl Acetate

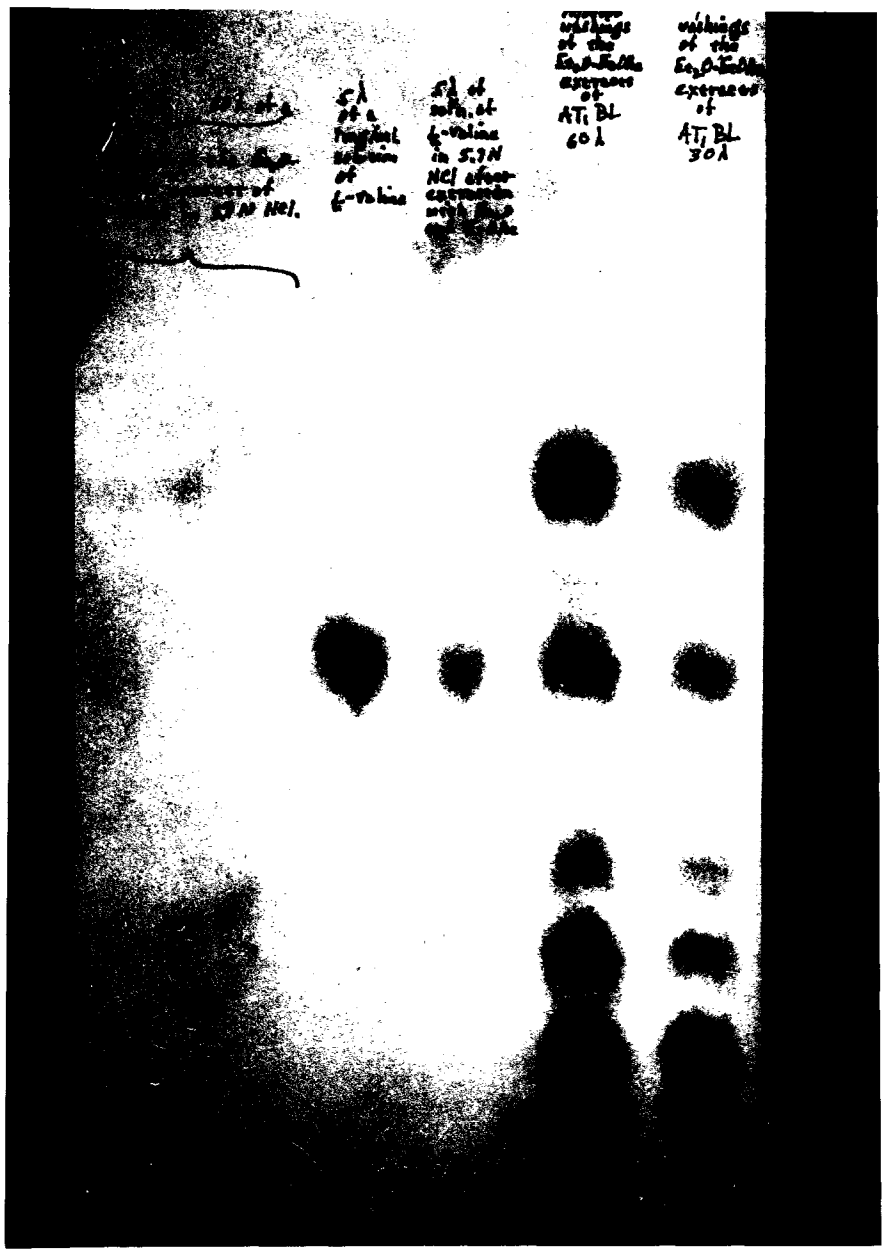
Calculations indicated that there were 585 gammas or approximately 0.6 mg. of L-valine in 6.0 mg. of insulin (molecular weight 12,000). Five times this amount, or 3.0 mg. of L-valine (Nutritional Biochemical Corporation) were dissolved in 5 ml. of 5.7 N hydrochloric acid. The solution was evaporated to dryness just as would be done with an insulin hydrolysate. The dry residue was dissolved in 10 ml. of 0.1 N hydrochloric acid and extracted ten times with ether and five times with ethyl acetate. The combined extracts were evaporated to dryness, redissolved in ether and

washed into a small test tube. The ether was evaporated. The dry residue was treated with 0.2 ml. of 0.25 N barium hydroxide and allowed to stand for two hours. The barium was precipitated with carbon dioxide and 60 and 120 lambda aliquots of the resulting solution were applied to adjacent chromatograms of a sheet of Whatman No. 1 chromatography paper. An adjacent chromatogram contained a 5 lambda aliquot of the L-valine hydrochloride solution remaining after the ether-ethyl acetate extraction and another adjacent chromatogram contained 5 gammas of L-valine. The developed and ninhydrin treated chromatogram showed a barely perceptible blue spot opposite the prominent L-valine standard and L-valine hydrochloride spots but only in the chromatogram of the 120 lambda application. Figure 11 is a photograph of the chromatogram.

Evidence was sought of the ether-ethyl acetate extraction of amino acids or the carrying-over of amino acids in the small amount of water which is always dissolved by the ether and ethyl acetate during the extraction of the PTH's from aqueous acid hydrolysates of PTC treated insulin. The aqueous washings of the ether-ethyl acetate extracts of Sample AT_1BL (p. 55) were evaporated to dryness, then dissolved in 0.5 ml. of water. Two adjacent chromatograms on a sheet of Whatman No. 1 chromatography paper were spotted,

Figure 11. A photograph of the paper chromatogram of the ether-ethyl acetate extract of a solution of L-valine hydrochloride and of the aqueous washings of the combined ether-ethyl acetate extracts of the PTH's of the N-terminal amino acids of Sample AT_1BL

Chromatogram No.	Material applied to the chromatogram
1 and 2	120 and 60 lambdas, respectively, of the ether-ethyl acetate extract of the L-valine hydrochloride solution
3	5 gammas of L-valine
4	5 lambdas of the L-valine hydrochloride solution remaining after the ether-ethyl acetate extraction
5 and 6	60 and 30 lambdas, respectively, of the aqueous washings of the combined ether-ethyl acetate washings of Sample AT_1BL

