# The Preparation and Properties of Some 2,4-Dinitrophenyl Peptides

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To a Sparkling Jem

ABSTRACT

DNP-glycylglycine, DNP-glycylglycylglycine, DNP-glycylglycylglycyl-glycine, DNP-L-alanylglycylglycine, DNP-L-leucylglycylglycine, and DNP-glycylglycyl-L-alanine were prepared by condensing 1-fluoro-2,4-dinitrobenzene with the appropriate peptide, using triethylamine as the condensing agent. In addition, the former three compounds were prepared by using sodium bicarbonate as the condensing agent. The optimum conditions for the reaction were determined for each compound.

The ultraviolet spectra of the DNP-peptides, as well as that of DNP-glycine, were determined in sodium bicarbonate solution and in glacial acetic acid solution, and the molar absorptivities at the wavelengths of maximum absorbance were calculated. The effect of light on solutions of DNP-glycine, and the effect of a basic medium on DNP-glycylglycine, DNP-glycylglycylglycine, and DNP-glycylglycylglycylglycine were also determined.

A partial separation of a mixture of the DNP-compounds was achieved by means of thin layer chromatography, using benzene-acetic acid, and benzene-acetic acid-95% ethanol developers. The main benzene-soluble impurity produced in the reaction of 1-fluoro-2,4-dinitrobenzene with glycine, and with the three glycyl peptides containing a varying number of glycyl residues, was separated by thin layer chromatography, and identified by its infrared spectrum.

The infrared spectra of all the DNP-compounds prepared were determined.

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# INTRODUCTION

In 1945, Sanger (1) introduced a new technique to the problem of determining the sequence of amino acid residues in proteins. He found that shaking the protein with 1-fluoro-2,4-dinitrobenzene (FDNB) in a basic water-alcohol medium resulted in a condensation between the FDNB and any free amino groups present in the protein. The free amino groups were then effectively labelled. The protein could then be hydrolyzed to yield a mixture of amino acids and 2,4-dinitrophenyl amino acids (DNP-amino acids). The latter were relatively resistant to hydrolysis, and could be separated and identified, thereby establishing the identity of those amino acid residues bearing free amino groups in the original protein molecule.

The DNP-amino acids produced by hydrolysis of the dinitrophenylated protein have been studied extensively. However, much less attention has been given to the DNP-peptides which are formed when hydrolysis of the DNP-protein is not carried to completion. Kruger (2) prepared several DNP-dipeptides and determined their melting points, neutralization equivalents, specific rotations, electrophoretic behaviors, and absorption spectra. In addition, he prepared and studied DNP-peptides with a varying number of glycyl residues. Of particular interest was the fact that the molar absorptivity of DNP-glycylglycylglycylglycine in 0.2 M sodium bicarbonate solution was greater than that of DNP-glycylglycylglycine in the region 350-355 mμ. Two possible explanations were suggested for this observation. Some impurity which absorbed radiation in the 350-355 mμ range may have been present in the DNP-tetrapeptide, thereby increasing its apparent molar absorptivity, or an impurity may have been present in the DNP-

tripeptide which either did not absorb in that range, or which decreased the molar absorptivity for some other reason. Alternatively, the conformation of the chain of glycyl residues of the DNP-tripeptide, ordinarily expected to be extended, may have been modified in some manner such as to decrease the molar absorptivity of the compound.

The purpose of the present work was to prepare and purify the DNP-tripeptide and DNP-tetrapeptide, and to measure their molar absorptivities at the wavelength of maximum absorbance. If the results obtained were in agreement with those obtained by Kruger, an attempt would be made to discover the reason for the difference in values. If the results were not in agreement with those previously obtained, an explanation would be suggested for the disagreement between the values obtained by Kruger and those obtained in the present investigation. In addition, the synthesis of some other DNP-tripeptides and DNP-glycylglycine was to have been carried out, using the methods employed by Kruger.

# LITERATURE SURVEY

## Introduction

Amino acid residues are the fundamental units of protein molecules. The two functional groups common to all amino acids are the α-amino group and the carboxyl group. This common feature gives the amino acids a common set of chemical properties, one of which is the ability to form the long polyamide chains that make up proteins. A peptide is an amide formed by the condensation of the amino group of one amino acid molecule and the carboxyl group of another, and therefore contains at least two amino acid residues. Peptides are intermediate in complexity between amino acids and proteins. They are frequently referred to as polypeptides if the molecular weight is less than 10,000, while heavier molecules are called proteins.

Peptides have been studied primarily as a step toward the understanding of proteins. In order to assign a structure to a particular peptide, it is necessary to know which amino acid residues make up the molecule, their relative amounts, and the sequence in which they occur in the chain.

The peptide chain in its general form may be represented by the structure

$$NH_2\text{-}CHR^1\text{-}CO\text{-}NH\text{-}CHR^2\text{-}CO\text{-}NH\text{-}CHR^3\text{-}.....\text{-}CO\text{-}NH\text{-}CHR^n\text{-}COOH$$

Two types of terminal amino acid residues are present in a peptide molecule, those with a free α-amino group, and those with a free carboxyl group. In accordance with a suggestion by Sanger (3), the amino acid residue bearing the free α-amino group is called the N-terminal residue, and that bearing the free carboxyl group is called the C-terminal residue. The terminal residues differ from the other residues in a peptide chain in that they contain free α-amino or free carboxyl groups, and this difference

has provided a means to their identification.

Methods of N-Terminal Residue Analysis

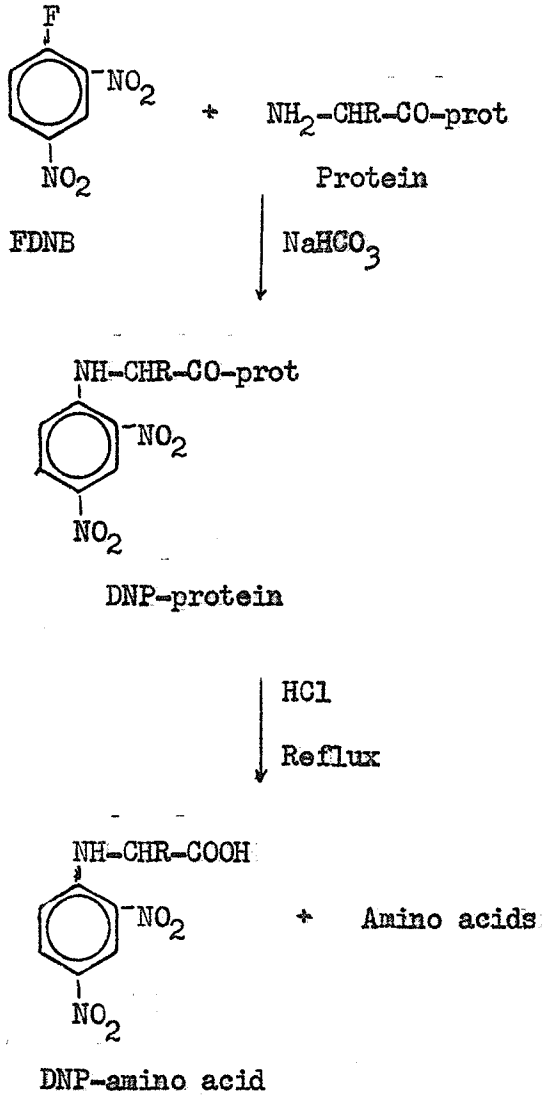
Many of the procedures employed for N-terminal residue analysis have depended upon a reaction in which an acylating agent combines with the N-terminal residue through a linkage which is more stable to subsequent hydrolysis of the peptide chain than are the other linkages in the chain. The derivative formed in the reaction must be separable and capable of identification. The dipolar ion character of the terminal amino acid is destroyed by reaction of the amino group with the acylating agent, and thus the derivative is soluble in non-polar solvents, unlike the remaining amino acids present in the hydrolysate. Therefore the compound may be extracted from the reaction mixture and identified chromatographically.

The first attempt at N-terminal residue analysis was by Fischer and Abderhalden (4), who coupled β-naphthalenesulfonyl chloride with a peptide presumed to be glycylalanine. On acid hydrolysis, the derivative yielded β-naphthalenesulfonylglycine, thus proving that glycine was the N-terminal residue.

Abderhalden and Blumberg (5) used 1-chloro-2,4-dinitrobenzene (CDNB) in a sodium bicarbonate medium to prepare a number of DNP-amino acids. Initial attempts to employ this reagent for the identification of N-terminal residues in a partial hydrolysate of silk fibroin (6) were unsuccessful, however, due to the presence of anhydrides in the hydrolysate and to difficulties in separating the products. Furthermore, CDNB reacted with amino groups in sodium bicarbonate medium only if heated, and this caused some

hydrolysis of the protein.

The modern aspects of this field began with the studies of Sanger (1) in 1945. FDNB was the reagent chosen to react with the N-terminal residues. The dinitrophenylation technique of labelling N-terminal amino acid residues played a central role in the elucidation of the structure of the peptide chains of insulin, and has since become a well-established procedure in protein chemistry. The principle of the method is summarized by the equation



where 'prot' represents the remainder of the protein molecule. The success of this procedure is based on the smooth, quantitative reaction of FDNB with the free amino groups of the protein, and on the stability of the DNP-amino acids to acidic or enzymatic hydrolysis.

Sanger's original procedure for synthesizing DNP-amino acids involved shaking the amino acid with a two-fold excess of FDNB and an equal weight of sodium bicarbonate in 67% ethanol (by volume) for two hours at room temperature. The ethanol was removed by vacuum distillation, and the excess FDNB was extracted with ether. Acidification with hydrochloric acid precipitated the DNP-amino acid.

With amino acids alone, FDNB combines not only with the free amino group, but also with the imino group of proline, the phenolic group of tyrosine, and the sulfhydryl group of cysteine. In its reaction with proteins, it combines not only with the free $\alpha$-amino groups, but also with the $\epsilon$-amino group of lysine residues. The estimation of $\epsilon$-DNP-lysine in a protein hydrolysate indicates how many $\epsilon$-amino groups are free in the intact protein.

## The Determination of Protein Structure by N-Terminal Residue Analysis

The primary goal of the FDNB method of analysis is the elucidation of the sequence of amino acid residues in peptides and proteins. The protein is treated with FDNB and the resulting DNP-protein hydrolyzed. Complete hydrolysis produces DNP-N-terminal amino acids (if present), various DNP-substituted internal residues, and free amino acids. Partial hydrolysis of the DNP-protein gives rise to a complex mixture of free amino acids and

peptides of various sizes, some of which will have been dinitrophenylated. The mixture may be separated into its constituent compounds, and each compound identified.

Schroeder's work on lysozyme (7) is an example of the determination of the sequence of amino acid residues in a peptide chain by means of a combination of N-terminal residue analysis and partial hydrolysis. It had been shown (8) that the terminal amino acid was lysine. Furthermore, DNP-peptides which yielded $\alpha,\epsilon$-di-DNP-lysine on complete hydrolysis could be isolated from partial hydrolysates of lysozyme. These facts suggested that the peptides were derived from the amino acid end of the lysozyme molecule. Schroeder determined the nature of this peptide, and devised a hydrolytic procedure which produced the long DNP-peptide from DNP-lysozyme, thus permitting determination of part of the amino acid sequence in the protein.

DNP-lysozyme was prepared according to the method used by Sanger, and the resulting DNP-protein was refluxed for various lengths of time in 6 N hydrochloric acid. By varying the length of time of refluxing, the extent of hydrolysis could be approximately regulated.

The hydrolysates were extracted with ether to remove the DNP-compounds, and the ether extracts were washed with water to remove any free amino acids or peptides. The ether was evaporated and the residue separated into its components by adsorption chromatography. Four ether-soluble compounds were found to be present in the partial hydrolysate: $\alpha,\epsilon$-di-DNP-lysine, and the DNP-derivatives of a dipeptide, tripeptide, and tetrapeptide.

Once the DNP-peptides had been separated, it was necessary to deter-

mine their constituent amino acids. This was accomplished by complete hydrolysis of each individual DNP-peptide, followed by extraction of the DNP-amino acid released, and dinitrophenylation of the remaining free amino acids. The DNP-amino acids produced were separated and identified chromatographically. They could be estimated quantitatively by spectrophotometric means, using a solution of the derivative in 1 N hydrochloric acid, 1% sodium bicarbonate solution, or glacial acetic acid. It was established that the N-terminal amino acid of each DNP-peptide was lysine. In addition to lysine, the most strongly adsorbed DNP-peptide on the chromatographic column contained valine, phenylalanine, and glycine. The second most strongly adsorbed compound contained valine and phenylalanine in addition to lysine, and the least strongly adsorbed compound contained only valine in addition to lysine. The sequence of amino acid residues in the peptide suggested by this information was lysylvalylphenylalanylglycine. It was confirmed by hydrolyzing the individual DNP-peptides isolated from the original partial hydrolysate of DNP-lysozyme. Partial hydrolysis of the most strongly adsorbed DNP-peptide, the DNP-tetrapeptide, gave the other two peptides and $\alpha,\epsilon$-di-DNP-lysine. The next most strongly adsorbed DNP-peptide gave a dipeptide and $\alpha,\epsilon$-di-DNP-lysine. The samples of dipeptide from the partial hydrolysate of the DNP-tripeptide and from the partial hydrolysate of DNP-lysozyme were identical. The identity of the constituent amino acids was based on a comparison of the chromatographic behavior of the DNP-derivatives with that of authentic DNP-derivatives. That the ratio of the constituent amino acids was unity could readily be seen from a spectrophotometric determination. Thus the sequence of the four amino acids in the peptide was established.

The reaction of a protein with FDNB to produce a DNP-protein is only the first step in the elucidation of the structure of the protein. In order to determine the amino acid residues present and their relative amounts, two techniques are used: chromatography and ultraviolet spectrophotometry respectively.

Sanger's original work on the free amino groups of insulin (1) included an attempt to separate the DNP-amino acids obtained on complete hydrolysis of the DNP-protein. He employed three chromatographic procedures. Little success was obtained with adsorption chromatography, since decomposition of the compounds occurred on the magnesium oxide or alumina columns used. Glycine and leucine derivatives in acid solution could be separated on a column of talc, and in ether solution on a calcium carbonate column. Partition chromatography on filter paper was unsuccessful due to tailing of the spots.

The most successful separations were obtained with column partition chromatography, using a stationary aqueous phase adsorbed on silica gel, and a moving organic phase. In some cases, glycol or aqueous ethanol or acetone was used as the stationary phase, and a non-polar solvent in equilibrium with it was used as the moving phase. Such a system gave a good separation of mono-amino acid derivatives, but the $R_f$ values of the different compounds varied with the batch of silica gel or solvent used, and with the distance travelled down the column. The $R_f$ values indicated the relative rates of movement of the different derivatives, but could not be used alone as a means of identification unless a parallel experiment were run using authentic derivatives.

A few years later, Blackburn (9) separated mixtures of the DNP-amino acids on a column of silica gel, using concentrated phosphate buffers instead of water as the stationary phase. By varying the pH, he found that the rate of movement of a band of a given DNP-amino acid could be varied within wide limits, the higher pH giving the slower rate. In general, the best separations occurred with the most alkaline buffers, although the rate of movement decreased and tailing of the bands became more pronounced. In practice, a compromise must be reached between the desired separation and the speed of the band.