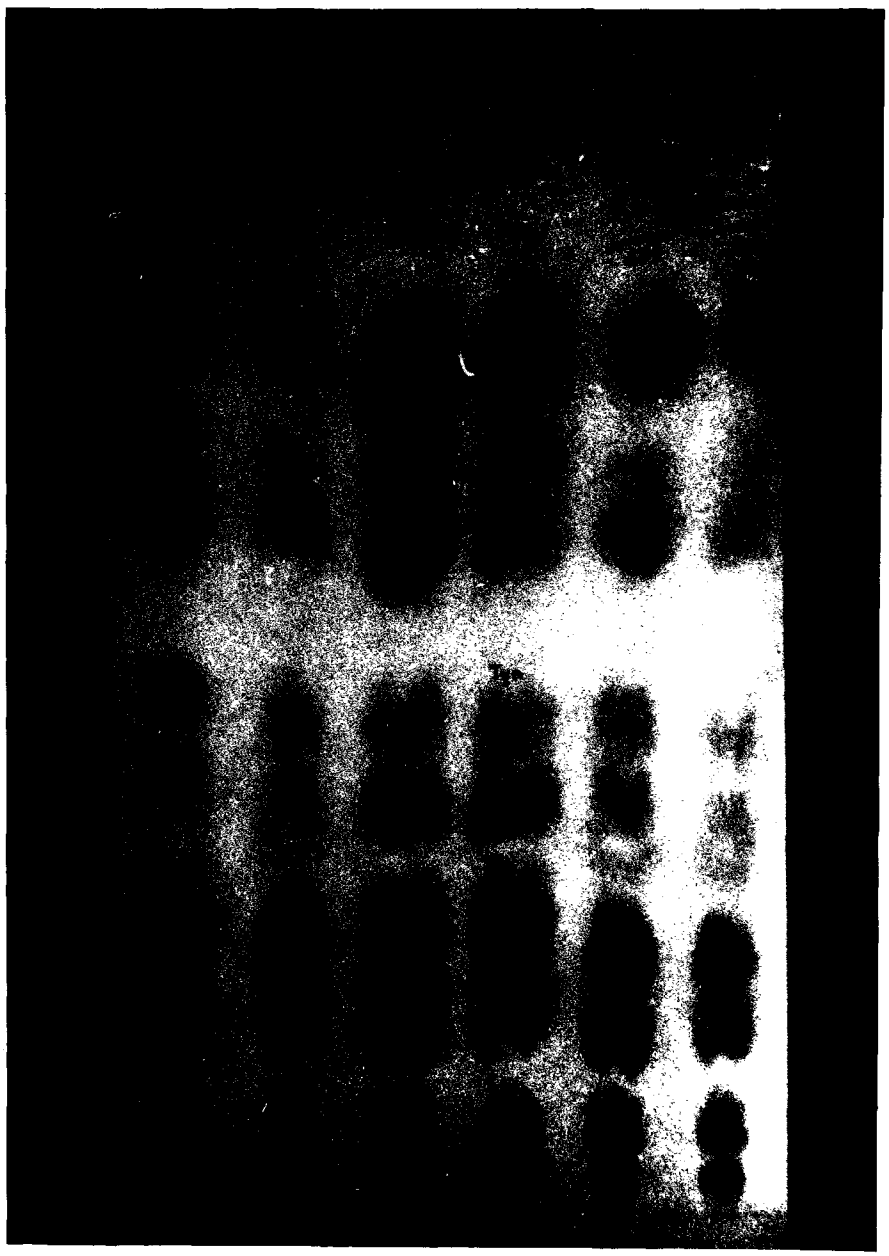

washings of the Et2O-EtOH extract of AT, BL 60 λ
washings of the Et2O-EtOH extract of AT, BL 30 λ

one with 30 lambdas and the second with 60 lambdas of this solution. The results are shown in Figure 11. The chromatogram was developed in the 4:1:1 n-butyl alcohol-acetic acid-water solvent system and sprayed with ninhydrin-acetic acid-collidine reagent. Very prominent spots were obtained for cystine, histidine and/or lysine, aspartic acid, glycine, glutamic acid and/or threonine, alanine, tyrosine, valine, phenylalanine, and leucine and/or isoleucine. These amino acids were determined by comparison of the positions and colors of their spots with amino acid standards run on previous paper chromatograms.

The paper chromatography of the distilled water washings of the ether-ethyl acetate extracts of the aqueous acid hydrolysate of a 10.0 mg. sample of insulin given one PTC treatment and of the 0.1 N hydrochloric acid washings of similar extracts of a 10.1 mg. sample of insulin (p. 56) yielded exactly the same results. Again prominent spots were obtained for all the amino acids known to be present in insulin. A photograph of the chromatogram is shown in Figure 12.

Figure 12. A photograph of the paper chromatograms of the aqueous washings of the ether-ethyl acetate extracts of the PTH's of the N-terminal amino acids of a 10.0 mg. sample of insulin and of the 0.1 N hydrochloric acid washings of the corresponding extracts of a 10.1 mg. sample of insulin

Chromatogram No.	Material applied to the chromatogram
1 and 2	60 and 30 lambdas, respectively, of the distilled water washings of the extracts of the 10.0 mg. insulin sample
3	5 gammas each of the amino acid standards of spot 4 plus 30 lambdas of the material on spots 5 and 6
4	5 gammas each of cystine, histidine, lysine, glycine, glutamic acid, threonine, alanine, proline, tyrosine, valine, phenylalanine, and isoleucine standards
5 and 6	60 and 30 lambdas, respectively, of the 0.1 N hydrochloric acid washings of the extracts of the 10.1 mg. insulin sample



Investigation of the Amino Acid Residue Sequence of Native Insulin by the DNFB Method

Sequence of Amino Acid Residues of Native Insulin

Investigation of the N-terminal amino acid residues.

A 6.159 mg. sample of native insulin was treated with DNFB in the manner described in the Methods section (p. 43). The yellow DNP-insulin derivative was hydrolyzed by refluxing eight hours in 20 percent (w/w) hydrochloric acid. The DNP-derivatives of the N-terminal amino acids were extracted with ether. The combined ether extracts were evaporated to dryness after washing with water. The dry DNP-amino acids were dissolved in a small amount of ether and washed into a 1 ml. volumetric flask. The volume was adjusted to the 1 ml. mark with ether. The aqueous phase of the hydrolysate was evaporated to dryness, washed into a 1 ml. volumetric flask and made up to volume with water. Aliquots (40 lambdas) of each were applied to sheets of Whatman No. 1 chromatography paper which had been buffered by dipping in 0.05 M potassium acid phthalate at pH 6.0 and dried. The paper was developed in a tertiary amyl alcohol-0.05 M potassium acid phthalate buffer solvent system (56) after equilibration for an hour in the presence of the lower solvent phase.

Two yellow spots were present in the area traversed by the ether extract of the hydrolysate, one diffuse, tailed spot opposite the DNP-phenylalanine standard and a compact, bright yellow spot opposite the DNP-glycine standard. DNP-Valine could have been present in the ether extract of the hydrolysate of the DNP-insulin because a mixture of DNP-phenylalanine and DNP-valine standards formed a long streak instead of separating.

A compact spot formed by ϵ-DNP-lysine was present in the area traversed by the aqueous phase of the DNP-insulin hydrolysate. A yellow streak extended about 2 cm. above the point of origin of the aqueous hydrolysate and may have been a mixture of incompletely hydrolyzed DNP-peptide derivatives of insulin.

The chromatograms were repeated twice more with 60, then 100 lambda aliquots of the ether extracts and the aqueous phase of the DNP-insulin hydrolysate. The results were always the same and no conclusions about the presence or absence of DNP-valine in the hydrolysate were obtained.

A sample of insulin weighing 29.753 mg. was treated with DNFB, hydrolyzed by refluxing 24 hours in 5.7 N hydrochloric acid, and extracted with ether. The ether extracts were washed into a 5 ml. volumetric flask and evaporated to

dryness. The dry DNP-amino acid derivatives were dissolved in and adjusted to 5 ml. with a 0.25 percent solution of ethanol in chloroform. Five-tenths ml. aliquots of the resulting solution were used for chromatography on buffered (13.8 g. monosodium phosphate monohydrate per 100 ml.) silica gel columns. A 0.25 percent solution of ethanol in chloroform was the moving phase.

The results of two runs indicated that only DNP-phenylalanine and DNP-glycine were present in the ether extract of the DNP-insulin hydrolysate. DNP-phenylalanine and DNP-valine were easily identified and separated because of their widely different R values (see p. 37, Methods) on silica columns prepared in this way.

An experiment was set up to determine whether the reaction medium for the PTC treatments denatured insulin or otherwise exposed an N-terminal valine residue. A sample of the insulin weighing 6.056 mg. was dissolved in 2 ml. of a 1:1 solution of pyridine and water. A few crystals of brom thymol blue were added and the color was adjusted to blue-green by the addition of four drops of a 1:1 solution of 0.05 N sodium hydroxide and pyridine. The solution was incubated for 25 hours at 37°C. No change in color occurred during this period. After evaporation to dryness and extraction of the residue with benzene, the dry residue was treated with DNFB

in the usual manner. The DNP-insulin was refluxed 24 hours with 6 N hydrochloric acid, extracted with ether, and the ether evaporated from the combined extracts. The dry residue was dissolved in 0.5 ml. of 25 percent ethanol in chloroform and applied to a 3.0 g. buffered (pH 3.7) silica gel column. Only DNP-phenylalanine and DNP-glycine were found to be present. No DNP-valine could be detected.

A sample of insulin was treated with quanidine-HCl, then with DNFB in order to determine whether denaturation would expose a "protected" N-terminal valine residue to reaction with DNFB. A 5.4 mg. sample of insulin was treated with 8 M quanidine-HCl (0.1430 g. in 0.25 ml. water) solution for 24 hours. The resulting solution was treated with DNFB in the usual way. The DNP-denatured insulin was hydrolyzed 24 hours with 5.7 N hydrochloric acid and extracted with ether. The ether extract, after concentration to 0.2 ml., was used for paper chromatography. The ether extract (30 lambdas) was spotted on Whatman No. 1 chromatography paper with the necessary DNP-amino acid derivatives and 2,4-dinitrophenol (DN-phenol) on adjacent chromatograms. The chromatograms were developed in a 2:1:3 n-butyl acetate-n-butyl alcohol-1 percent ammonia in water solvent system (58). DNP-Phenylalanine, DNP-glycine, and DN-phenol were identified in the ether extract of the hydrolysate of the DNP-derivative

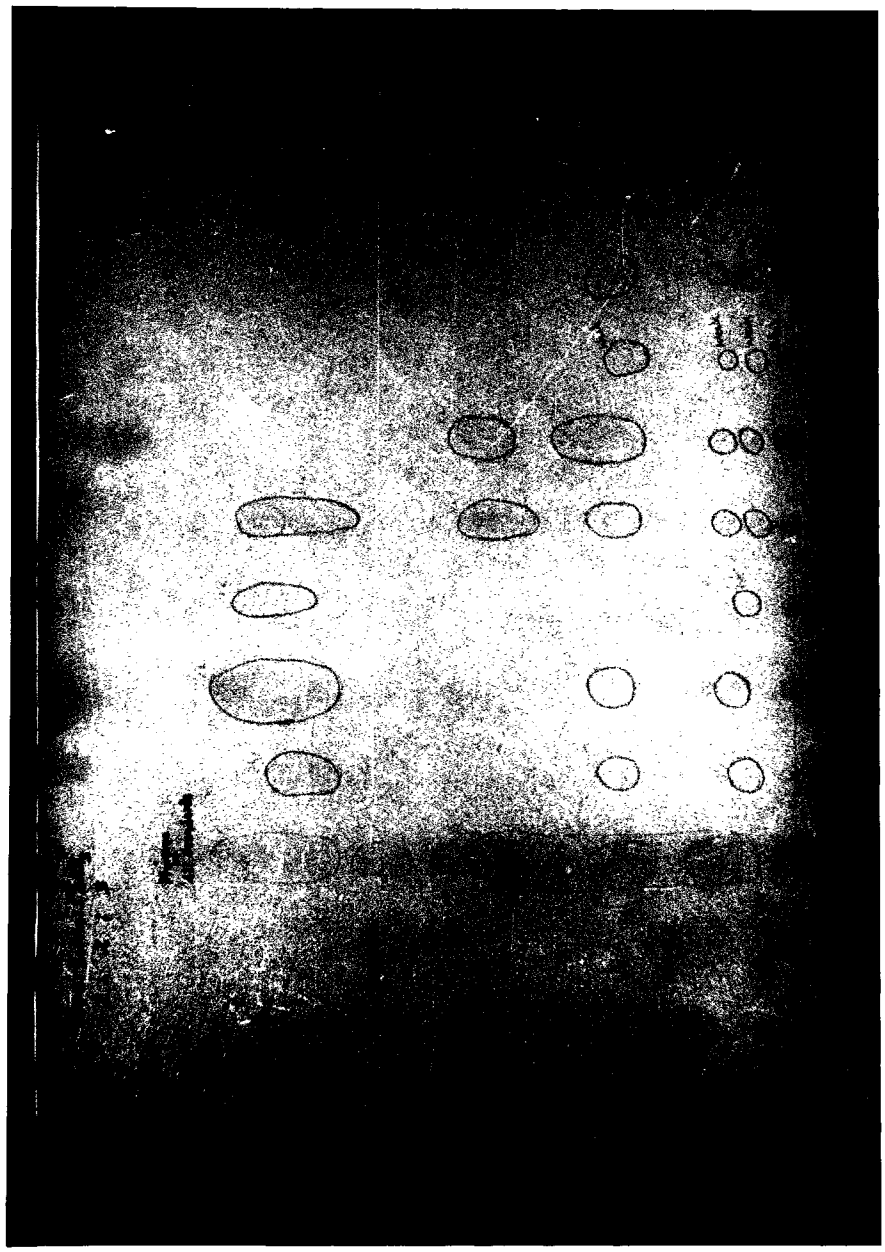
of guanidine-HCl treated insulin. Figure 13 is a photograph of the chromatogram.

Sequence of amino acid residues in penultimate, third, fourth, and fifth positions. The dry residues of samples of native insulin given one PTC and dioxane-HCl treatment (Sample 1-N, p. 45), two PTC and dioxane-HCl treatments (Sample 2-N, p. 62), three PTC and dioxane-HCl treatments (Sample 3-N, p. 64), and four PTC and dioxane-HCl treatments (Sample 4-N, p. 67) were each treated with DNFB in the usual way. The DNP-insulin residues were refluxed for 24 hours with 5.7 N hydrochloric acid. The DNP-amino acids were extracted with ether and used for ascending paper chromatography in the 2:1:3 n-butyl acetate-n-butyl alcohol-1 percent ammonia in water solvent system (58). A portion of the ether extract of the DNP-insulin hydrolysate used for silica column chromatography (p. 96) was also placed on a chromatogram adjacent to those of the above samples on the same sheet of Whatman No. 1 chromatography paper. A photograph of the chromatograms will be found in Figure 13.

DNP-Phenylalanine, DNP-glycine, and DN-phenol were present in the ether extracts of the DNP-insulin hydrolysate. DNP-Isoleucine, DNP-valine, DN-phenol and two spots, one with an R_f slightly above DNP-glycine, were found in DNP-1-N. DNP-Aspartic acid, DNP-valine, DN-phenol, and the

Figure 13. A photograph of a paper chromatogram of the DNP-amino acid derivatives of native, denatured, and oxidized insulin, and insulin samples after one, two, three, and four PTC treatments and dioxane-HCl cleavages

Chromatogram No.	Material applied to the chromatogram
1	DNP-aspartic acid (contained DN-phenol as an impurity), DNP-glycine, DNP-valine, and DNP-phenylalanine
2	DN-phenol (streaked, excessive amount applied)
3	DNP-alanine and DNP-isoleucine
4	DNP-glutamic acid, DNP-proline, bis-DNP-histidine
5	All of the above DNP-amino acids and derivatives
6	N-terminal DNP-amino acids of DNP-native insulin
7	N-terminal DNP-amino acids of the DNP-derivative of insulin denatured with quanidine-HCl
8	N-terminal amino acids of the DNP-derivative of performic acid oxidized insulin
9	DNP-amino acid derivatives of DNP-1-N
10	DNP-amino acid derivatives of DNP-2-N
11	DNP-amino acid derivatives of DNP-AT_2Ox
12	DNP-amino acid derivatives of DNP-3-N
13	DNP-amino acid derivatives of DNP-3-Ox
14	DNP-amino acid derivatives of DNP-4-N
15	DNP-amino acid derivatives of DNP-4-Ox



two spots noted in DNP-1-N with R_f's near the R_f of glycine were found in DNP-2-N. A bright yellow spot of DNP-glutamic acid, a faint spot of DNP-valine, and DN-phenol were present in DNP-3-N. A weak DNP-glutamic acid spot and one containing DN-phenol were present in the DNP-4-N. Attempts to chromatograph the aqueous phase of DNP-4-N failed because some orange-yellow substance present diffused out into the paper ruining all results.