The advantage of using phosphate buffers rather than water as the stationary phase is that on a given buffered column, no significant variation in the rate of movement of the band of a given DNP-amino acid is found between different batches of silica gel. Therefore the difficulty in preparing a suitable gel does not arise. By using one solvent in conjunction with columns buffered to different pH values, the number of DNP-amino acids which can be separated is greater than that with an unbuffered column using the same solvent.

When the substance used for supporting the stationary phase holds the more polar solvent preferentially, a satisfactory separation of those compounds with predominantly water-soluble characteristics can be obtained. However, full advantage cannot be taken of small differences in partition coefficients if these are in the range which give $R_f$ values greater than 0.5. Therefore, compounds having similar partition coefficients and being more soluble in the organic phase are difficult to separate. In order to effect a separation of such compounds, Partridge and Swain (10) developed

a reversed-phase technique in which the commercial chlorinated rubber 'Alloprene' was used as a support for butanol, the organic stationary phase. By varying the pH of the moving phase, an aqueous citrate-phosphate buffer, the DNP-amino acids could be satisfactorily separated. Their recovery, as estimated by ultraviolet spectrophotometry, was almost quantitative. DNP-tyrosine and DNP- phenylalanine, which were not fully eluted from the column, and DNP-glycine, which was partly decomposed, were the only derivatives giving unsatisfactory results.

During the early studies of chromatographic separations of DNP-amino acids, when the techniques being investigated left much to be desired, several methods of regenerating the amino acid from the DNP-derivative were developed. Mills (11) brought about regeneration by heating the DNP-derivative in dilute sulfuric acid containing hydrogen peroxide. In addition, 2,4-dinitrophenol was produced. This method was not well adapted to the micro scale; a more satisfactory technique involved heating the compound in a sealed tube with saturated baryta water. Although good results were obtained with relatively long-chain amino acids, the method proved unsatisfactory for regenerating glycine, serine, threonine, and aspartic acid from their DNP-derivatives. It was found that either short-chain or long-chain amino acids could be obtained by heating the DNP-derivative with ammonia in a sealed tube (12). The advantage of converting the DNP-compounds to the amino acids was that the chromatographic behavior of the acids was well-known and more reliable than that of the DNP-compounds. However, as further work with the DNP-derivatives progressed, separations became better and the regeneration of amino acids became

obsolete.

Green and Kay (13) devised a systematic scheme for the qualitative separation and identification of sixteen ether-soluble DNP-amino acids. They used a column of silic acid mixed with 0.5 parts by weight of Celite to act as a filter aid. In most cases, the sample was placed on the column in an acetone-ligroin mixture. Studies using ligroin-acetic acid (94:6) as the developer indicated that certain groups of derivatives had similar adsorption affinities, and were separable from one another. Individual members of each group were studied with acetic acid-acetone and acetic acid-ethyl acetate developers, in which the ratio of the volume of acetic acid to that of the other component was varied in order to determine how such variations affected the relative adsorption affinities of the compounds. From studies on the development behavior of the ether-soluble DNP-amino acids with a variety of developing solvents, a scheme was devised for the isolation and identification of each derivative. Only DNP-isoleucine and DNP-leucine could not be separated from one another. The best separation of groups was obtained by using ligroin-acetic acid-acetone (88:8:4) as the developer. Each of the groups could be further analyzed by other developers. Tentative identification of zones was made by comparing the development behavior of the unknown with plots of known compounds under the same conditions. The distance from the top of the column was plotted against the volume of developer used for each DNP-amino acid. Since the derivatives showed great variation in behavior with different developers, the identity of a zone which behaved in the same way as a known compound with several different developers was established with considerable certainty.

One of the first chromatographic investigations of DNP-peptides arose as a result of the studies made on the lysozyme hydrolysates. This study showed that the separation of unknown peptides would be facilitated by a knowledge of the chromatographic behavior of known DNP-peptides. Accordingly, Schroeder and Honnen (14) chromatographed a variety of known compounds, and were able to deduce some generalizations which permitted the prediction of the behavior of known DNP-peptides, and which aided in the identification of tentatively identified compounds by a comparison of determined and predicted behavior. Identification of an unknown could not be made on this basis alone, however. Using a column and developers similar to that of Green and Kay, they found that no correlation existed between the behavior of dissimilar DNP-peptides and the length of the peptide chain. Dipeptide and tripeptide derivatives did not fall into distinct groups. Rather, a range of adsorption affinities was shown within each group, some DNP-dipeptides being as strongly adsorbed as DNP-tetrapeptides. While chromatographic behavior was not conditioned by the presence of a given amino acid residue in a certain position, some correlation existed between the relative adsorption affinity of a DNP-peptide and the type of constituent amino acid residues. If the adsorption affinities of the DNP-derivatives of the amino acids A, B, C, D, ... increased in that order, then the adsorption affinities of the DNP-derivatives of the peptides XA, XB, XC, XD, ..., or AX, BX, CX, DX, ..., where X was any given amino acid residue, also increased in that order. The arrangement of the amino acid residues could affect the behavior of the DNP-peptide. Thus, whereas DNP-alanylglycine

and DNP-glycylalanine could not be separated by any developer used, DNP-glycylleucine and DNP-leucylglycine were readily distinguishable. The chromatographic behavior of a DNP-peptide was found to be related to that of the DNP-derivatives of the constituent amino acids of the peptide. Since DNP-glycine was more strongly adsorbed than DNP-alanine, it would be expected, and was in fact observed, that DNP-glycylglycine was more strongly adsorbed than DNP-alanylalanine. Furthermore, the DNP-peptide was more strongly adsorbed than the DNP-derivative of any constituent amino acid.

In addition to column chromatography, paper chromatography has been used extensively in the separation and identification of DNP-amino acids. Early workers found difficulty in separating the compounds because of tailing of the spots. Blackburn and Lowther (15) separated DNP-amino acids by one-dimensional paper chromatography, using strips of paper soaked in phthalate buffer and dried at room temperature. When tertiary amyl alcohol was used as the developer, all DNP-amino acids moved as well-defined spots. Characteristic $R_f$ values were obtained for each compound, but since they varied with the pH of the buffer, as well as with the temperature, the identity of a DNP-amino acid was never based on the $R_f$ value alone; a control DNP-amino acid was always run on the same sheet as the unknown.

It has been found that DNP-amino acids run best on paper in solvents in which the mobile phase has a high water content (15). Water may prevent association of the DNP-compounds, which leads to tailing of the spots. The presence of water keeps the spots compact, but if too much water be present, the $R_f$ values are too large, and the differences in values become

insufficient for satisfactory separation.

Once the constituent amino acids of a peptide or protein have been separated and identified, the problem remains of determining their relative abundance. Ultraviolet spectrophotometry is the usual means of quantitatively estimating DNP-amino acids. The DNP group largely determines the absorption spectra of these compounds. Molar absorptivities may be obtained by dissolving the compound in sodium bicarbonate solution or in glacial acetic acid, and measuring the absorbance at the wavelength of maximum absorption, 360 mμ or 340 mμ respectively. At 360 mμ, the molar absorptivity of the DNP group in 1% sodium bicarbonate solution is approximately 16,000. The amount of substance recovered from a chromatographic column may be estimated by comparison with a calibration curve obtained from known concentrations of the compound.

Ramachandran (16) developed a method for the colorimetric determination of DNP-amino groups. Treatment of the DNP-compound in bicarbonate solution with sodium borohydride produced a brick red color which could be used in a quantitative estimation of the number of DNP groups in a dinitrophenylated protein. Calibration curves were obtained by using graded concentrations of DNP-amino acids. For concentrations ranging from 0.01 to 0.05 μmole/ml, measurements made with standard solutions of DNP-glycine indicated a precision of ±2%. Owing to the red color of the product and its stability under acetylating conditions, it was assumed that the reduction produced an azo compound rather than an amino group.

In connection with the spectrophotometric analysis of DNP-compounds, it had been observed that the molar absorptivities in aqueous bicarbonate

solutions were higher than those in glacial acetic acid solutions, and that the maximum absorbance in bicarbonate solution occurred at approximately 360 mμ, while in acetic acid it occurred at 340 mμ. Ramachandran and Sastry (17) used these differences to evaluate an approximate pK of the carboxyl group from a curve relating absorbance to pH.

The DNP-amino acids were dissolved in a series of buffers whose pH decreased from alkaline values to acidic values until changes in the spectra ceased, that is, until the lowest absorbance at 360 mμ had been reached. Measurements of absorbances were made at 360 mμ since at that wavelength, marked differences in molar absorptivities, $\epsilon_M$, existed between the ionized and unionized forms of the compounds. Curves were drawn for each compound, relating absorbance to pH, and points on the curves were chosen corresponding to $1/2\,\Delta\epsilon_M$, where $\Delta\epsilon_M$ was the difference in molar absorptivity between the ionized and unionized compound. The pH corresponding to this point was taken as the pK of the carboxyl group (Fig. 1).

The sensitivity of DNP-arginine to a change in hydrogen ion concentration in the pH range 2 to 5, at which the carboxyl group usually titrates, warranted the assumption that the curves obtained were due to the change from the anionic form to the acidic form as the pH decreased. Values of the pK obtained by the spectrophotometric method were in agreement with those measured potentiometrically. Furthermore, DNP-aminoethanol, which had no carboxyl group, showed no change in molar absorptivity with change in pH.

The spectrophotometric method may be used to supplement or replace potentiometric determinations of the pK of DNP-amino acids, which are

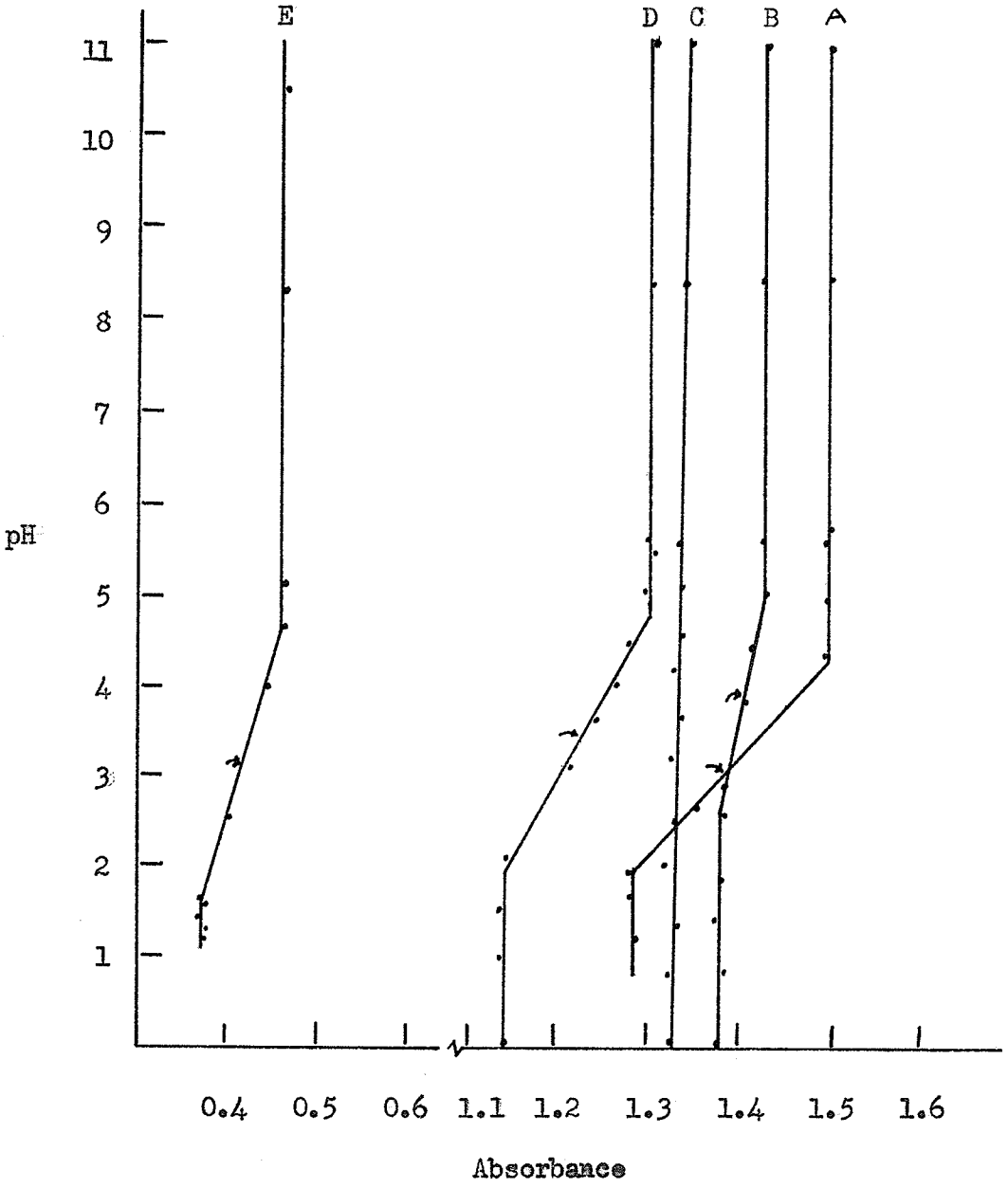



Figure 1. Spectrometric titration curves of DNP-amino acids. A, DNP-$\alpha$-aminobutyric acid; B, DNP-$\beta$-aminobutyric acid; C, DNP-$\gamma$-aminobutyric acid; D, DNP-$\alpha$-aminoisobutyric acid; E, DNP-valine. Arrows point to the pK, corresponding to the pH at 1/2 $\Delta\epsilon_M$. Measurements were made at 360 mμ.

rather difficult, owing to the low solubility of DNP-amino acids in water. Its use is restricted, however, since the change in $\epsilon_M$ on going from the ionized to the unionized forms decreases rapidly with increasing separation of the chromophore and carboxyl group (Fig.1). Thus it may be used only in the case of $\alpha$-amino acid derivatives.

The FDNB method of N-terminal residue analysis has the advantages that (1) condensation with the reagent is almost quantitative and relatively free from side reactions, and (2) that the DNP-amino acids produced are bright yellow, the latter factor being significant in their chromatographic detection and spectrophotometric estimation. One of the main disadvantages is that the DNP-amino acid residues suffer destruction, which may in some cases be extensive, under the conditions of hydrolysis necessary to cleave them from the DNP-peptides or DNP-proteins. Precise correction for the destruction is difficult. Although reproducible in a given case, the extent of destruction is different with each protein, and is generally greater than would be expected for the free DNP-amino acids under the same hydrolyzing conditions in the absence of protein. Less vigorous hydrolysis conditions are required for DNP-peptides and DNP-derivatives of smaller proteins, and the use of nearly anhydrous acidic mixtures results in less destruction of the DNP-amino acids. Destruction can be prevented in some cases by first treating the DNP-protein with xanthydrol. It had been observed that DNP-amino acids were partially destroyed by acid hydrolysis in the presence of the amino acid tryptophan, or the protein lysozyme, which contains 10.6% tryptophan (18). The destruction of $\epsilon$-DNP-lysine and $\alpha,\epsilon$-di-DNP-lysine obtained from DNP-lysozyme hydrolysates was greatly

reduced by treating the DNP-lysozyme with xanthydrol before hydrolysis, as demonstrated by Dickmans and Asplund (19). The increased recovery of the DNP-lysines after xanthylation of the DNP-protein was likely due to formation of dixanthyltryptophan in the intact protein, as indicated by the characteristic purple color of that compound in the solutions. Dixanthyltryptophan was shown to be non-destructive of DNP-alanine under conditions of protein hydrolysis.