The results of the sequence studies of native insulin by the DNFB method are summarized in Table 4 and compared with those expected from Sanger's structure (1, 2).

Table 4. Amino acid residues found in the first five positions of the insulin chains

Sample	Amino acids identified from DNP-derivatives	Expected from Sanger's structure (1, 2)
DNP-insulin	phenylalanine	phenylalanine
	glycine	glycine
DNP-1N (Two chromatograms)	valine	valine
	isoleucine	isoleucine
DNP-2-N (Two chromatograms)	aspartic acid	aspartic acid
	valine	valine
DNP-3-N	glutamic acid	glutamic acid
DNP-4-N	glutamic acid	histidine
		glutamic acid

Sequence of Amino Acid Residues of Oxidized Insulin

A 6.2 mg. sample of oxidized insulin (p. 68) was treated with DNFB and hydrolyzed in the usual way. The DNP-derivatives of the N-terminal amino acids were extracted with ether and used for paper chromatography. One paper chromatogram produced a prominent spot of DNP-phenylalanine and a weak spot of DNP-glycine. A second paper chromatogram of the ether extract produced very weak spots for both DNP-phenylalanine and DNP-glycine.

The dry residues of the PTC treated samples, 1-Ox, 2-Ox, 3-Ox, and 4-Ox were also treated with DNFB as usual. The ether extracts of the hydrolysates gave very disappointing results when chromatographed on paper. DNP-1-Ox and DNP-2-Ox produced long yellow streaks which could not be interpreted. DNP-glycine appeared to be present as a weak spot and large amounts of DN-phenol were evident. DNP-3-Ox and DNP-4-Ox both appeared to have very weak brownish yellow DNP-glutamic acid spots and prominent spots for DN-phenol (see Figure 13).

Photography of Paper Chromatograms

A Voigtlander Vito 35 mm. camera with a 2 Eastman Kodak portrait lens was used to photograph the chromatograms

in Figures 1 through 13. Plus-X film (Eastman Kodak Company, Rochester, N. Y.) was chosen because it is an easily obtained, fine-grain film which is reasonably sensitive to blue colors.

The chromatograms were placed upon the milk-glass top of a viewing box. The photographs were usually taken in a darkened room with the fluorescent lamps in the viewing box as the only light source. A meter stick was used for all measurements of distance from camera to the chromatogram. A Weston Junior photoelectric exposure meter was used to calculate all exposures. The photographs were usually taken with an f:3.5 lens opening at one twenty-fifth second.

DISCUSSION

The Appearance of Valine among the N-Terminal Amino Acid Residues of Insulin

Vigorous vs. Mild Cleavage of Peptide Bonds

Sanger (4) and Sanger and his co-workers (1, 2) found only phenylalanine and glycine as N-terminal residues of the peptide chains of insulin. The appearance of an apparent N-terminal valine residue in addition to the expected phenylalanine and glycine residues in seven experiments of the present investigation and in two during Blaney's investigation (5) disagrees with Sanger's results. Valine, however, appeared only as the result of vigorous aqueous acid hydrolysis (5.7 N hydrochloric acid at autoclave temperatures and pressures for 16 hours). Anhydrous dioxane-HCl cleavage produced the N-terminal phenylalanine and glycine residues expected from Sanger's structure (1, 2). The results of the present investigation thus indicate that valine appears among the N-terminal residues, when determined by the PTH method, only after vigorous aqueous acid hydrolysis. All other investigations of the N-terminal amino acid residues of insulin by the PTH method have provided negative evidence

to support this finding. Other investigators used mild aqueous acid cleavages, namely, 0.6-1.2 N hydrochloric acid (Fraenkel-Conrat and Fraenkel-Conrat, 27), vapors of glacial acetic acid and 5.7 N hydrochloric acid at reduced pressure (Fraenkel-Conrat, 28), and 0.1 N hydrochloric acid at 70°C. (Christensen, 29). Such mild cleavages apparently produced only the PTH's of phenylalanine and glycine. Even when somewhat more vigorous hydrolytic conditions were used, such as 2 N hydrochloric acid in a sealed tube in boiling water for one hour, (Kaiser et al., 30) or 2 N hydrochloric acid at 70°C. for two hours (Landmann et al., 32), only phenylalanine and glycine were reported. The valine found in the present investigation was apparently linked in some bond which could not be cleaved by mild conditions of hydrolysis. Other investigators who used the PTH method therefore were not able to detect its presence.

Seven possible explanations for the presence of valine among the amino acids regenerated from PTH's of the N-terminal amino acids of insulin have been considered. These are, (1) a possible third peptide chain or a side chain in insulin, (2) the cleavage of the N-terminal and adjacent peptide bonds, (3) proteolysis of the N-terminal bond by an enzyme provided by a contaminating microorganism, (4) the incomplete hydrolysis of the N-terminal peptide bond, (5) the extraction

or carrying-over of valine by the ether-ethyl acetate extraction, (6) the presence of an atypical derivative of valine, and (7) the presence of an atypical ring structure.

The Possibility of a Third Peptide Chain in Insulin

The strong valine spot on chromatograms of the barium hydroxide hydrolysate of the PTH's of the N-terminal amino acid residues of insulin can be interpreted to mean that insulin contains a third peptide chain with an N-terminal valine residue. This interpretation receives support in the work of Chibnall (40) and of Crowfoot (41). Chibnall (40) found 18 free amino groups in addition to the -amino groups of lysine per insulin molecule of molecular weight 35,500 by the Van Slyke amino nitrogen determination. The X-ray data of Crowfoot (41) showed the presence of 18 peptide chains per insulin molecule of molecular weight 36,000. The presence of six free amino groups and six peptide chains for an insulin molecule of molecular weight 12,000 is indicated by the results of the two investigations. It should further be noted that Sanger's fractionation of the performic acid oxidized insulin produced four precipitates (9). The fractionation of oxidized insulin by Andersen produced two fractions from Sanger's fraction B (44). No data concerning

the nature of Sanger's fractions X and M (9) and of Andersen's second "B fraction" (44) have been published other than to say that these were considered to be shorter peptides derived from the A and/or B chains of insulin. Comparison of the sequences of these peptides with those of the A and B fractions to determine whether these represent additional peptide chains of insulin is thus impossible. The method of precipitation of the A and B chains of insulin by Sanger (9) also made no provision for isolating any short, soluble peptides or single amino acids. It is possible that one or more soluble peptides or amino acids escaped isolation. Evidence can thus be accumulated which would support the presence of a third peptide chain in insulin, or which would at least show how a third peptide chain could have escaped detection in other investigations.

The present investigation, on the other hand, has also provided evidence that the insulin sample studied contained no peptide chain with an N-terminal valine residue. An N-terminal valine, either attached to a peptide chain, or attached to the A or B chains of insulin as a single amino acid side chain, would be expected to react with PTC. Previous investigations indicate that the PTH of valine would be formed by dioxane-HCl fission. Valine residues have been shown to react readily and quantitatively with PTC and to be

cleaved by dioxane-HCl in whole number increments by Fox, Hurst, and Itschner (6). These authors investigated the PTC reaction with several valine containing peptides, two of which contained N-terminal valine. The penultimate valine residue of lysozyme was shown by De Fontaine and Fox (63) to react quantitatively with PTC after the N-terminal lysine residue had been removed. One mole of valine was found to be unavailable for microbiological assay after cleavage by dioxane-HCl in an investigation of the amino acid residue sequence of that protein. Furthermore, DNFB studies of the N-terminal residues of insulin would be expected to show the presence of any N-terminal valine residues because valine reacts readily with DNFB to form DNP-valine. N-Terminal valine in proteins has been quantitatively determined as the DNP-valine derivative by Middlebrook (50). Another indication of the ready reaction of N-terminal valine with DNFB is that the N-terminal valine residue of the residual B chain of insulin after one PTC treatment and dioxane-HCl cleavage, and also the N-terminal valine residue in the residue of the A chain of insulin after two PTC treatments and dioxane-HCl cleavages reacted with DNFB as would be expected. The possibility that an N-terminal valine residue of insulin could escape reaction with DNFB cannot, however, be entirely discounted until Fletcher, Lowther, and Reith (23) determine the nature of the unknown DNP-derivative found as the result

of their investigation of the N-terminal amino acid residues of insulin.

Porter (64) found that DNFB would not react with all the free amino groups of native β-lactoglobulin and native serum globulin. All of the free amino groups could, however, be acetylated with ketene or acetic anhydride. DNFB reacted with all of the free amino groups when the proteins were denatured with urea or guanidine-HCl. These results suggested that a free amino group could be protected from reacting with the bulky DNFB by the folding of the protein molecule. The nitrous acid of the Van Slyke amino nitrogen determination of Chibnall (40) would also be expected to react with amino groups which the bulky DNFB could not reach. PTC contains no bulky nitro groups. In addition, the isothiocyanate group of PTC is probably no more bulky than ketene or acetic anhydride and therefore may be able to "reach into" the folds of the insulin molecule to react with an N-terminal valine residue which is protected from reaction with DNFB. Experiments were set up to determine whether such a possibility existed in insulin.

Only DNP-phenylalanine and DNP-glycine were isolated by silica column chromatography when an insulin sample was dissolved and allowed to stand for 25 hours in 1:1 pyridine-water (the solvent system used for the PTC treatment), then

treated with DNFB. The same DNP-amino acid derivatives were obtained when insulin, denatured in 8 M guanidine-HCl, was treated with DNFB.

Oxidation of the disulfide bonds of insulin with performic acid to form cysteic acid residues would be expected to release all peptide chains held together by these bonds. An N-terminal valine residue of a third peptide chain would thus become more susceptible to reaction with DNFB. If the peptide chain existed as a side chain of one of the longer A or B chains, it still might become more available to reaction with DNFB after performic acid oxidation. When, however, performic acid oxidized insulin was treated with DNFB, only DNP-phenylalanine and DNP-glycine were isolated.

On the basis of the evidence accumulated during the present investigation, there does not appear to be a third peptide chain having an N-terminal valine residue, nor does there appear to be a single valine residue as a side chain to one of the long peptide chains. The evidence presented does not rule out the possibility of a third peptide chain or single residue side chain having an amino acid residue with a free amino group which breaks down to a valine residue. Such a possible structure will be discussed later.

Cleavage of the N-Terminal and the Adjacent Peptide Bonds

Fraenkel-Conrat reported that direct paper chromatography of the PTH's of PTC treated insulin, oxytocin, and alpha corticotropin indicated that "the N-terminal and adjacent bonds were split through the first five to seven steps with little evidence of non-specific splitting" (28). Fraenkel-Conrat appears to mean that the cleavage reaction used in his investigation not only splits the N-terminal peptide bond, to form the PTH's of the N-terminal amino acids, but also splits the adjacent bonds to release the penultimate residues as free amino acids. The data presented in the paper do not support any but the expected cleavages. Because the author has provided such meager information in his brief report (28), it is possible that his statement has been misinterpreted. The results of the present investigation were examined to determine whether such a cleavage could be a possible explanation for the apparent appearance of an N-terminal valine residue. An examination of the N-terminal amino acid sequences for the two peptides of insulin as proposed by Sanger (1, 2) and apparently confirmed by the work of Fraenkel-Conrat (28) shows that isoleucine must be released as well as valine if the N-terminal and adjacent bonds are split. Furthermore, the free valine hydrochloride

Phe.-Val.-Asp.-Glu.-His.-

Gly.-Ileu.-Val.-Glu.-Glu.-

would have to be extracted from the aqueous solution of the hydrolysate. The investigation of the ether-ethyl acetate extraction of L-valine hydrochloride, under the same conditions used to extract PTH's and chromatograph the regenerated amino acids of the PTH's, resulted in a very faint blue spot indicative of L-valine. The initial quantity of L-valine hydrochloride was five times that of a 6 mg. insulin sample, and, in addition, twice the usual amount of solution was applied to the chromatogram. It is thus unlikely that free valine hydrochloride can be extracted with the PTH's in sufficient quantity to be visible on chromatograms.

The method of cleavage used by Fraenkel-Conrat (28) was to place the filter paper containing the PTC treated protein in the bottom of a desiccator which also contained a beaker of glacial acetic acid and one of 5.7 N hydrochloric acid. The desiccator was then evacuated to 100 mm. Under such conditions, the cleavage of bonds other than the N-terminal bonds is not surprising because this is essentially a mild aqueous acid hydrolysis. Perhaps glacial acetic acid saturated with hydrogen chloride or anhydrous dioxane-HCl would have given selective fission of the N-terminal bond.

The DNFB treated residues of each step of the PTC treatments in the present investigation yielded only the expected DNP-amino acid derivatives. It is evident, therefore, that the anhydrous dioxane-HCl cleavage gives selective fission of the N-terminal peptide bond to release only the amino acid residue containing the attached phenylthiocarbamyl group (see p. 40).

Proteolytic Enzyme Provided by a Contaminating Microorganism

The possibility was suggested (50) that the insulin preparation used in this investigation was contaminated with a minute amount of a microorganism which provided a proteolytic enzyme to cleave some of the N-terminal phenylalanine and/or the N-terminal glycyl-isoleucine from the B and A chains, respectively, of insulin. Valine residues would thus become N-terminal amino acids of one or more peptide chains. No growth of a contaminating microorganism was obtained when some of the insulin sample was streaked on nutrient agar and incubated for six days at 37°C. Furthermore, any such enzymatic hydrolyses would have become apparent when a native insulin sample and the residues of the first PTC treatments of insulin were analyzed for terminal residues by

the DNFB method. Only the expected DNP-amino acid derivatives were isolated.

The Incomplete Hydrolysis of the N-Terminal Peptide Bond of the Insulin B Chain

The possibility was suggested (50) that the peptide bond between the N-terminal phenylalanyl-valine dipeptide of the insulin B chain was not completely cleaved during the aqueous acid hydrolysis after PTC treatment. This incomplete hydrolysis would have left a phenylthiocarbamyl-L-phenylalanyl-L-valine residue in the aqueous acid hydrolysate. The phenylthiocarbamyl derivatives of short peptides have been found by Dahlerup-Petersen *et al.* (65) to be soluble in the solvents used to extract PTH's from aqueous solutions. DNP-Aspartic acid should have been isolated from the hydrolysate of the DNP-derivative of the residue remaining after one PTC treatment and dioxane-HCl cleavage if such a partial hydrolysis had occurred. DNP-Aspartic acid was not isolated and therefore such an incomplete cleavage could not have occurred.

The Extraction or Carrying-Over of Valine and Other Amino Acids

The ether-ethyl acetate extraction used was investigated when numerous amino acid residues appeared on the chromatograms of the hydrolysates of PTH's extracted from the second, third, and fourth PTH treatments and dioxane-HCl fissions of insulin (see Figures 4, 5, and 6). The 0.1 N aqueous acid solution from which the PTH's of the N-terminal amino acid residues were extracted always decreased in volume during the ten to 20 ether and two to five ethyl acetate extractions. The ether, and especially the ethyl acetate, dissolved and thus carried over a certain amount of the aqueous solution on each extraction. Some of the aqueous acid solution of the continuous ether extraction was also carried over into the ether extracts. The volume of aqueous acid solution carried over in the latter case was much less than in the former.