Side reactions during dinitrophenylation are not a serious problem, but several are known to occur. 2,4-Dinitrophenol is formed to some extent in the basic reaction medium, and will interfere with the spectrophotometric estimation of the DNP-amino acids unless removed. Isherwood and Cruickshank (20) separated 2,4-dinitrophenol from DNP-amino acids by treating them with strong sulfuric acid and extracting the 2,4-dinitrophenol with benzene.

Investigations by Heikens, Hermans, and van Velden (21) showed that polymerization could occur, particularly in molecules in which the amino and carboxyl groups were widely separated. Since most amino acids of interest have the amino group on the $\alpha$-carbon, the polymerization side reaction was of little importance insofar as amino acids were concerned, except in the case of $\omega$-amino acids, such as aspartic acid and glutamic acid, in which considerable amounts of DNP-polymers were formed (22). With peptides, however, the reaction may be of greater concern; reaction of FDNB with diglycylglycine produced significant amounts of polymer (22).

Modifications of Sanger's Procedure

Several modifications of Sanger's original reaction conditions have been introduced. Schroeder and LeGette (23) conducted an investigation to ascertain the conditions necessary for quantitative dinitrophenylation of amino acids and peptides. They found that the proportions of reagents used by Sanger did not give quantitative yields of the DNP-derivatives. Substitution of sodium carbonate for sodium bicarbonate improved the yields, but larger amounts of 2,4-dinitrophenol were produced in the more basic medium. Increasing the reaction time had little effect, while an increase in the amino acid concentration gave somewhat better yields. They observed that yields were higher when the reagents were present in lower concentrations, and suggested that when the reaction mixture was homogeneous, the actual concentrations of reagents were greater than when the mixture was heterogeneous because the sodium bicarbonate had not salted out the FDNB. Less 2,4-dinitrophenol was obtained from the homogeneous reaction medium.

Levy and Chung (24) achieved a more rapid reaction, and eliminated the evaporation of ethanol and the extraction of FDNB with ether. They carried out the reaction at 40° in aqueous sodium carbonate solution, using an equal number of moles of FDNB and amino acid. Greater control of the reaction could be effected by maintaining the reaction mixture at pH 9.0 through addition of 2 N sodium hydroxide, the rate of addition indicating the rate of reaction. The addition of base also showed that the reaction resulted in the consumption of the theoretical two equivalents of base per amino group reacted, provided that the alkali needed to titrate the amino acid from its isoelectric point to pH 9.0 was included. The time of reac-

tion seldom exceeded one hour, except for complete substitution of the imidazole group of histidine, which required two moles of FDNB per mole of amino acid. The resulting DNP-derivatives were purer than those prepared by former methods.

A procedure which involves the use of trimethylamine rather than sodium bicarbonate as the condensing agent has been applied to the reaction of protein fragments with FDNB in order to avoid the subsequent desalting required prior to paper chromatography, or to separation on columns of ion exchange resins (25,26). The trimethylamine can be removed readily in vacuo. It has been found that the reaction of FDNB with serine or seryl residues in the presence of triethylamine (but not sodium bicarbonate) may lead to substitution not only on the amino group, but also on the hydroxyl group, to give N,O-di-DNP-derivatives (27).

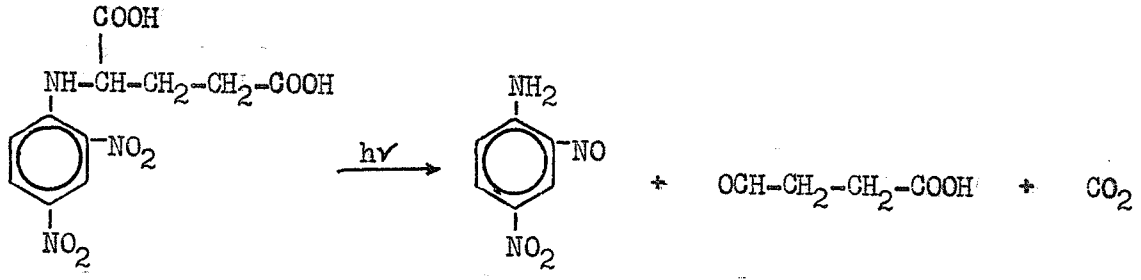
Photosensitivity of DNP-Compounds

DNP-amino acids have been known to be light-sensitive for a number of years (9, 28). Rao and Sober (29) reported that the yields of DNP-glutamic acid and DNP-aspartic acid were increased if light were excluded at all stages of preparation. In general, most operations with DNP-derivatives can be carried out successfully under subdued laboratory lighting conditions. However, paper or column chromatography of these compounds must be carried out in the dark, since prolonged exposure to light will result in decomposition.

Pollara and Von Korff (30) showed through the use of radioactively-labelled compounds that samples in the solid state underwent photo-induced

decarboxylation, yielding the N-alkyl-2,4-dinitroaniline corresponding to the original DNP-amino acid.

Russell (31) irradiated a solution of DNP-leucine in dilute sodium bicarbonate solution. On extracting the solution with ethyl acetate, he obtained an unknown compound which he later proved to be 4-nitro-2-nitroso-aniline (32). This same compound was also obtained from an irradiated solution of DNP-glutamic acid. Subsequent acidification and extraction of the aqueous phase yielded 3-formylpropionic acid, isolated as its 2,4-dinitrophenylhydrazone. The photolysis reaction therefore appeared to be an intramolecular oxidation-reduction accompanying decarboxylation:



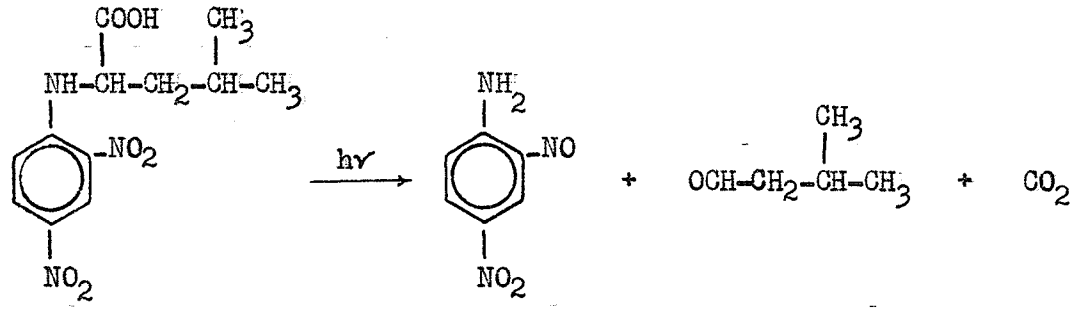

DNP-glutamic acid

4-nitro-2-nitroso-aniline

3-formylpropionic acid

The characteristic absorption spectrum of 4-nitro-2-nitrosoaniline was also observed in the irradiated solutions of a number of other DNP-α-amino acids, but no change was observed for those compounds such as ε-DNP-lysine, in which the DNP-substituent was not located on an α-amino group.

Further work on DNP-leucine showed that irradiation produced carbon dioxide and 3-methylbutyraldehyde in addition to 4-nitro-2-nitrosoaniline.



DNP-leucine

4-nitro-2-nitroso-aniline

3-methyl-butyraldehyde

Thus the reduction of the 2-nitro group was accompanied by oxidation of the amino acid residue at the $\alpha$-carbon, as had been observed with DNP-glutamic acid under the same conditions. DNP-leucine was decomposed rapidly by light at any pH, but 4-nitro-2-nitrosoaniline was formed only in solutions having a pH greater than 6. The reaction was found to be fairly general for DNP-$\alpha$-amino acids, although not all gave the same product. The presence of hydroxyl, sulfoxide, etc. groups in the amino acid side chain permitted side reactions, so that the usual product was formed to a lesser extent, or not at all. Similar compounds, in which the carboxyl group was not both free and in the $\alpha$-position with respect to the DNP-amino group, were relatively stable to light. DNP-peptides which were studied decomposed much more slowly than DNP-$\alpha$-amino acids.

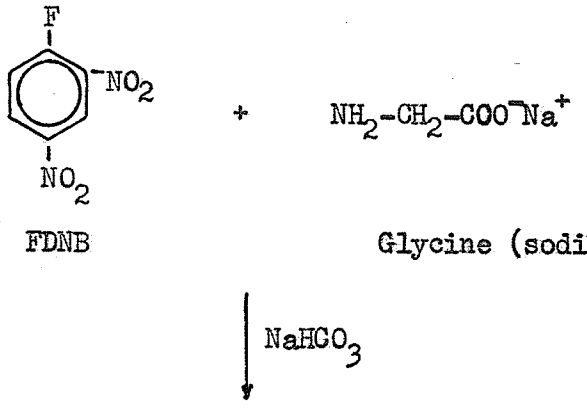
# EXPERIMENTAL AND RESULTS

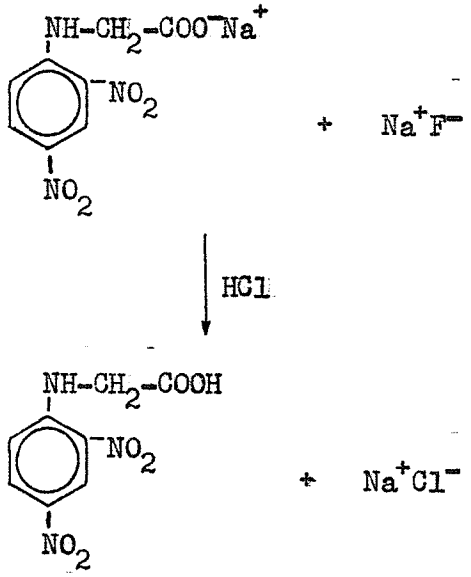
## Preparation of DNP-Glycine

The preparation of DNP-glycine, although not originally intended to be an integral part of the present work, was necessary in order to obtain a sample for the determination of the infrared spectrum and the molar absorptivity. Therefore, its preparation was studied in a manner similar to that of the DNP-peptides.

The method of synthesis employed incorporated the suggestions of several workers. In accordance with Levy and Chung (24), an equal number of moles of FDNB and glycine were reacted. The reaction vessel was wrapped in paper during the actual reaction period, and the compound was exposed to subdued laboratory light only when necessary, in order to keep photo-induced decomposition to a minimum. The weight of sodium bicarbonate used was varied, in order to discover whether such variations affected the yield of DNP-glycine. Although most preparations were carried out at room temperature, the reaction mixture was maintained at 40° during three runs, as suggested by Levy and Chung (24).

The reaction may be summarized by the equation






DNP-glycine

Materials:

| | |
|---|---|
| FDNB | 1.00 gm (0.00538 mole) |
| Glycine | 0.404 gm (0.00538 mole) |
| Sodium bicarbonate | 3.9 gm or 7.1 gm |
| Ethanol | 40 ml |
| Water | 45 ml or 85 ml |
| Hydrochloric acid | 4 ml or 8 ml |

FDNB was dissolved in 95% ethanol and added to a solution of glycine and sodium bicarbonate in water. The reaction vessel was wrapped in paper and the mixture was stirred for two hours. The ethanol was removed by vacuum distillation from a water bath, the temperature of which was maintained at 40 to 43°. Distillation was continued until the pressure of the system had decreased to 25 to 30 mm, as indicated by a manometer. DNP-glycine was precipitated by adding concentrated hydrochloric acid to the residual solution; overnight refrigeration ensured complete crystallization

of the product. It was filtered with suction, washed with ice water to remove excess hydrochloric acid, and dried at room temperature. Washing with benzene removed any unreacted FDNB, as well as any 2,4-dinitrophenol produced during the reaction period. The product was dried overnight in the vacuum desiccator, or for one hour at 100° before weighing the crude yield. In order to obtain pure DNP-glycine, the compound was dissolved in a minimum amount of glacial acetic acid (approximately 125 ml/gm), filtered, diluted with ice water until the total volume was six times the volume of acetic acid solution, and refrigerated overnight. It was filtered with suction and dried for two hours at 100°. The bright yellow solid obtained melted at 205-205.5°. Literature, 193° (13), 205° (5), 206° (33). The yields of crude product obtained by the various methods of preparation, as well as the final purified yields, are summarized in Table 1. A Gallenkamp Melting Point Apparatus was used to determine the M.P. (uncorrected)

TABLE 1

| Number of Preparations | Weight of $NaHCO_3$ (gm) | Temperature | Crude Yield (%) | Crude M.P. | Pure Yield (%) |
|---|---|---|---|---|---|
| 4 | 7.1 | Room | 72-79 | 203-204.5 | 57-68 |
| 3 | 3.9 | Room | 83-86 | 203-205 | 70-73 |
| 3 | 7.1 | 40±1 | 85-93 | 203-205 | 72-79 |

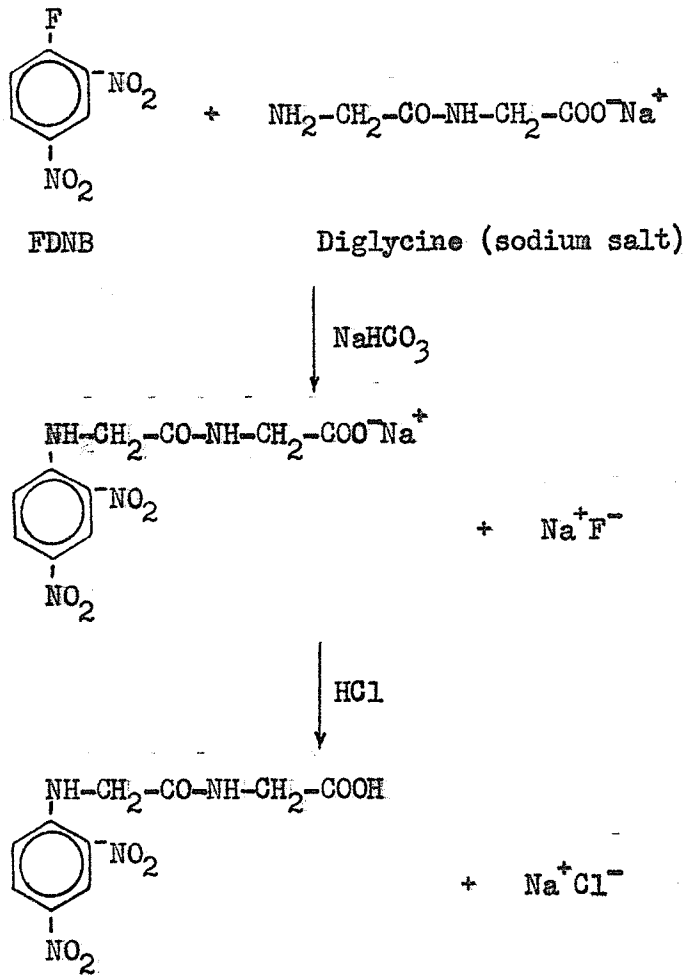
Preparation of DNP-Glycylglycine (DNP-Diglycine)

DNP-diglycine was prepared by reacting equimolar quantities of FDNB and diglycine in a basic medium. In one series of preparations, sodium

bicarbonate served as the condensing agent. The duration and temperature of the reaction were varied in order to determine which conditions produced the highest yield of DNP-diglycine. In another series of preparations, the condensing agent used was triethylamine; the reaction period and temperature were fixed, and the amount of base was varied.