The paper chromatograms of the aqueous washings of the combined ether-ethyl acetate extracts of the N-terminal amino acid residues of Sample AT_1BL and of a 10.0 mg. sample of insulin, and the 0.1 N hydrochloric acid washings of the similar extracts of a 10.1 mg. sample of insulin (see pp. 56-61 and 92, and Figures 11 and 12) showed quite conclusively

that the aqueous solution carried over during these extractions did contain free amino acids. Unfortunately, no more continuous ether extractions were available to determine whether free amino acids were carried over during continuous extraction or not.

These results emphasize the need for care in the way the ether-ethyl acetate extracts are handled. If the unwashed extracts had been transferred directly into a test tube for barium hydroxide hydrolysis, sufficient amounts of all the amino acid residues found in insulin would have been carried along to give easily visible spots on paper chromatograms. In the present investigation, however, the ether-ethyl acetate extracts were always evaporated to complete dryness. The dry residue was then redissolved in dry ether (dried over sodium). The resulting ether solution was transferred to the test tube for barium hydroxide or hydrobromic acid hydrolysis. It would therefore be necessary for the free amino acids (present as their hydrochlorides) to dissolve in the dry ether. This is highly unlikely. The results of the attempt to extract L-valine hydrochloride from 0.1 N hydrochloric acid solution with ether and ethyl acetate demonstrated that the evaporation to dryness and redissolving in dry ether would not transfer sufficient L-valine hydrochloride to be visible on the paper chromatogram

unless much larger quantities of insulin were used. Starting with five times the quantity of L-valine hydrochloride present in insulin, approximately twice the amount of solution usually applied to paper chromatograms was necessary on the chromatogram shown in Figure 11 to give a barely visible valine spot. The very faint spot, too faint to photograph well, indicated that the volume of the extract applied to the paper contained a concentration of valine barely over the threshold quantity necessary to produce a visible spot. The apparent appearance of an N-terminal valine residue is not likely to be due to extraction of this particular amino acid residue by ether and/or ethyl acetate. If the appearance of an apparent N-terminal valine residue were due to the carrying-over of the amino acid with the aqueous phase during ether-ethyl acetate extraction, all the other amino acids present in insulin would be expected to appear on the chromatograms also. Their spots on the paper chromatograms would be expected to be as intense as valine. This does not happen (Figures 2 and 3).

The Presence of an Atypical Derivative of Valine

One explanation for the appearance of an apparent N-terminal valine residue by the PTC method might be that an

atypical derivative of valine is bound in the insulin peptide chains by means of its carboxyl group and a reactive group of its side chain leaving its alpha amino group free. This atypical valine derivative, though it would thus be free to react with the PTC, would have to be bound in the insulin molecule by a linkage which would not be split by mild aqueous acid hydrolysis or dioxane-HCl cleavage. The bond would have to be one which could be split by vigorous acid hydrolysis with either degradation of the resulting PTH to that of valine or the degradation of the PTH or its regenerated amino acid to valine during barium hydroxide hydrolysis. Penicillamine, for example (and offered only as an example), could be attached through a disulfide linkage as a side chain. The penicillamine carboxyl group could be attached to serine, threonine, tyrosine or cysteine by an ester linkage. The disulfide linkage would be stable to acid hydrolysis but would be broken and the penicillamine would undergo dismutation to valine upon the alkaline hydrolysis of the PTH's. Another atypical residue which might be considered is -, '-dimethyl aspartic acid. One carboxyl group of such an amino acid could be attached through a

$$HOOC - C(CH_3)_2 - CH(NH_2)-COOH$$

peptide linkage to one of the insulin chains. The second carboxyl group could be attached through an ester linkage to a hydroxyl group of a serine, threonine, or tyrosine residue. The amino group would be free to react with PTC. The gamma carboxyl group of this amino acid would decarboxylate easily to form valine. However, one would expect, in this case, to isolate DNP-valine on treatment with DNFB. Other similar linkages could no doubt be suggested. No positive evidence for the presence of such atypical substances can, however, be offered as the result of this investigation.

The direct paper chromatography of PTH's was unsuccessful. The results of such chromatograms could very well have established the reason for the appearance of valine among the N-terminal amino acid residues under the conditions used in this investigation. The failure of these paper chromatograms was most likely due to failure to form and/or extract the PTH's of the N-terminal amino acids. The successful use of direct paper chromatography of PTH's has been reported by four investigators (28, 32, 55, and 66). Further work with the PTC reaction and the better methods of extraction used later in the investigation would probably have given successful results if the trials had been repeated.

The fact that no evidence for atypical amino acid residues has been found as the result of DNFB studies during the present and other investigations may be offered as evidence against such a structure. There are, however, no data concerning the reactions of DNFB with any substances other than the typical amino acid residues. To carry on the example of the penicillamine structure proposed above, Porter (48) reports a 75 percent destruction of bis-DNP-cystine during hydrolysis in boiling 5.7 N hydrochloric acid for 12 hours. It is quite possible that nearly all of a DNP-derivative of the proposed structure would be destroyed during the hydrolysis of the DNP-insulin. If any remained, the quantity could quite conceivably be below the visible threshold for both paper and silica column chromatography. Furthermore, the bulky side chain of penicillamine may hinder the reaction with DNFB. There is also no evidence to show how the DNP-derivative of penicillamine and other atypical amino acids would react on paper and silica column chromatography. Again, one must consider the findings of Fletcher, Lowther, and Reith (23) of a third, and as yet, unknown DNP-derivative in their determination of the N-terminal amino acid residues of insulin by preparing the methyl esters of the DNP-amino acids. Much more research would have to be done before the possible

presence of an atypical amino acid in insulin could either be proved or disproved.

The Presence of an Atypical Ring Structure

The possibility should be considered, during further investigations, of the presence of some atypical ring structure with no free amino group in the insulin molecule, which, though stable to acid hydrolysis, is freed from the amino acid residues of the insulin chains by vigorous aqueous acid hydrolysis. Such a structure would need to be extracted during the ether-ethyl acetate extraction of the PTH's, and then be hydrolyzed or degraded by the barium hydroxide to give a free valine residue. An atypical ring would be undetected during DNFB studies because there is no free amino group to react with the reagent. A ring with the proposed properties would probably remain undetected by DNFB studies as they are usually performed. The structure would have to be extractable from aqueous acid solution with ether and/or ethyl acetate in order to be carried over for alkaline degradation during the PTH hydrolysis.

Ring structures having no free amino groups and which are present in biologically active compounds are not uncommon. Examples are the thiazole nucleus of thiamine, and the

thiazolidine ring of penicillin and perhaps of bacitracin (67). Penicillin, of course, contains penicillamine which could be degraded to valine. Further evidence of unexpected linkages in proteins is found in the report of Viswanatha and De (68). These authors found evidence for the presence of a nitrogen-sulfur complex in soybean protein which resists enzymatic hydrolysis unless the protein is first heated. Resistance or susceptibility of the bond to acid hydrolysis was not discussed in the abstract of the paper. The occurrence of such a linkage demonstrates the inadvisability of discounting any logical linkage one might suspect. Again, the present investigation provides no data to support such a structure. The possibility must, however, be considered until it is disproved.