Method 1

The preparation of DNP-diglycine is given by the equation




DNP-diglycine

Materials:

| | |
|---|---|
| FDNB | 0.47 gm (0.0025 mole) |
| Diglycine | 0.33 gm (0.0025 mole) |
| Sodium bicarbonate | 3.3 gm |
| Ethanol | 15 ml |
| Water | 35 ml |
| Hydrochloric acid | 4 ml |

FDNB was dissolved in 95% ethanol and added to a solution of diglycine and sodium bicarbonate in water. The reaction vessel was wrapped in paper to minimize photo-decomposition, and the mixture was stirred vigorously, followed by removal of ethanol by vacuum distillation from a water bath. The bath temperature was maintained at 40 to 43°; distillation was stopped when the pressure had decreased to approximately 25 mm. DNP-diglycine was precipitated by acidifying the residual solution with concentrated hydrochloric acid; overnight refrigeration ensured complete crystallization of the compound. The product was filtered with suction, washed with ice water to remove excess hydrochloric acid, and dried at room temperature. 2,4-Dinitrophenol and any unreacted FDNB were removed by washing with benzene. The product was dried overnight in the vacuum desiccator, or for one-half hour at 80°. Recrystallization was carried out by dissolving the compound in glacial acetic acid (approximately 45 ml/gm), filtering to remove any insoluble material, diluting with ice water until the total volume was seven to nine times that of the acetic acid solution, and refrigerating overnight. The product was filtered, then dried at room temperature for several hours, and at 80° for one hour. The yields of DNP-diglycine obtained by this

method are given in Table 2 (a).

Method 2

A stock solution of FDNB was prepared by dissolving FDNB (1.54 gm) in 95% ethanol (50 ml). A stock solution of triethylamine was prepared by adding triethylamine (1.20 gm) to distilled water (100 ml). In the following list of materials, the number of equivalents of base per equivalent of peptide is given in brackets following the volume of triethylamine solution used.

Materials:

| | |
|---|---|
| FDNB solution | 3 ml (0.00048 mole) |
| Diglycine | 0.064 gm (0.00048 mole) |
| Triethylamine solution | 4.0 ml (1), 8.1 ml (2), 12.1 ml (3) |
| Hydrochloric acid | 1 ml |

FDNB solution was added to diglycine dissolved in the aqueous triethylamine solution. The mixture was stirred at room temperature for three hours, care being taken to protect the reaction vessel from strong light. The excess triethylamine was removed by vacuum distillation from a water bath at 41 to 43°, discontinuing the distillation when the pressure had decreased to 25 mm. DNP-diglycine was precipitated by acidifying the residual solution with concentrated hydrochloric acid and refrigerating overnight. The product was filtered with suction, washed with ice water, and dried at room temperature. Any unreacted FDNB was removed by washing with benzene. The DNP-diglycine was dried for one-half hour at 80° before weighing. The product was recrystallized as outlined in Method 1. The yields obtained are summarized in Table 2 (b).

TABLE 2 (a)

Preparation of DNP-Diglycine using Sodium Bicarbonate as Condensing Agent

| Number of Preparations | Reaction Time (Hr) | Temperature | Crude Yield % | Crude M.P. | Pure Yield % |
|---|---|---|---|---|---|
| 5 | 2 | Room | 20-29 | 200-202 | 16-23 |
| 4 | 4 | Room | 36-50 | 200-202 | 29-40 |
| 3 | 2 | 40±1 | 55-56 | 199-201 | 44-45 |
| 2 | 4 | 40±1 | 67 | 200-201 | 54 |

TABLE 2 (b)

Preparation of DNP-Diglycine using Triethylamine as Condensing Agent

| Number of Preparations | Equiv. of $(C_2H_5)_3N$/ Equiv. of peptide | Crude Yield % | Crude M.P. | Pure Yield % |
|---|---|---|---|---|
| 3 | 1 | 29-39 | 201-203 | 23-31 |
| 3 | 2 | 72-76 | 201-203 | 58-61 |
| 3 | 3 | 70-77 | 200-203 | 56-62 |

The purified DNP-diglycine was a yellow-orange solid, melting at 201.5-202.5°.

Analysis

(a) The compound was dried for one-half hour at 80° before analysis.

Found: C, 36.24%; H, 4.36%; N, 16.60%.

Calculated for DNP-diglycine dihydrate, $C_{10}H_{14}N_4O_9$: C, 35.93%; H, 4.22%; N, 16.76%.

(b) The compound was dried for one hour at 100° before analysis.

Found: C, 39.87%; H, 3.90%; N, 18.86%.

Calculated for DNP-diglycine, $C_{10}H_{10}N_4O_7$: C, 40.28%; H, 3.38%; N, 18.79%.

The existence of a hydrate was based on the following observation. When a sample of DNP-diglycine which had been dried at 80° for one-half hour was placed in the melting point apparatus (preheated to 190°), moisture condensed on the upper part of the melting point tube. When a sample which had been dried for one-half hour at 100° was treated in a similar way, no moisture condensed on the upper part of the melting point tube. Furthermore, the sample took on a deeper orange color, and became more brittle after drying at the higher temperature, suggesting that some change had taken place.

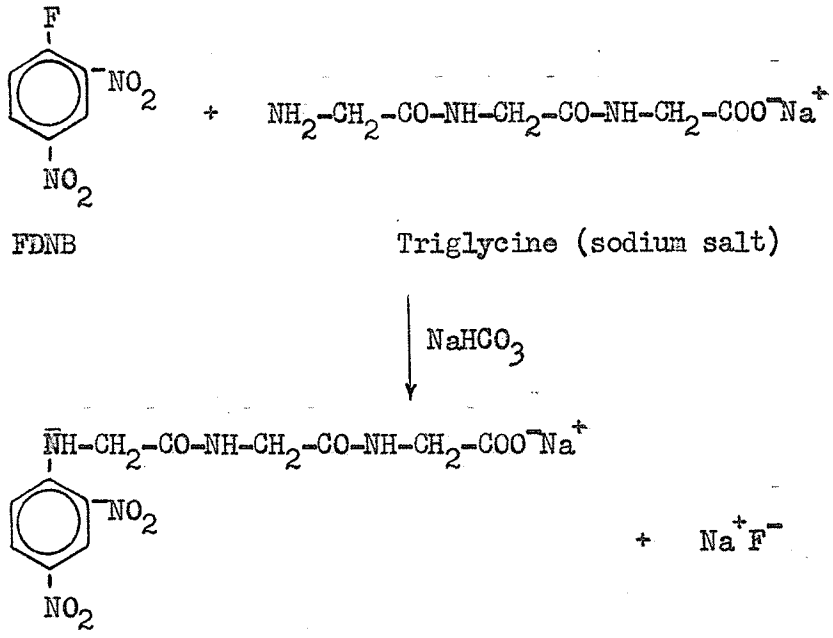
Preparation of DNP-Glycylglycylglycine (DNP-Triglycine)

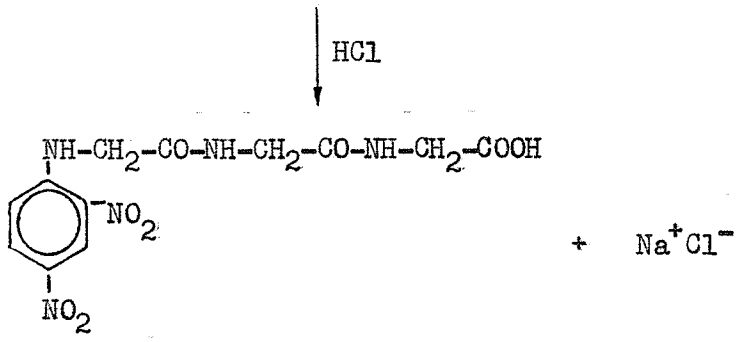
DNP-triglycine was prepared by a method similar to that used for DNP-diglycine. Two series of preparations were carried out, one using sodium bicarbonate as the condensing agent, and another using triethylamine.

For the preparation of DNP-triglycine and all subsequent DNP-peptides in this investigation, a stock solution of FDNB in ethanol was prepared by dissolving FDNB (1.53 gm) in 95% ethanol (50 ml). The solution was added directly to a solution of the peptide in aqueous sodium bicarbonate or triethylamine. This procedure was more convenient than weighing the FDNB required for each preparation, and it enabled greater reproducibility of the quantities of reagents used. However, it was necessary to use the FDNB solution within a few days of its preparation; decreased yields of the DNP-compounds were obtained by using older solutions. In the series of reactions in which triethylamine was used as the condensing agent, a stock solution of the base was prepared by dissolving triethylamine (1.20 gm) in distilled water (100 ml). The peptide was then dissolved in the desired volume of triethylamine solution.

Method 1

The preparation of DNP-triglycine involves the condensation of FDNB and triglycine:






DNP-triglycine

Materials:

| | |
|---|---|
| FDNB solution | 10 ml (0.00161 mole) |
| Triglycine | 0.305 gm (0.00161 mole) |
| Sodium bicarbonate | 2.10 gm |
| Water | 30 ml |
| Hydrochloric acid | 3 ml |

FDNB solution was added to a solution of triglycine and sodium bicarbonate in water. The reaction vessel was wrapped in paper, and the solution was stirred. Ethanol was removed by vacuum distillation from a water bath maintained at 37 to 40°, the distillation being continued until the pressure had decreased to 20 to 25 mm. The residual solution was acidified with concentrated hydrochloric acid and refrigerated overnight. The bright yellow solid which precipitated was filtered with suction, washed with ice water to remove excess hydrochloric acid, and dried at room temperature. 2,4-Dinitrophenol and any unreacted FDNB were then removed by washing with benzene. The compound was dried overnight in the vacuum desiccator before weighing.

Two methods of recrystallization were employed in the purification of DNP-triglycine. In the first method, the crude product was dissolved

in 95% ethanol, filtered to remove any insoluble material, then diluted with an equal volume of cold water, and allowed to evaporate at room temperature. The impurities, which appeared as a dark red ring on the upper portion of the evaporating dish, could be removed, leaving the purer product on the sides and bottom of the dish. The procedure was repeated as often as necessary in order to obtain a constant melting point.

A second, and more satisfactory method of purification, involved recrystallization from glacial acetic acid and water. The crude product was dissolved in glacial acetic acid (approximately 200 ml/gm), filtered to remove any insoluble material, diluted with ice water until the total volume was five to nine times that of the acetic acid solution, then refrigerated for two hours. The bright yellow product was collected on a filter and dried at 100° for one hour. The solid melted at 204-205°. The yields of DNP-triglycine obtained by this method are listed in Table 3 (a).

Method 2

Triethylamine was employed as the condensing agent. In the following list of materials, the number of equivalents of base per equivalent of peptide is given.

Materials:

| | |
|---|---|
| FDNB solution | 5 ml (0.00081 mole) |
| Triglycine | 0.152 gm (0.00081 mole) |
| Triethylamine | 1, 2, or 3 equivalents |
| Hydrochloric acid | 1-2 ml |

FDNB solution was added to a solution of triglycine in aqueous triethylamine. The mixture was stirred for six hours, after which time any

TABLE 3 (a)

Preparation of DNP-Triglycine using Sodium Bicarbonate as Condensing Agent

| Number of Preparations | Reaction Time (Hr) | Temperature | Crude Yield % | Crude M.P. | Pure Yield % |
|---|---|---|---|---|---|
| 3 | 4 | Room | 21-22 | 199-202 | 18-19 |
| 3 | 12 | Room | 50-53 | 198-200 | 43-46 |
| 2 | 24 | Room | 60-61 | 198-201 | 52-53 |
| 3 | 4 | 40-41 | 58-61 | 198-200 | 50-53 |
| 3 | 12 | 40-41 | 58-68 | 193-200 | 50-59 |

TABLE 3 (b)

Preparation of DNP-Triglycine using Triethylamine as Condensing Agent

| Number of Preparations | Equiv. $(C_2H_5)_3N$/ Equiv. peptide | Temp. | Crude Yield % | Crude M.P. | Pure Yield % |
|---|---|---|---|---|---|
| 3 | 1 | Room | 42-49 | 202-203 | 37-43 |
| 3 | 2 | Room | 67-68 | 202-203 | 58-59 |
| 3 | 3 | Room | 76-81 | 199-201 | 66-70 |
| 6 | 2 | 40±1 | 60-80 | 202-204 | 52-70 |

excess triethylamine was removed by vacuum distillation from a water bath maintained at 40 to 45°. The product was precipitated by acidifying the residual solution with concentrated hydrochloric acid, and refrigerating overnight. The product was collected on a suction filter, washed with ice water and dried at room temperature. Excess FDNB was removed by washing with benzene. The product was dried overnight in the vacuum desiccator, or at 80° for one-half hour before weighing. It was recrystallized as outlined in Method 1. The yields of DNP-triglycine obtained by this method are summarized in Table 3 (b).

Analysis

The compound was dried for one hour at 100° before analysis.

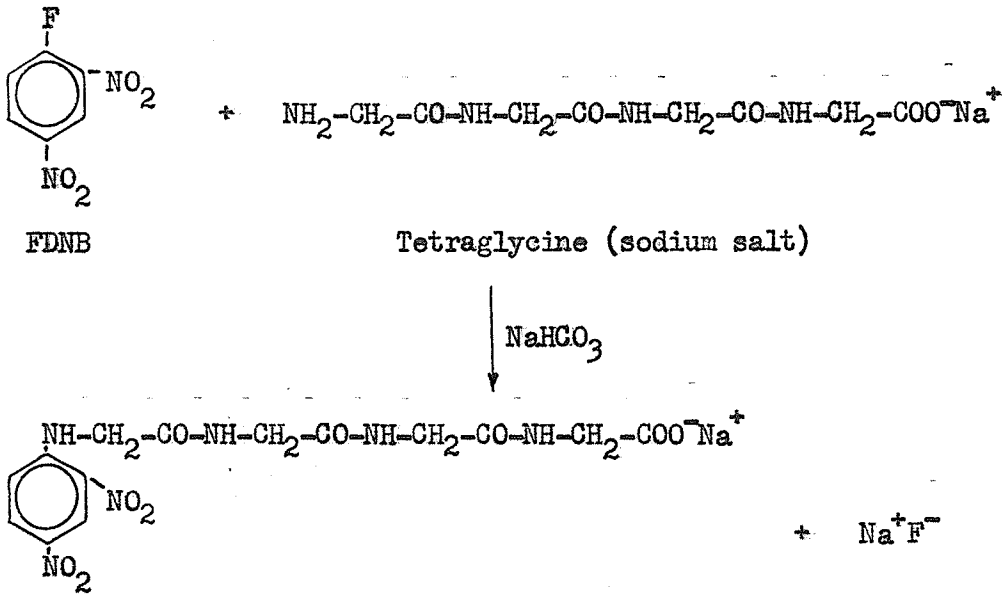
Found: C, 40.71%; H, 3.88%; N, 19.87%.