The Second, Third, Fourth, and Fifth Positions in the Insulin Peptide Chains

The Use of the PTH Method

The investigation of the amino acids in the second, third, and fourth positions in the insulin chains by the PTH method is far from complete because major emphasis was placed upon the investigation of the N-terminal amino acid residues

of these chains. The appearance of the numerous ninhydrin reactive spots on the paper chromatograms of the amino acids regenerated from the PTH's of residues in the second, third, and fourth positions has been puzzling. It may be that some spots do not represent amino acid residues because their R_f's do not agree with those of the amino acid residues reported to be in insulin. Hydrobromic acid (48 percent) was used in the hydrolysis of these PTH's. This method of PTH hydrolysis may produce numerous ninhydrin reactive degradation products. Dismutation of the regenerated amino acids to glycine and/or alanine would explain the very intense spots obtained for glycine in most chromatograms and for alanine in some. Degradation and dismutation would also explain the numerous colored spots obtained which either do not agree with the amino acid standards in position and/or color produced. Turner and Schmertzler (22) and De Fontaine (51), however, found the parent amino acids and no other spots on paper chromatograms of the amino acids regenerated from PTH's by hydrobromic acid hydrolysis.

The presence of spots other than for the parent amino acids has been interpreted by Thompson (66) and Christensen (29) to mean that linkages within the protein molecule have been broken. If linkages within the protein molecule had been broken, other amino acids would have appeared during

subsequent steps of the sequence determination. The DNFB studies of the residues of each successive step in the PTH method of sequence determination showed that no bonds other than those expected from Sanger's structure (1, 2) were cleaved by the dioxane-HCl. The ninhydrin reactive spots could not therefore have arisen from amino acids which had become N-terminal residues due to non-specific cleavage of peptide bonds in other portions of the insulin chains during a previous step.

The possible carrying over of free amino acid residues and peptides by the wet ether during the continuous extraction of the weak acidic suspension of the insulin residues of each successive PTC treatment is also not indicated. The presence of numerous free amino acids and/or peptides after dioxane-HCl cleavage would require that bonds other than the N-terminal bonds were split. Such non-specific cleavages would have provided numerous DNP-amino acids as the result of the DNFB studies of the residues after each PTC and dioxane-HCl treatment. The excess ninhydrin reactive spots in the paper chromatograms of the amino acids regenerated from the PTH's of the residues in the second, third, and fourth positions of insulin cannot be explained on the basis of the results obtained in the present investigation.

The Use of DNFB on the Residues of Stepwise PTC Treatments

The paper chromatography of the DNP-derivatives of the amino acid residues in the N-terminal positions in native, denatured, and oxidized insulin, and the amino acid residues in the N-terminal positions of each residue remaining after the first, second, third, and fourth PTC treatment and dioxane-HCl cleavage of insulin has provided complete agreement with the structure proposed by Sanger (1, 2). The paper chromatogram reproduced in Figure 13 is one of those prepared during the DNFB studies.

The DNP-isoleucine of the DNP-1-N insulin residue (Chromatogram no. 9) is streaked enough on the reproduced chromatogram so that identification would be uncertain. The DNP-isoleucine of the same DNP-1-N insulin preparation on a previous chromatogram was much more compact and better separation from DNP-phenylalanine was obtained than in the reproduced chromatogram.

The DNP-derivatives of the residues of oxidized insulin after the various PTC treatments did not give any conclusive results. The shorter, more soluble peptides were probably lost during the isolation of the DNP-oxidized insulin derivatives.

The DN-phenol standard solution applied to the chromatogram in Figure 13 had been applied in such excessive quantities that a large sword-shaped streak was obtained. When, however, a subsequent paper strip chromatogram was run with much smaller quantities of DN-phenol and with some of the DNP-aspartic acid standard on an adjacent spot, it was found that the impurity in the DNP-aspartic acid standard (the third spot from the bottom of the sheet in the area traversed by the material applied to chromatogram no. 1) and the DN-phenol had the same R_f values. This indicates that the substances having the same R_f as the impurity in the DNP-aspartic acid standard and found in chromatograms no. 6, 7, 9, 10, 11, 12, 13, 14, and 15 are also DN-phenol. Such a product of the degradation of DNP-amino acids during hydrolysis would be expected in every case where a DNP-derivative of a protein is hydrolyzed in aqueous acid solution.

The failure of bis-DNP-histidine (fifth position in the B chain of insulin) to appear on the reproduced chromatogram in the area traversed by the ether extracts of the hydrolysate of DNP-4-N is not surprising. Bis-DNP-histidine is soluble in aqueous solutions and ethanol but not in ether. This derivative of histidine should appear in paper chromatograms of the aqueous phase of the DNP-4-N (1). However, the attempts to chromatograph the aqueous phase of the various

hydrolysates failed due to a substance which diffused very rapidly throughout the paper during equilibration of the paper chromatogram. No interpretation of results was possible.

The Investigation of the Barium Hydroxide Hydrolysis of PTH's

The barium hydroxide hydrolysis of the PTH's of phenylalanine and glycine was investigated in order to determine whether the appearance of valine was a result of this hydrolysis. The results obtained by paper chromatography of the first hydrolysates of the PTH of phenylalanine and a mixture of the PTH's of phenylalanine and glycine synthesized by Serat (37) were thought to indicate that valine was produced during this hydrolysis. A blue area at the base of the large blue-green phenylalanine spot was thought to represent the presence of valine. Later chromatograms of the barium hydroxide hydrolysates of the PTH of phenylalanine and mixtures of the PTH's of phenylalanine and glycine indicated that no valine was present in the hydrolysate. The blue color was probably due to a particular concentration of phenylalanine at the base of the major portion of the amino acid because

such bluish areas were found in other positions in the subsequent chromatograms.

The investigations of the barium hydroxide hydrolysis of the PTH's of phenylalanine and glycine, both separately and in a mixture of the two, indicate that the amounts of amino acid regenerated from any two hydrolyses can vary greatly even though the initial quantities and the conditions of the hydrolyses of the PTH's are the same. The difference in the intensities of the phenylalanine and glycine spots in the two paper chromatograms in Figure 7 shows this variability. The amount of PTH used for each hydrolysis was 0.3 g. The recrystallized PTH of phenylalanine, however, gave the weaker phenylalanine spots even though it was presumably the purer preparation. An equal amount of 0.25 N barium hydroxide was added to each, and the two test tubes in which the PTH's were hydrolyzed were hung side-by-side in the same oil bath. There should thus have been no significant difference in the conditions of hydrolysis.

If one compares Figures 8 and 9, it is quite evident that alanine occurs in every chromatogram of a hydrolysate of the PTH of phenylalanine in Figure 8 while alanine occurs in none of those in Figure 9. The dilutions of the hydrolysates of the PTH's in chromatograms 4, 5, and 6 of Figure 8 are the same as those used for the chromatograms in Figure 9,

although more hydrolysate was applied to chromatogram 2 of Figure 9 than was applied to chromatograms 4, 5, and 6 of Figure 8. The apparent variability in the appearance of alanine and of the ninhydrin reactive spot having an R_f greater than that of any amino acid residue known to be present in insulin was also noticed during the investigations of the N-terminal amino acids of insulin. The latter unknown ninhydrin reactive spot was present on the paper chromatograms of the hydrolysates of the PTH's of the N-terminal amino acids of Sample AT_1BL (p. 55) and of the 8.8 mg. sample of insulin (Figure 3). An alanine spot was present on all chromatograms but varied in intensity. The combined ether-ethyl acetate extracts of Sample AT_1BL had been washed three times with distilled water, evaporated to dryness, then redissolved in ether for transfer to a test tube for subsequent hydrolysis of the extracted PTH's. The corresponding extracts of the 8.8 mg. sample had not been washed but were evaporated to dryness, then redissolved in ether for transfer to the hydrolysis tube. Yet the unknown compound appeared in both hydrolysates but did not appear in those of the washed 10.0 mg. samples (Figure 2). The unknown compound apparently is a product of the barium hydroxide hydrolysis of the PTH of phenylalanine because it is present only in chromatograms containing the hydrolysate of the PTH of phenylalanine.