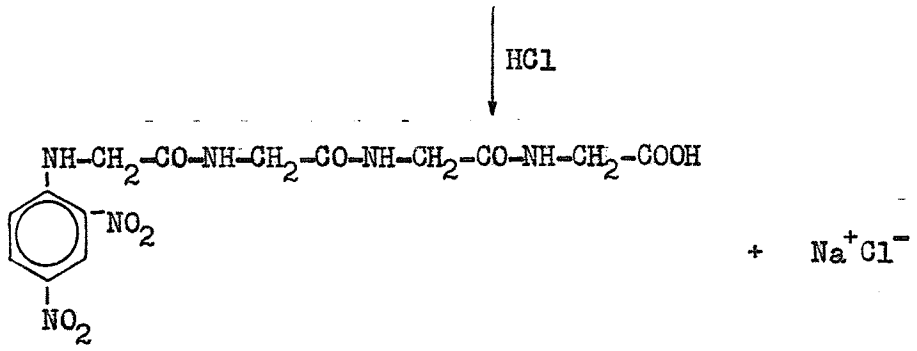
Calculated for DNP-triglycine, $C_{12}H_{13}N_5O_8$: C, 40.57%; H, 3.69%; N, 19.72%.

## Preparation of DNP-Glycylglycylglycylglycine (DNP-Tetraglycine)

### Method 1

DNP-tetraglycine may be prepared according to the equation






DNP-tetraglycine

Materials:

| | |
|---|---|
| FDNB solution | 5 ml (0.00081 mole) |
| Tetraglycine | 0.198 gm (0.00081 mole) |
| Sodium bicarbonate | 1.05 gm |
| Water | 15 ml |
| Hydrochloric acid | 2 ml |

FDNB solution was added to a solution of tetraglycine and sodium bicarbonate in water. The mixture was stirred under subdued lighting conditions in order to minimize photo-decomposition. The ethanol was removed by vacuum distillation from a water bath maintained at 38 to 40°. Distillation was continued until the pressure of the system had been reduced to 20 to 25 mm. The product was precipitated by acidifying the residual solution with concentrated hydrochloric acid, and refrigerating it overnight. It was filtered with suction, washed with ice water to remove excess hydrochloric acid, and dried at room temperature. 2,4-Dinitrophenol and any unreacted FDNB were removed by washing with benzene. The product was dried overnight in the vacuum desiccator, or at 80° for one-half hour before

weighing.

DNP-tetraglycine was purified by dissolving the solid in a minimum amount of hot water (85°), filtering out any insoluble material, and cooling the solution in an ice bath. The bright yellow solid was filtered and dried for one hour at 100°; it melted at 225-226°. The yields obtained by this method are summarized in Table 4 (a).

Method 2

Triethylamine was employed as the condensing agent. In the following list of materials, the number of equivalents of base per equivalent of peptide is given.

Materials:

| | |
|---|---|
| FDNB solution | 3 ml (0.000484 mole) |
| Tetraglycine | 0.119 gm (0.000484 mole) |
| Triethylamine | 1, 2, or 3 equivalents |
| Hydrochloric acid | 2 ml |

FDNB solution was added to a solution of tetraglycine in aqueous triethylamine, and the mixture was stirred for six hours. Any excess triethylamine was removed by vacuum distillation from a water bath maintained at 40 to 42°. DNP-tetraglycine was isolated and purified by the procedure outlined in Method 1. The yields obtained by this method are given in Table 4 (b).

Analysis

The compound was dried for one hour at 100° before analysis.

Found: C, 39.01%; H, 4.22%; N 19.22%.

Calculated for DNP-tetraglycine monohydrate, $C_{14}H_{18}N_6O_{10}$: C, 39.06%;

TABLE 4 (a)

Preparation of DNP-Tetraglycine using Sodium Bicarbonate as Condensing Agent

| Number of Preparations | Reaction Time (Hr) | Temperature | Crude Yield % | Crude M.P. | Pure Yield % |
|---|---|---|---|---|---|
| 3 | 4 | Room | 13-17 | 208-213, 222-223 | 9-14 |
| 4 | 12 | Room | 25-42 | 222-224 | 21-36 |
| 3 | 24 | Room | 40-49 | 218-219, 222-223 | 34-42 |
| 2 | 4 | 40±1 | 44-47 | 220-223 | 37-40 |
| 3 | 12 | 41±1 | 51-59 | 202-204, 213-219 | 37-43 |

TABLE 4 (b)

Preparation of DNP-Tetraglycine using Triethylamine as Condensing Agent

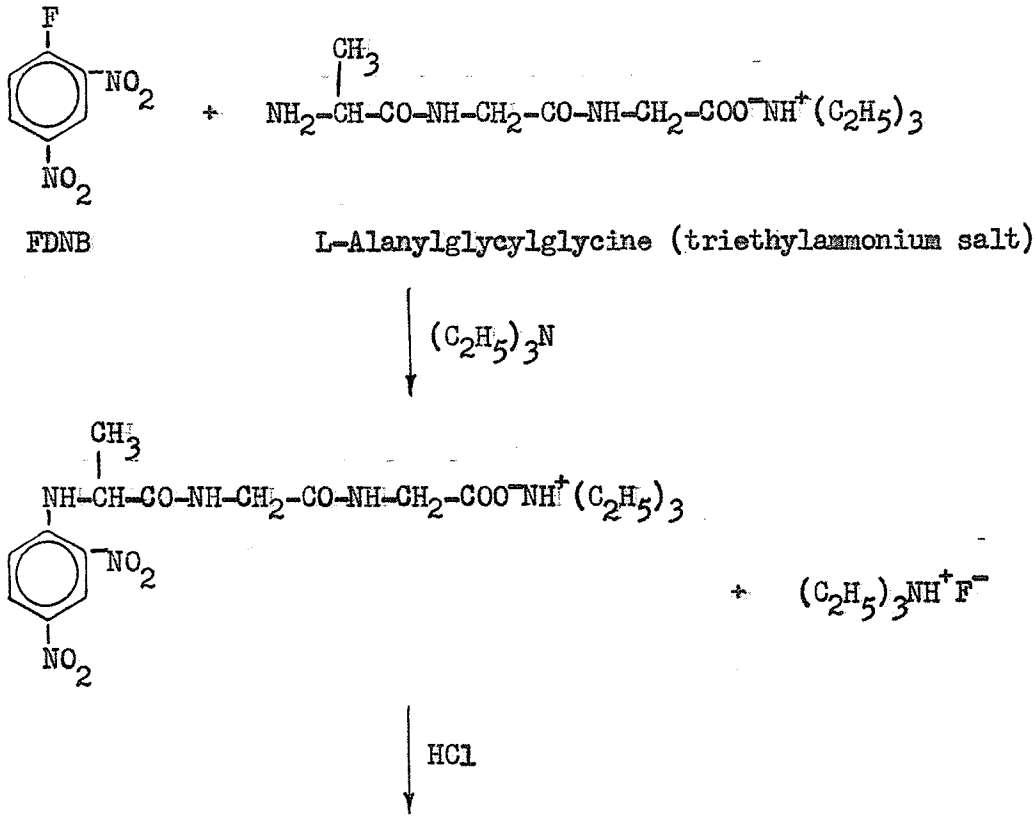
| Number of Preparations | Equiv. $(C_2H_5)_3N$/ Equiv. peptide | Temp. | Crude Yield % | Crude M.P. | Pure Yield % |
|---|---|---|---|---|---|
| 3 | 1 | Room | 38 | 212-216, 225 | 27-32 |
| 5 | 2 | Room | 66-74 | 212-218, 222-224 | 48-63 |
| 3 | 3 | Room | 51-55 | 209-215 | 37-40 |
| 3 | 2 | 40±1 | 73-74 | 220-223 | 62-63 |
| 3 | 3 | 40±2 | 50-59 | 198-205, 210-213 | 36-43 |

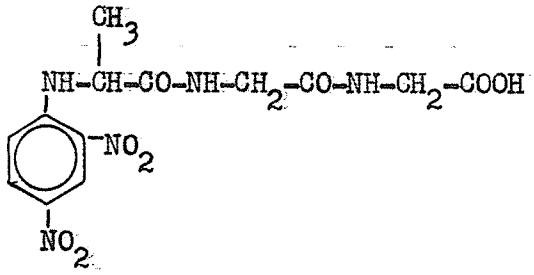
H, 4.22%; N 19.53%.

The presence of a hydrate was suggested by the fact that, when DNP-tetraglycine was place in the melting point apparatus (preheated to 190°), moisture condensed on the upper part of the melting point tube, even after the compound had been dried at 100° for one hour.

Preparation of DNP-L-Alanylglycylglycine

The preparation of DNP-L-alanylglycylglycine may be summarized by the equation:





DNP-L-alanylglycylglycine

Materials:

| | |
|---|---|
| FDNB solution | 5 ml (0.00081 mole) |
| L-Alanylglycylglycine | 0.164 gm (0.00081 mole) |
| Triethylamine | 2, 3, or 4 equivalents |
| Hydrochloric acid | 1 ml |

L-Alanylglycylglycine was dissolved in triethylamine solution and the FDNB solution was added. The reaction vessel was wrapped in paper and the mixture was stirred for six hours, at the end of which time, any excess triethylamine was removed by vacuum distillation from a water bath maintained at 41 to 43°. The distillation was continued until the pressure of the system had decreased to 25 to 30 mm. The residual solution was acidified with concentrated hydrochloric acid and refrigerated overnight. The product was filtered with suction, washed with ice water to remove excess hydrochloric acid, and dried at room temperature. It was washed with benzene to remove any unreacted FDNB, then dried for one-half hour at 80° before weighing. Purification of the crude product was by recrystallization from acetic acid and water. It was dissolved in glacial acetic acid, filtered to remove any insoluble impurities, diluted with ice water until the total volume was six times that of the acetic acid solution, then refrige-

rated overnight. More than one recrystallization was necessary in order to obtain a constant melting point. Ultimately, a yellow-orange solid melting at 197-198° was obtained. The yields of DNP-Lalanylglycylglycine obtained are summarized in Table 5.

Analysis

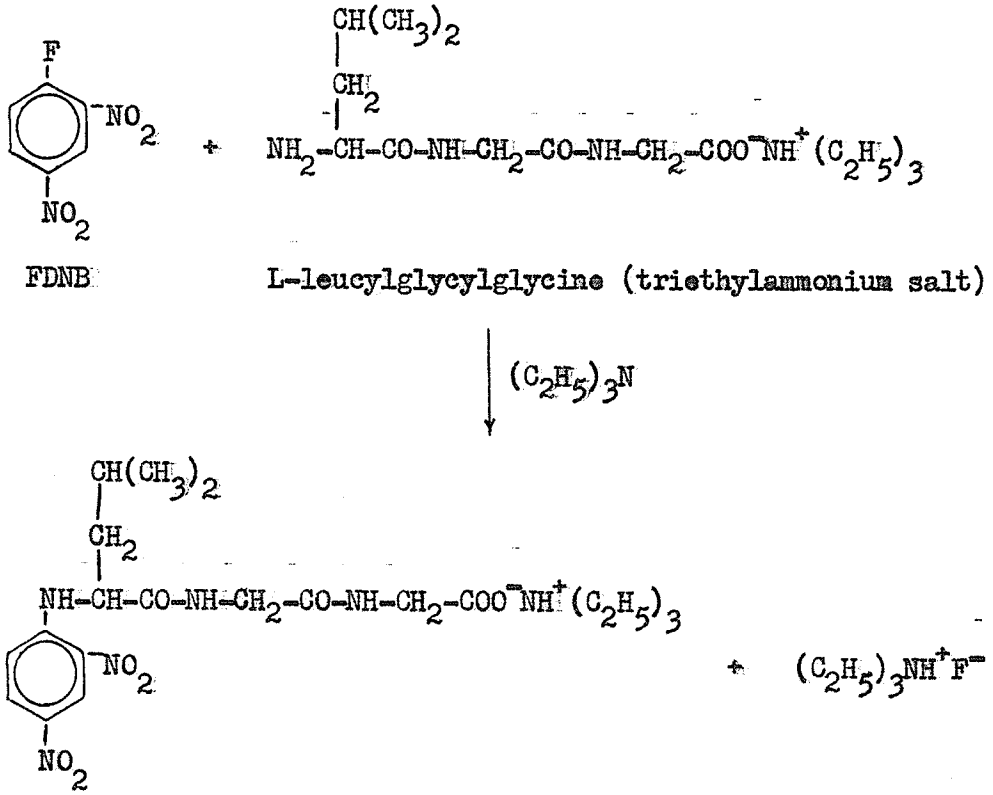
The compound was dried at 100° for one hour before analysis.

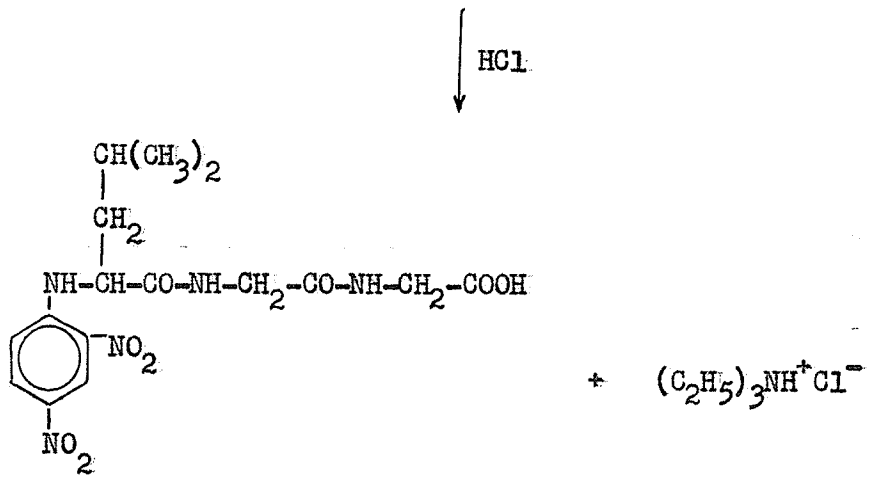
Found: C, 41.95%; H, 4.64%; N, 18.69%.

Calculated for DNP-L-alanylglycylglycine, $C_{13}H_{15}N_5O_8$: C, 42.28%; H, 4.09%; N, 18.97%.

Preparation of DNP-L-Leucylglycylglycine

The preparation of DNP-L-leucylglycylglycine may be summarized by the equation:






DNP-L-leucylglycylglycine

Materials:

| | |
|---|---|
| FDNB solution | 1.5 ml (0.00024 mole) |
| L-leucylglycylglycine | 0.059 gm (0.00024 mole) |
| Triethylamine | 2, 3, or 4 equivalents |
| Hydrochloric acid | 1 ml |

L-Leucylglycylglycine was dissolved in triethylamine solution, FDNB solution was added, the reaction vessel was wrapped in paper to reduce photodecomposition, and the mixture was stirred for six hours. Excess triethylamine was removed by vacuum distillation from a water bath maintained at 40 to 41°. The product was precipitated by addition of concentrated hydrochloric acid and overnight refrigeration. It was filtered with suction, washed successively with ice water and benzene, dried for one-half hour at 80°, and weighed.

DNP-L-leucylglycylglycine was recrystallized by dissolving the solid in a minimum amount of glacial acetic acid, filtering, and diluting with

ice water until the total volume was eight times that of the acetic acid solution. It was filtered immediately and dried at 80° for one-half hour. The purified compound was a bright yellow solid melting at 183.5-184.5°. The yields obtained are listed in Table 6.

Analysis

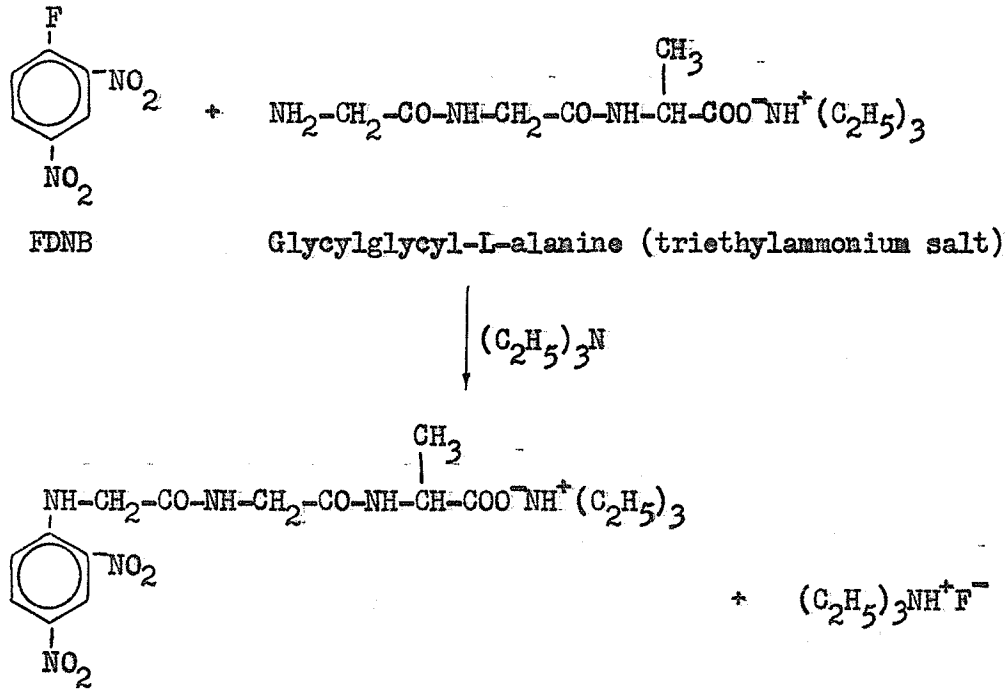
The compound was dried at 100° for one hour before analysis.

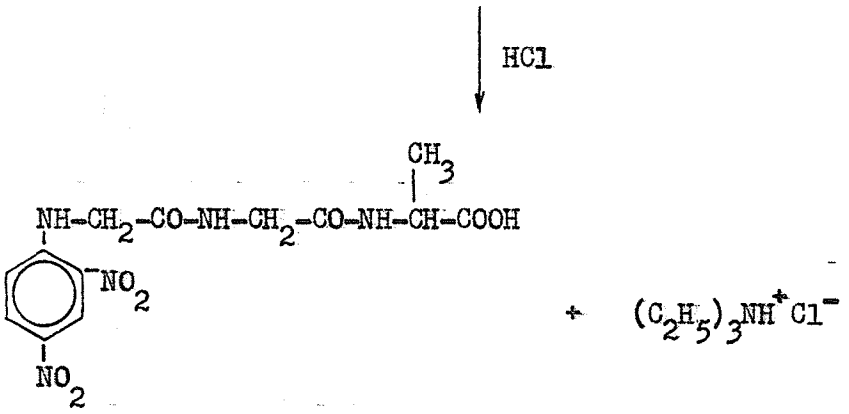
Found: C, 45.01%; H, 5.46%; N, 16.26%.

Calculated for DNP-L-leucylglycylglycine monohydrate, $C_{16}H_{23}N_5O_9$: C, 44.75%; H, 5.40%; N, 16.31%.

Preparation of DNP-Glycylglycyl-L-alanine

The preparation of DNP-glycylglycyl-L-alanine is summarized by the equation:






DNP-glycylglycyl-L-alanine

Materials:

| | |
|---|---|
| FDNB solution | 1.9 ml (0.00031 mole) |
| Glycylglycyl-L-alanine | 0.062 gm (0.00031 mole) |
| Triethylamine | 2, 3, or 4 equivalents |
| Hydrochloric acid | 1 ml |

Glycylglycyl-L-alanine was dissolved in triethylamine solution, and the FDNB solution was added. The reaction vessel was wrapped in paper, and the mixture stirred for six hours. Any excess triethylamine was removed by vacuum distillation. The crude product was recovered in a manner identical to that used in preparing DNP-L-leucylglycylglycine. It was purified by recrystallization from hot water (85°). The yellow product melted at 213.5-215°. The yields of impure DNP-glycylglycyl-L-alanine obtained are given in Table 7. The yields of pure product could not be obtained, since the available supplies of the peptide were exhausted during the preliminary work, the results of which are given in Table 7. Time did not permit the ordering of new supplies with which to complete the investigation.

Analysis

Found: C, 41.99%; H, 4.13%; N, 18.76%.

Calculated for DNP-glycylglycyl-L-alanine, $C_{13}H_{15}N_5O_8$: C, 42.28%; H, 4.09%; N, 18.97%.

TABLE 5

Preparation of DNP-L-Alanylglycylglycine

| Number of Preparations | Equiv. $(C_2H_5)_3N$/ Equiv. peptide | Temp. | Crude Yield % | Crude M.P. | Pure Yield % |
|---|---|---|---|---|---|
| 4 | 2 | Room | 30-35 | 191-194 | 19-22 |
| 3 | 3 | Room | 41-49 | 185-192 | 25-30 |
| 2 | 4 | Room | 40 | 165-196, 140-192 | - |
| 3 | 2 | 40±1 | 32-34 | 192-194 | 20-21 |

TABLE 6

Preparation of DNP-L-Leucylglycylglycine

| Number of Preparations | Equiv. $(C_2H_5)_3N$/ Equiv. peptide | Temp. | Crude Yield % | Crude M.P. | Pure Yield % |
|---|---|---|---|---|---|
| 2 | 2 | Room | 46-50 | 171-178, 179-183 | 29-32 |
| 2 | 3 | Room | 62-64 | 181-185 | 50-51 |
| 2 | 4 | Room | 43-46 | 179-182, 183-185 | 28-37 |
| 1 | 2 | 40±3 | 49 | 177-182 | 31 |
| 1 | 3 | 40±1 | 63 | 185-187 | 50 |

TABLE 7

Preparation of DNP-Glycylglycyl-L-alanine

| Number of Preparations | Equiv. $(C_2H_5)_3N$/ Equiv. peptide | Temp. | Crude Yield % | Crude M.P. |
|---|---|---|---|---|
| 2 | 2 | Room | 56-59 | 208-210 |
| 2 | 3 | Room | 61-71 | 209-211 |
| 1 | 4 | Room | 45 | 206-208 |
| 1 | 2 | 40±2 | 58 | 208-210 |
| 1 | 3 | 41±1 | 68 | 209-210 |

## Thin Layer Chromatography

Thin layer chromatography was used in an attempt to separate a mixture of seven DNP-compounds into its individual components. In addition, it was employed in an investigation of the benzene-soluble impurities which were formed during the reaction of FDNB with glycine and with the three peptides containing a varying number of glycyl residues. Several developers were tested in order to find one in which the samples moved as well-defined spots with suitable $R_f$ values.

### Apparatus

The apparatus used in all thin layer chromatography studies was that contained in the Mallinckrodt Chroma-Kit supplied by Mallinckrodt Chemical Works, St. Louis, Mo. It consisted of six 8" x 8" x 1/8" glass plates, and two aluminum sheets which could be assembled to form a rack on which the plates were placed during development of the chromatogram. The sorbent, one pound of SilicAR™ TLC-7GF, was composed of approximately 80% active hydrated silica of controlled particle size, 14% calcium sulfate binder, and 6% inorganic phosphor which fluoresced white under 2540 Å ultraviolet radiation. A roll of pressure-sensitive filament tape was supplied to enable the preparation of sorbent layers of uniform thickness. The tape, 0.5" wide and 0.25 ± 0.002 mm thick, was printed in 25.5 cm repetitive portions, 20 cm of which contained index marks at 0.5 and 1.0 cm intervals, and 5.5 cm of which contained no printing. It was contained in a tape-dispenser with a sharp cutting edge to ensure that the end of the strip of tape was cut smoothly. Five micropipettes, each of 5 μl capacity, and an applicator bulb were used in the application of samples to the layer.

A polypropylene boat was supplied to contain the developing solvent.

Purification of Solvents

(a) Benzene was purified by shaking the organic liquid (1 liter) successively with portions of concentrated sulfuric acid (100 ml) until free of thiophene, then with water until the washings were neutral to litmus. The water was removed by shaking the benzene with anhydrous calcium chloride, followed by refluxing for six hours over sodium metal. The benzene was distilled, the initial and final 50 ml of distillate being discarded, and the dry solvent was stored over sodium. It was distilled just prior to use.

(b) Glacial acetic acid was purified by the method of Vogel (34). Approximately 600 gm of A.R. glacial acetic acid was partly frozen, and the liquid was decanted. The solid (300 gm) was melted, mixed with A.R. potassium permanganate (6 gm), and distilled from a round-bottom flask fitted with a fractionating column. The fraction boiling at 115.5 to 116.4° was collected, partly frozen, and the liquid decanted. The solid was melted and fractionally distilled, the fraction boiling at 115.8 to 116.8° being collected and stored in a glass-stoppered flask. Precautions were taken to prevent the ingress of moisture during the fractional distillation.

(c) Ethanol was prepared by distilling 95% ethanol (200 ml) from a round-bottom flask fitted with a fractionating column. The initial 25 ml of the distillate were rejected, and the middle fraction (150 ml) was collected, leaving the residual 25 ml in the flask. This procedure was repeated once in order to obtain 95% ethanol used in the chromatographic developer.

Preparation of the Plates

The glass plates were cleaned by treatment with sulfuric acid-potassium dichromate cleaning solution, rinsed thoroughly with distilled water, and dried. It was imperative that all grease and dirt be removed from the glass surface; otherwise, the sorbent layer would not adhere properly.

The tape was trimmed carefully to the zero index mark. A strip was pulled off and cut precisely at the 20 cm mark, then placed along one edge of the glass plate, care being taken to place the zero index point exactly at the bottom edge of the glass. The 5.5 cm section of unprinted tape was cut off and saved. Another 20 cm section of tape was affixed in the same fashion to the parallel edge of the glass plate.

In order to prepare the sorbent layer, distilled water (12 ml) was added to the active hydrated silica (6 gm) and shaken vigorously for 15 to 25 seconds in a stoppered Erlenmeyer flask. The resulting slurry was poured across one untaped edge of the plate from tape to tape. A glass rod was placed across the plate with the ends resting on the tape. The slurry was levelled by moving the rod smoothly in a direction parallel to the taped edges of the plate, while pressing firmly on both ends. A smooth, uniform layer 0.25 mm thick was obtained. The plate was air-dried until the layer had set. Before placing the plate in the oven for activation, the tape was split lengthwise and the inner half removed from the plate, leaving a gap of approximately 1/4" between the remaining tape and the sorbent layer. In this way, solvent contact with the tape during development of the chromatogram was eliminated.

A cover plate was prepared by splitting two 20-cm lengths of tape lengthwise, and applying double thicknesses (0.50 mm) to two opposite edges of a glass plate. The plate was turned over, and another 20-cm strip of tape was applied along one edge at right angles to the first strips. The plate was used both as a spotting template and as a developing chamber cover for spotted plates.

The sorbent layer was activated by placing the plate in the oven for one hour at the desired temperature. In order to spot the samples on the coated plate, the cover plate was placed on the coated plate, resting tape against tape in order to avoid disturbing the sorbent layer. The samples to be chromatographed were dissolved in a suitable solvent and applied on a line parallel to, and at least 2 cm from, the edge of the coated plate. In addition, a margin of at least 2 cm was maintained between any spot and a taped edge. The samples were applied to the coated plate by means of a 5 μl micropipette. Approximately 2 μl was applied at once, allowing time for solvent evaporation between successive applications, in order to keep the spot size small.

After the spots had been applied, the cover plate was adjusted until it just covered the spots. The plates were taped together at both edges near the top with short unprinted pieces of tape. The tape at the bottom edge of the coated plate was peeled away and bent back over the edge of the cover plate. Unprinted tape applied at this point held the peeled portion away from the solvent, in addition to holding the plates together. The tape at both the edges of the coated and cover plates functioned as a gasket, sealing the chamber so that it could be saturated by a small volume of the

developing solvent.

The chromatogram was developed by filling the solvent boat to a depth of about 1 cm with the appropriate solvent, and placing the plate carefully in the solvent. The chamber was rested against the aluminum rack provided for that purpose. The solvent was allowed to move up through the layer to a height of 10 to 15 cm. The chamber was removed from the solvent boat, the cover plate removed, the position of the solvent marked, and the solvent allowed to evaporate. The distances moved by the samples were measured, and their $R_f$ values calculated.

The preceeding method was used when the developing solvent consisted of only one liquid. In most of the investigations, however, a mixture of two or three liquids was employed as a developer, and a slightly modified procedure was adopted. For mixed-solvent systems, it was necessary that the atmosphere of the developing chamber be presaturated with the solvent, in order that the composition of the developer did not change due to rapid evaporation of the more volatile component, as it rose through the sorbent layer. For this purpose, a coated plate was prepared in the usual fashion, and activated at 60° for one hour. The tape thickness at the edge of the coated cover plate was built up to 1 mm by adding three additional 1/4" wide strips of tape. The coated cover plate was placed on the bench top with one end slightly elevated, and thoroughly saturated with the developing solvent by pouring approximately 10 ml across the elevated end. The spotted plate was covered immediately with the saturated coated cover plate, and the chromatogram was developed as explained previously, except that the cover plate, rather than the spotted plate, was placed toward the aluminum

rack, in order to allow the course of spot movement to be followed.

Thin Layer Chromatography of Benzene-Soluble Impurities Produced during Dinitrophenylation in a Bicarbonate Medium

The DNP-derivatives of glycine and three peptides containing a varying number of glycyl residues were precipitated from the aqueous sodium bicarbonate medium by acidifying with concentrated hydrochloric acid. The compounds were filtered with suction, washed with ice water to remove excess hydrochloric acid, and dried at room temperature. Benzene-soluble impurities were removed by washing with benzene. The washings were concentrated by allowing the benzene to evaporate partially at room temperature, and the solutions were applied to the chromatographic plate in amounts sufficient to produce distinct yellow spots (2 to 4 $\mu$l). When an adsorbent layer of 0.25 mm thickness was activated at 60° for three hours and developed with a benzene-acetic acid mixture (98:2), the benzene washings obtained from DNP-glycine were shown to contain two yellow compounds, one having an $R_f$ value of 0.40, and the other having an $R_f$ value of 0.03. The washings from DNP-triglycine and DNP-tetraglycine also contained two yellow compounds, one having an $R_f$ value of 0.40, and the other, 0.10, while the washings from DNP-diglycine contained only one yellow compound, the $R_f$ value being 0.40. The faster moving compound obtained from DNP-glycine produced a less intense spot than the slower moving compound, but in the cases of DNP-triglycine and DNP-tetraglycine, the faster moving compound was more pronounced. Further experiments, in which the DNP-compounds in 95% ethanol solution were spotted to the plate and developed under the same conditions, showed that the more intensely colored compound found in the

benzene washings of DNP-glycine was probably DNP-glycine itself, since the $R_f$ value obtained was 0.03. The slower moving spots found in the washings of DNP-triglycine and DNP-tetraglycine were definitely not the DNP-peptides, since the latter remained at the origin under the developing conditions employed. The spots were not identified.