The barium hydroxide hydrolysis of PTH's causes not only degradation of the PTH to its parent amino acid, but also degrades the amino acid to other amino acids. Thus the PTH of phenylalanine, including the recrystallized PTH, as well as the PTH synthesized from chromatographically pure phenylalanine, is not only hydrolyzed to phenylalanine, but apparently the phenylalanine undergoes dismutation to alanine, glycine, and a third ninhydrin reactive compound. The appearance of alanine and glycine indicates that phenyl and benzyl groups are removed, either from the PTH, or from the regenerated phenylalanine. However, the appearance of alanine in easily visible quantities during the barium hydroxide hydrolysis of the PTH of glycine may be due to the presence of some PTH of alanine as a contaminant of the PTH of glycine. Such a possibility exists because no recrystallization or synthetic studies of this PTH were made.

Fraenkel-Conrat and Fraenkel-Conrat (27) found phenylalanine, glycine, and a small amount of alanine during their determination of the N-terminal amino acid residues of insulin by the PTH method. The small amount of alanine found on their paper chromatograms very likely was formed during the barium hydroxide hydrolysis of the PTH's of the N-terminal amino acids of insulin. The results found by the Fraenkel-Conrats (27) therefore appear to confirm those obtained in

the present investigation of the barium hydroxide hydrolysis of the synthetic PTH's of phenylalanine and glycine.

The investigation of the barium hydroxide hydrolysis of the PTH's of phenylalanine and glycine did not provide, as was hoped, an answer to the problem of the N-terminal valine residue. The results, however, indicate that one must use extreme caution in interpreting the results obtained by this method. Indeed, it would be most advisable to use the extraction and hydrolysis of PTH's only in conjunction with a second method of analysis such as the subtractive microbiological assay method of Fox, Hurst, and Warner (33) or a subtractive method using the Dowex-50 column separation of amino acids in protein hydrolysates as was applied to the amino acid residue sequence determination of bacitracin by Griffith (69). In conjunction with the subtractive method, the hydrolysate of PTH's can, if carefully interpreted, provide qualitative information about which amino acid residues are present and their order in the sequence. Such information would reduce the number of microbiological assays which must be made. Here again, however, direct paper chromatography of the extracted PTH's can further reduce the work involved and would probably not be subject to the limitations of the appearance of degradation products as is the case when the PTH's are hydrolyzed.

The Lack of Evidence for Non-Specific Cleavages

The report of the investigation by Thompson (66) of the N-terminal amino acid residue sequence of serum albumins appears to be critical of the PTH method of sequence determination. Thompson treated human serum albumin with PTC, then cleaved portions of the phenylthiocarbamyl serum albumin by one of four methods, namely, acetic acid-HCl (70), dioxane-HCl (33), formic acid (66), and 0.05 M citrate buffer at pH 4.5 (64). When the residues remaining after the cleavage reactions were treated with DNFB, alanine (0.5 mole per mole albumin), serine (0.1 mole per mole albumin), threonine (0.2 and 0.15 moles, respectively, per mole albumin), and aspartic acid (0.1 mole per mole albumin) were obtained from the residues of the acetic acid-HCl and dioxane-HCl cleavages. Alanine (0.6 and 0.25 moles, respectively, per mole albumin) and aspartic acid (0.1 mole per mole albumin) were obtained when the cleavage reagents were formic acid and citrate buffer. Since the N-terminal amino acid residue of human serum albumin is aspartic acid (25, 71) and the N-terminal dipeptide is aspartylalanine (66), Thompson did not obtain complete reaction of the PTC with the N-terminal amino acid. His results also indicate that non-specific cleavage occurred at serine and threonine residues. The present investigation,

which also utilized the independently conceived method of first reacting the insulin with PTC, cleaving with dioxane-HCl, then treating the residue with DNFB to obtain the DNP-residue of the next amino acid residue, provides no evidence of incomplete reaction of PTC with any N-terminal amino acid. The DNP-amino acid derivatives isolated after each successive PTC reaction and cleavage were those expected from Sanger's structure and no others. The cleavages of only the terminal blocked units of proteins by dioxane-HCl were also reported by Fox et al. (33) and Landmann et al. (32). Since an aspartic acid residue was in the third position of the insulin phenylalanine chain according to Sanger's structure, this residue must have also reacted quantitatively with the PTC. These results also gave no indication of splitting at serine and/or threonine bonds or any other than the expected terminal bond of the blocked residue. The results of Fox et al. (33), Landmann et al. (32), and Harris and Li (72) with ACTH with its N-terminal Ser.-Tyr.-Met.-Glu.-His.-Phe.- sequence also indicate no unexpected cleavages. Explanations which can be offered for Thomoson's results after a comparison of his method of PTC treatment with that used in the present investigation are that the reaction medium he used (1:1 dioxane-water, pH adjusted with triethylamine) and/or the conditions of the reaction used (1 to 2 hours at 40°C. and pH 8.5) may

not have allowed the reaction to go to completion. In addition, anhydrous conditions of cleavage may not have been rigidly maintained by Thompson (66) so that the labile serine and threonine bonds were split during his cleavage reactions. The observation of Thompson (66) that the 3-phenyl-2-thiohydantoins of amino acids are difficult to recover quantitatively has been observed by Blaney (5) and was apparent during the present investigation.

The Use of an Air-Conditioned Room for Paper Chromatography

The use of an air-conditioned room for paper chromatography was found to be highly desirable, if not essential, for obtaining reproducible results. The temperature of the room used was found to be $25^{\circ} \pm 0.5^{\circ}C$. Conditions of constant humidity were also maintained. The chromatograms developed in this room, then dried for at least one half hour in a stream of air before spraying with the ninhydrin reagent, were found to be completely reproducible. The R_f values and the colors of the amino acid standards did not vary noticeably during any of the work carried on in this room. Paper chromatograms developed in the laboratory and subject to fluctuations in temperature and humidity often

could not be interpreted due to poor separation of the substances being chromatographed. Laboratory temperature and humidity conditions may very well have contributed to the failure of the direct paper chromatography of PTH's and the first attempts to separate DNP-amino acid derivatives by paper chromatography.

The Photography of Paper Chromatograms

The photography of paper chromatograms, especially those of amino acids treated with the quickly fading ninhydrin reagents, is highly recommended. The use of Microfile film (Eastman Kodak Company, Rochester, N. Y.) would undoubtedly provide greater contrast than was obtained with Plus-X film (Eastman Kodak). Not only does the photograph of the chromatogram provide an easily filed, permanent record, but a film which is highly sensitive to the blue end of the spectrum will often bring to the attention of the investigator spots of ninhydrin reactive compounds which are below the visible threshold of the eye. Indications of such spots are found in the chromatogram in Figure 3 between the alanine and valine spots.

SUMMARY

The sequence of the amino acid residues in the polypeptide chains of insulin have been studied by the PTH and DNFB methods and by a combination of the two.

The amino acid residues sequence predicted by the insulin structure proposed by Sanger and his co-workers (1, 2) has been verified by a combination of the PTH and DNFB methods.

When the N-terminal amino acid residues of insulin were determined by the PTH method using anhydrous dioxane-HCl for cleavage of the N-terminal peptide bonds or by the DNFB method, only phenylalanine and glycine residues were indicated at N-terminal residues. When, however, the N-terminal amino acid residues are determined by the PTH method and all peptide bonds in the insulin molecule were cleaved by vigorous aqueous acid hydrolysis, phenylalanine, glycine, and valine residues appeared as N-terminal residues. Some possible explanations for the appearance of valine by the latter method were discussed.

An investigation of the barium hydroxide hydrolysis of the PTH's of phenylalanine and glycine was begun. The results showed that not only the parent but one or more other amino acid residues were produced during the reaction. The

results of such hydrolyses must be interpreted with caution and should preferably be used in conjunction with other methods of sequence determination.

Some observations on the advisability of using an air-conditioned room for paper chromatography and of photographing the completed paper chromatogram to provide a permanent record were given.

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