Preparative thin layer chromatography was employed in isolating the compound which had an $R_f$ value of 0.40, and which appeared as an impurity in all the DNP-compounds prepared by using sodium bicarbonate as a condensing agent. A layer of sorbent 1 mm thick was prepared by mixing the activated silica (30 gm) with water (51 ml) according to the method of Honegger (35), and spreading the slurry on the plate in the usual manner. In order to achieve the required thickness, four layers of tape, rather than only one, were affixed to the edges of the plate. The plate was activated at 60° for two hours, and the benzene solution of the impurities was applied in a narrow band by means of a clean glass tube drawn out to a fine capillary. By using a thicker layer of adsorbent, and by applying the sample as a band rather than as a spot, a sufficient amount of the impurity could be separated for identification. The chromatogram was developed with a benzene-acetic acid mixture (98:2). Three bands were obtained, the most intense having an $R_f$ value of approximately 0.40, the next, much less intense band, having an $R_f$ value of approximately 0.10, and a third very weak band, previously undetected, having an $R_f$ value of approximately 0.03. Each band was scraped from the plate and eluted with benzene. Only the most intense band yielded enough compound for identification. The pale yellow solid recovered had a melting point of 103.5-108° after recrystallization

from hot water. Its infrared spectrum was taken and compared to that of 2,4-dinitrophenol, since that compound was known to be produced in the reaction of FDNB with amino acids. The spectra were identical, proving that 2,4-dinitrophenol was the main benzene-soluble impurity produced during dinitrophenylation of the peptides studied under the conditions employed.

The appearance of 2,4-dinitrophenol in a sodium bicarbonate medium containing FDNB was not dependent on the presence of the amino acid or peptide. When FDNB, 95% ethanol, sodium bicarbonate, and water were reacted for two days in the same proportions as those used in the preparation of the DNP-compounds, a compound was produced which could be extracted into benzene from the acidified reaction mixture, and which had an $R_f$ value identical to that of the most intense spot obtained from the benzene washings of DNP-triglycine and DNP-tetraglycine, when the samples were chromatographed under identical conditions. Therefore, 2,4-dinitrophenol was produced in the basic medium in the absence of any amino acid or peptide.

Other investigations with the benzene-soluble impurities produced during the synthesis of DNP-triglycine and DNP-tetraglycine showed that, using benzene as the developer, tailing of the spots was much less pronounced on activated plates than on plates which were only air-dried. By using a benzene-acetic acid mixture (98:2) as the developing solvent, tailing was reduced on an air-dried plate and virtually eliminated on an activated plate.

The identity of two samples of 2,4-dinitrophenol could not be established on the basis of $R_f$ values alone, unless approximately the same amount of the compound were spotted in each case. If the amounts of com-

pound spotted to the plate were very different, the sample present in the higher concentration showed a larger $R_f$ value than that present in the smaller amount.

Separation of DNP-Peptides by Thin Layer Chromatography

Each of the DNP-compounds prepared was dissolved in 95% ethanol and spotted to a plate which had been activated at 60° for one hour. An attempt to develop the chromatogram with a benzene-acetic acid solvent (98:2) indicated that a much more polar developer would be required, since only DNP-glycine moved from the origin, and its $R_f$ was only 0.02. The use of a benzene-acetic acid mixture (22:3) moved all the samples except DNP-tetraglycine from the origin, but only DNP-glycine had separated completely from the other six compounds. By using a mixture of benzene and acetic acid (10:3), DNP-glycine and DNP-diglycine could be separated from each other and from DNP-triglycine and DNP-tetraglycine, but the DNP-tripeptide and DNP-tetrapeptide could not be separated from one another. DNP-L-leucylglycyl-glycine could be separated from all the other compounds, except DNP-di-glycine and DNP-glycylglycyl-L-alanine. DNP-L-alanylglycylglycine could be separated from DNP-glycine, DNP-L-leucylglycylglycine, and DNP-tetraglycine. DNP-L-leucylglycylglycine could be separated from DNP-glycylglycyl-L-ala-nine and DNP-diglycine by using benzene-acetic acid (2:1) as the develo-ping solvent; however, the spots began to show some tailing as the pro-portion of acetic acid was increased.

The two developing solvents which produced the most satisfactory separ-ations were benzene-acetic acid (7:3) and benzene-acetic acid-95% ethanol (20:6:2). The separations achieved are shown in Figures 2 and 3. The

mixture of DNP-glycine and the three DNP-peptides containing a varying number of glycyl residues was readily separated into its constituent compounds by either developer. DNP-L-leucylglycylglycine separated from the other six compounds when the mixture was developed with either solvent. DNP-L-alanylglycylglycine could not be separated from DNP-diglycine or DNP-glycyl-glycyl-L-alanine by either developer, but each of these was readily separated from DNP-triglycine, DNP-tetraglycine, and DNP-glycine when developed by either solvent. The $R_f$ values obtained for the individually spotted DNP-compounds are listed in Table 8 (a) and Table 8 (b).

TABLE 8

$R_f$ VALUES OF DNP-COMPOUNDS

(a) Benzene-Acetic Acid (7:3) Developer

| Compound | $R_f$ |
|---|---|
| DNP-glycine | 0.41 |
| DNP-diglycine | 0.20 |
| DNP-triglycine | 0.10 |
| DNP-tetraglycine | 0.02 |
| DNP-L-alanylglycylglycine | 0.18 |
| DNP-L-leucylglycylglycine | 0.27 |
| DNP-glycylglycyl-L-alanine | 0.16 |

(b) Benzene-Acetic Acid-95% Ethanol (20:6:2) Developer

| Compound | $R_f$ |
|---|---|
| DNP-glycine | 0.46 |
| DNP-diglycine | 0.32 |
| DNP-triglycine | 0.23 |
| DNP-tetraglycine | 0.10 |
| DNP-L-alanylglycylglycine | 0.29 |
| DNP-L-leucylglycylglycine | 0.35 |
| DNP-glycylglycyl-L-alanine | 0.27 |

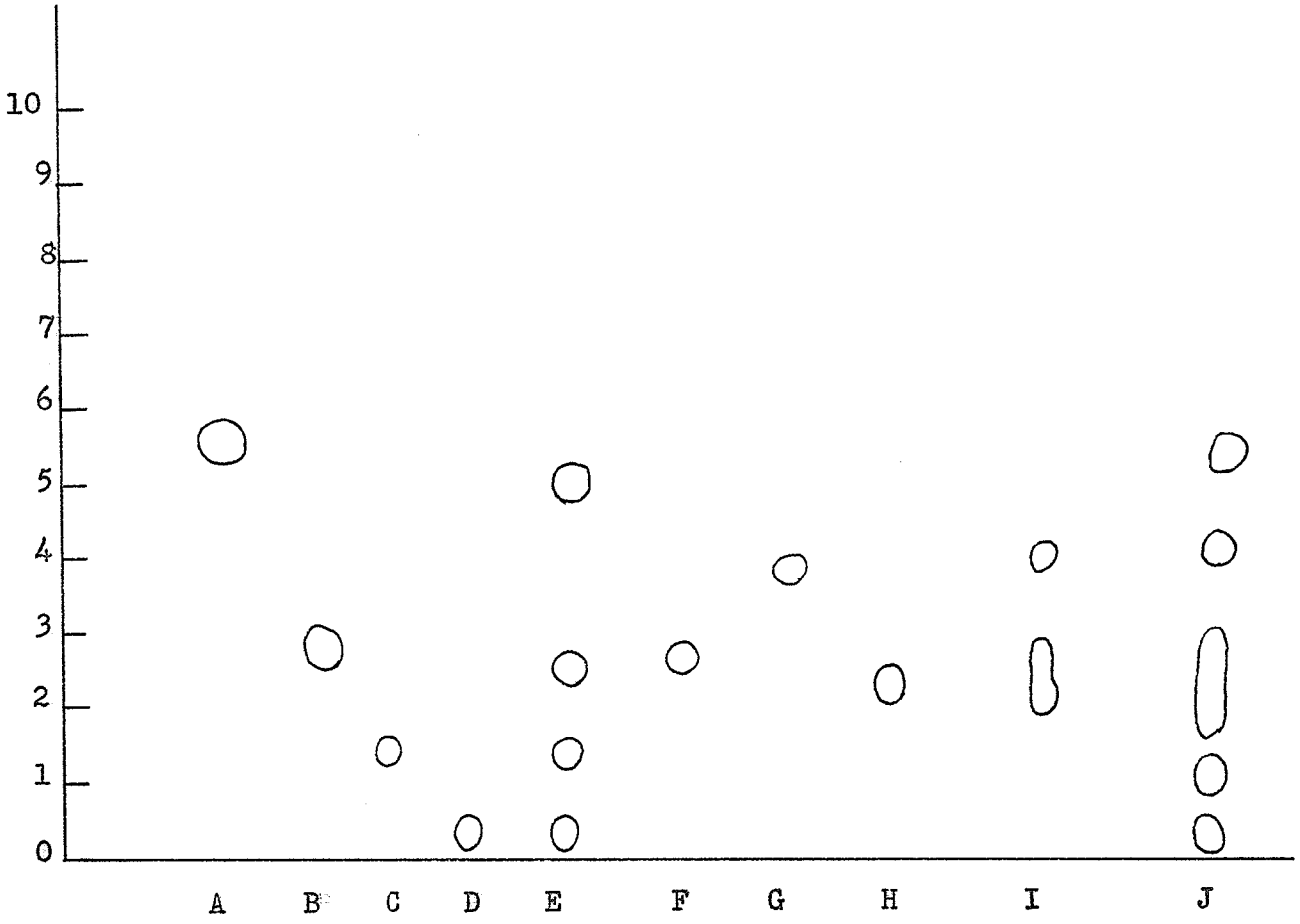



Figure 2. Thin layer chromatography of DNP-compounds using benzene-acetic acid (7:3) as developer. A, DNP-glycine; B, DNP-diglycine; C, DNP-triglycine; D, DNP-tetraglycine; E, a mixture of A, B, C, D; F, DNP-L-alanylglycylglycine; G, DNP-L-leucylglycylglycine; H, DNP-glycylglycyl-L-alanine; I, a mixture of F, G, and H; J, a mixture of A, B, C, D, F, G, and H.

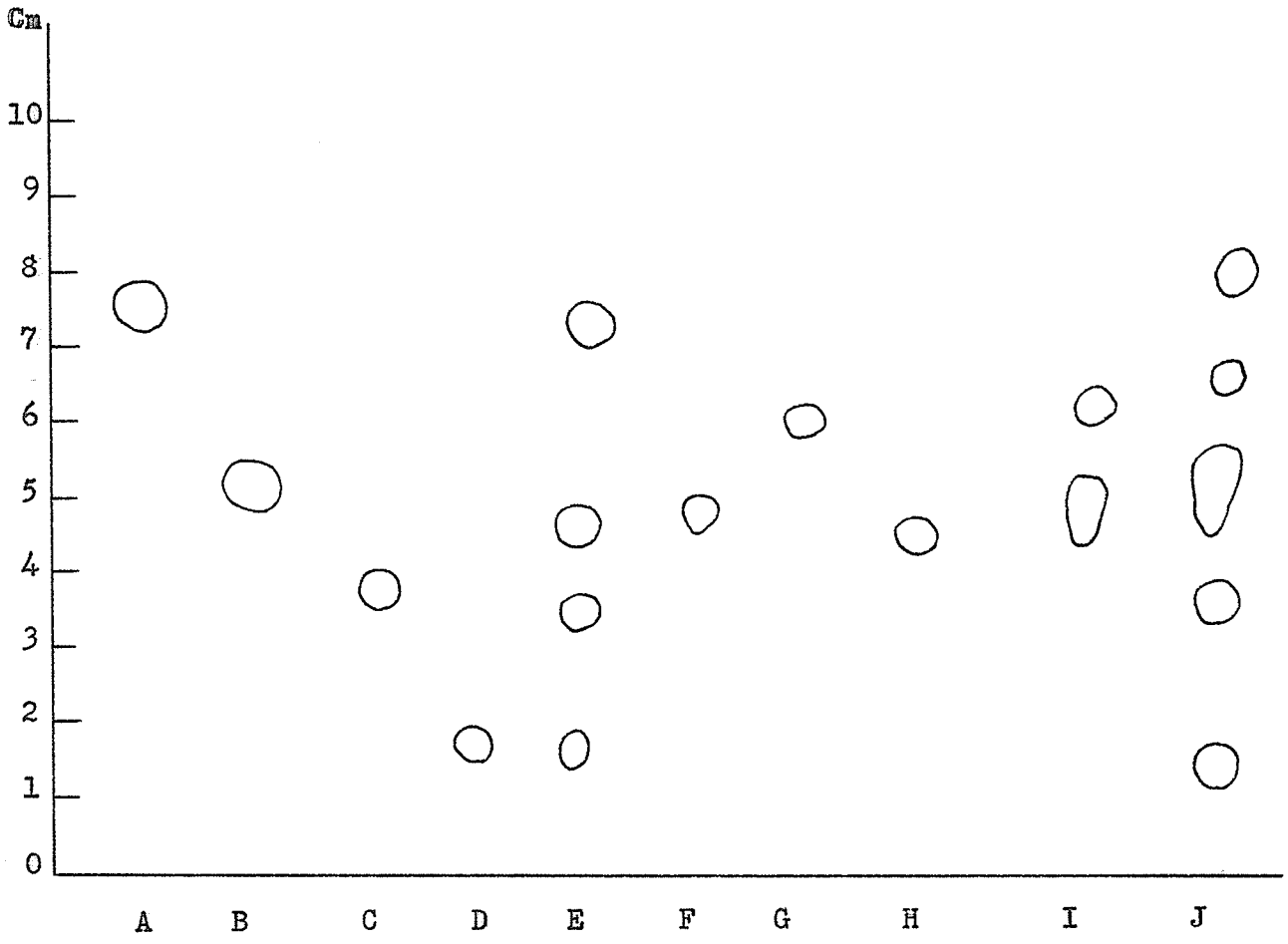



Figure 3. Thin layer chromatography of DNP-compounds using benzene-acetic acid-95% ethanol as developer. A, DNP-glycine; B, DNP-diglycine; C, DNP-triglycine; D, DNP-tetraglycine; E, a mixture of A, B, C, and D; F, DNP-L-alanylglycylglycine; G, DNP-L-leucylglycylglycine; H, DNP-glycylglycyl-L-alanine; I, a mixture of F, G, and H; J, a mixture of A, B, C, D, F, G, and H.

Ultraviolet Spectrophotometry

A Beckman Model DK recording spectrophotometer attached to a Brown recorder, and quartz cells of 1 cm path length were employed in the ultraviolet studies of all the DNP-compounds prepared. The ultraviolet spectrum of each compound was determined in 0.2 M sodium bicarbonate solution and in glacial acetic acid solution.

A stock solution of the compound was prepared by dissolving the sample (approximately 0.015 gm) in the solvent (50 ml). Two solutions suitable for study of the spectrum were prepared by diluting two 5 ml aliquots of the stock solution with volumes of solvent sufficient to reduce the concentration of the DNP-compound to approximately $3 \times 10^{-5}$ and $6 \times 10^{-5}$ respectively. The transmittance of the less concentrated solution was in the 30 to 40% range at 340 to 360 mμ, and that of the more concentrated solution was in a similar range at 260 to 270 mμ. It was desirable to arrange the concentrations to values which would give minimum transmittances between 30% and 40%, since the per cent relative analysis error per 1% photometric error is a minimum at 37% transmittance.

The wavelengths at which the absorption maxima, $\lambda_{max}$, occurred were determined for each compound in sodium bicarbonate solution and in glacial acetic acid solution, and the molar absorptivity, $\epsilon_M$, at those wavelengths was calculated. The results of the determinations are summarized in Table 9 and Table 10.

The sensitivity of DNP-amino acids to light is well known (9, 28, 29, 31, 32). A study of the DNP-peptides containing a varying number of glycyl residues was undertaken in order to determine their behavior under the

TABLE 9

(a) Molar Absorptivity of DNP-Compounds in 0.2 M Sodium Bicarbonate Solution in the Range 350 to 360 mμ

| Compound | $\lambda_{max}$ (mμ) | $\epsilon_M$ at $\lambda_{max}$ |
|---|---|---|
| DNP-glycine | 360 | 17,000 |
| DNP-diglycine | 354 | 18,200 |
| DNP-triglycine | 352 | 15,800 |
| DNP-tetraglycine | 351 | 16,100 |
| DNP-L-alanylglycylglycine | 353 | 19,300 |
| DNP-L-leucylglycylglycine | 352 | 17,000 |
| DNP-glycylglycyl-L-alanine | 352 | 16,000 |

(b) Molar Absorptivity of DNP-Compounds in Glacial Acetic Acid Solution in the Range 335 to 345 mμ

| Compound | $\lambda_{max}$ (mμ) | $\epsilon_M$ at $\lambda_{max}$ |
|---|---|---|
| DNP-glycine | 338 | 15,700 |
| DNP-diglycine | 339 | 17,300 |
| DNP-triglycine | 341 | 14,600 |
| DNP-tetraglycine | 341 | 15,500 |
| DNP-L-alanylglycylglycine | 339 | 18,800 |
| DNP-L-leucylglycylglycine | 338 | 16,700 |
| DNP-glycylglycyl-L-alanine | 340 | 15,800 |

TABLE 10

(a) Molar Absorptivity of DNP-Compounds in 0.2 M Sodium Bicarbonate Solution in the Range 260 to 270 mμ

| Compound | $\lambda_{max}$ (mμ) | $\epsilon_M$ at $\lambda_{max}$ |
|---|---|---|
| DNP-glycine | 265 | 8700 |
| DNP-diglycine | 265 | 9800 |
| DNP-triglycine | 264 | 8500 |
| DNP-tetraglycine | 265 | 8900 |
| DNP-L-alanylglycylglycine | 264 | 10,500 |
| DNP-L-leucylglycylglycine | 265 | 9000 |
| DNP-glycylglycyl-L-alanine | 264 | 8700 |

(b) Molar Absorptivity of DNP-Compounds in Glacial Acetic Acid Solution in the Range 255 to 265 mμ

| Compound | $\lambda_{max}$ (mμ) | $\epsilon_M$ at $\lambda_{max}$ |
|---|---|---|
| DNP-glycine | 260 | 9200 |
| DNP-diglycine | 261 | 9400 |
| DNP-triglycine | 260 | 8000 |
| DNP-tetraglycine | 260 | 8600 |
| DNP-L-alanylglycylglycine | 260 | 10,600 |
| DNP-L-leucylglycylglycine | 261 | 8600 |
| DNP-glycylglycyl-L-alanine | 260 | 8600 |

influence of light. The four solutions of each compound, as well as those of DNP-glycine, which were prepared for the measurement of molar absorptivities were employed in the investigation. Of the two solutions of each compound in glacial acetic acid, one was stored in darkness, and the other was allowed to stand in the laboratory, unprotected from the light. The two solutions of each compound in sodium bicarbonate solution were treated in an identical manner. The spectrum of each solution was determined periodically in order to detect any changes which occurred.

DNP-glycine was stable over a period of several weeks in both acetic acid solution and sodium bicarbonate solution, if the solutions were protected from light. When the sodium bicarbonate solution of DNP-glycine was exposed to light, a gradual change in the absorption spectrum occurred. The original absorbance maximum occurring at 360 mμ shifted slowly to a lower wavelength and decreased in intensity. After five weeks, the maximum had shifted to 352 mμ. The secondary maximum which occurred at 264 mμ in a freshly prepared solution, had shifted to 287 mμ and increased slightly in intensity after five weeks. DNP-glycine in glacial acetic acid also showed a sensitivity to light. The absorbance maximum occurring at 338 mμ in the freshly prepared solution had shifted to 352 mμ and decreased considerably in intensity after five weeks. The changes in the spectrum of DNP-glycine in each solution are shown in Fig. 4 and Fig. 5.

DNP-diglycine, DNP-triglycine, and DNP-tetraglycine in glacial acetic acid solution were relatively stable over a period of five weeks, whether the solution was exposed to light or stored in darkness. The absorbance maximum of DNP-diglycine, which occurred at 339 mμ, showed a very slight decrease in intensity, while the absorbance of DNP-triglycine and DNP-tetra-

glycine had increased slightly at wavelengths shorter than 300 mμ.

The spectra of the three DNP-peptides studied changed radically when the compounds were dissolved in sodium bicarbonate solution. The change was independent of the lighting conditions to which the solution was exposed. The absorbance maximum occurring at 350 to 355 mμ in the freshly prepared solution was replaced by a maximum of somewhat greater intensity at 295 mμ. The change was complete in two to three weeks. Furthermore, the reaction rate was strongly dependent on the pH of the solution. It was followed by periodic measurements of the absorbance at 295 mμ, since it was at this wavelength that the greatest change was observed. It was observed that, whereas the reaction required two to three weeks to reach completion in 0.2 M sodium bicarbonate solution, having a pH of approximately 8.4, only a slight change occurred after two hours in a solution of pH 11, and the reaction was virtually complete in four hours. When the pH was increased to 12, reaction was complete in fifteen minutes. Studies with identical solutions stored in the dark yielded identical results. The reaction therefore appears to be dependent on the pH of the solution rather than on exposure to light. The change in the absorbance curve is shown in Fig. 6.

Acidification of the yellow sodium bicarbonate solutions of each of the three DNP-peptides, after the change in their spectra had reached completion, resulted in a colorless solution. In the acidic medium, the absorbance maximum had been shifted to 280 mμ, and the absorbance at 400 mμ was virtually eliminated. The shape of the absorbance curve in both the basic and acidic media was essentially the same.

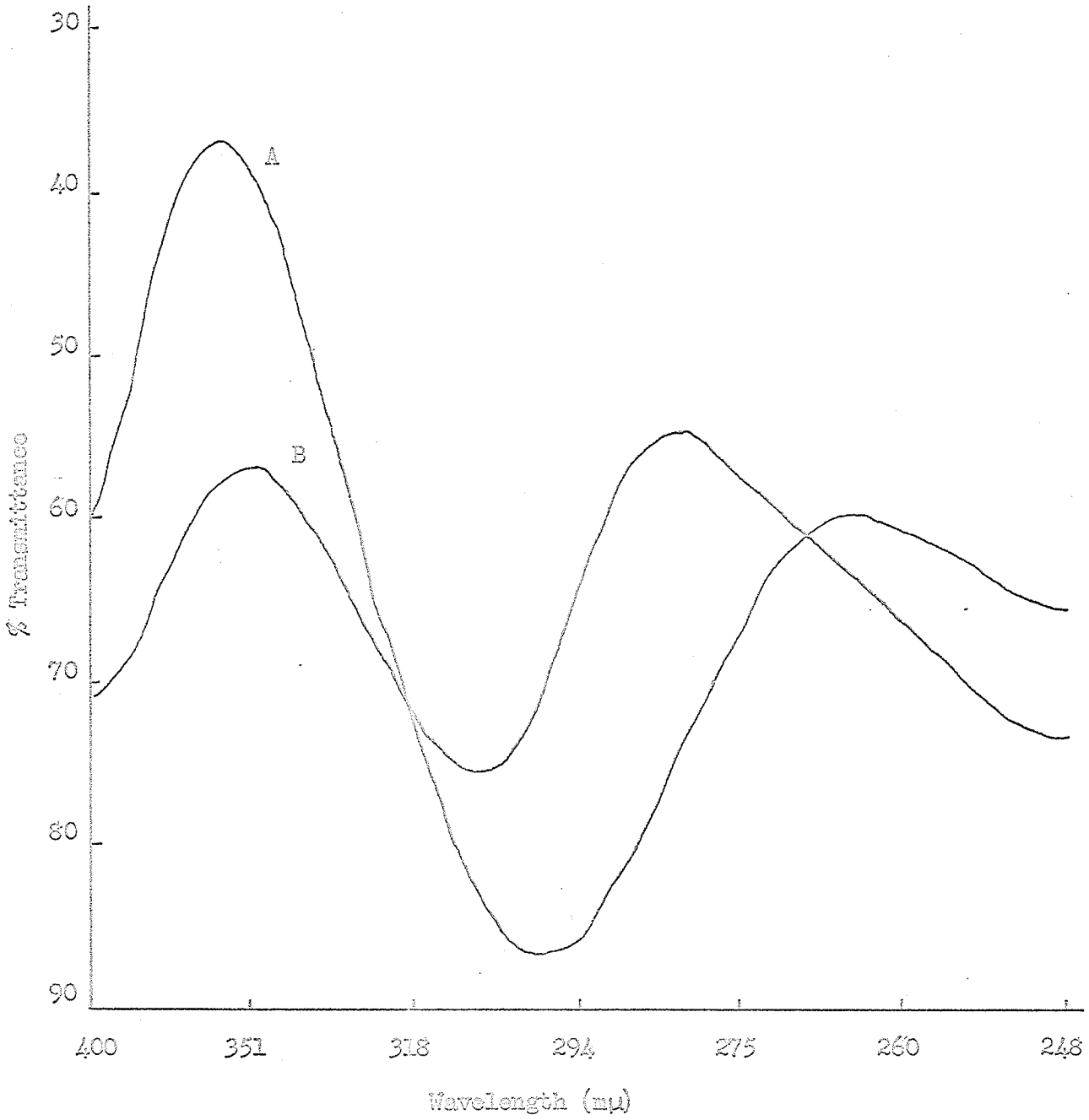



Figure 4. DNP-glycine in 0.2 M sodium bicarbonate solution. A, spectrum of a freshly prepared solution; B, spectrum of a solution exposed to light for 5 weeks.

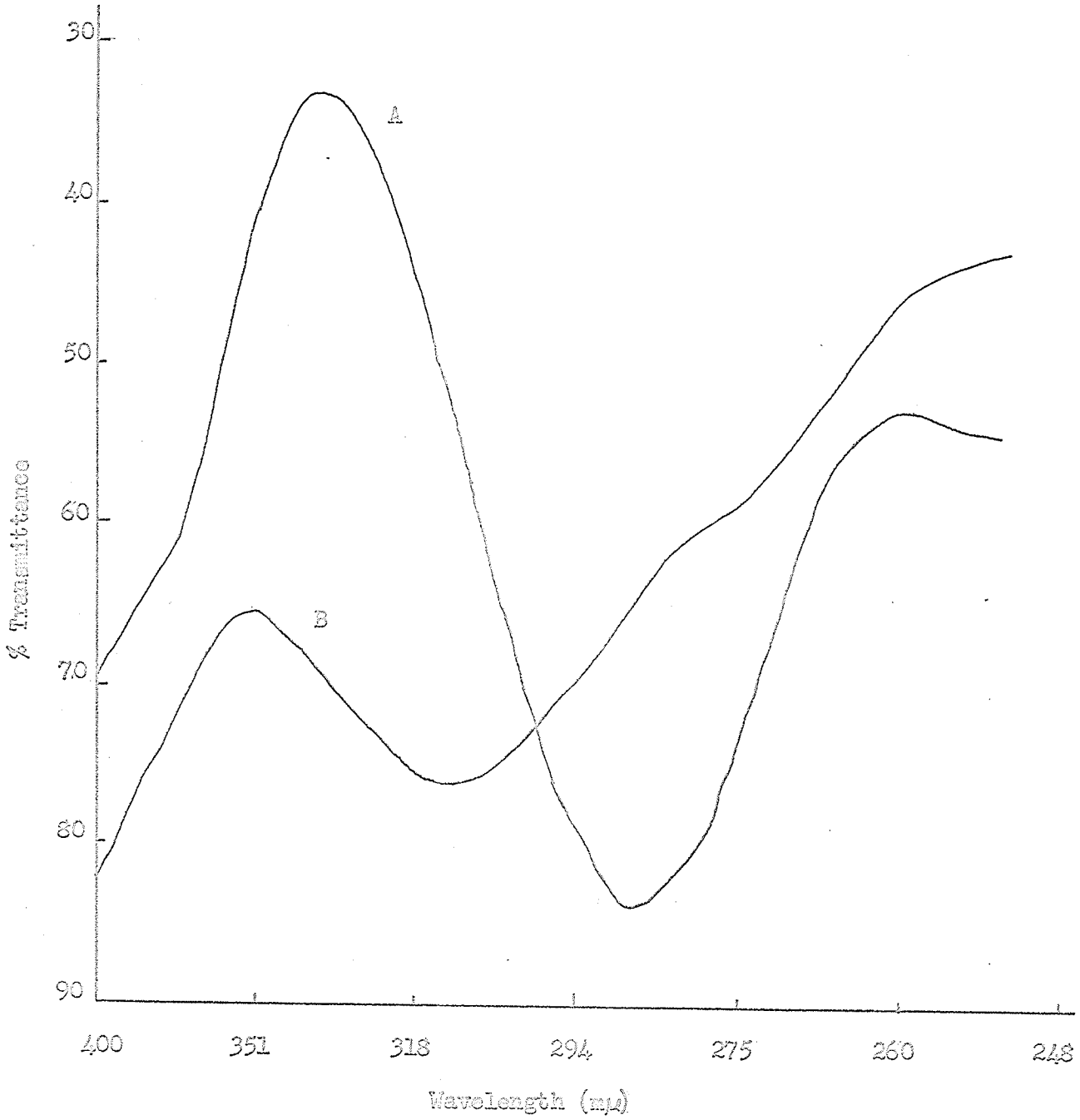



Figure 5. DNP-glycine in glacial acetic acid solution. A, spectrum of a freshly prepared solution; B, spectrum of a solution exposed to light for 5 weeks.

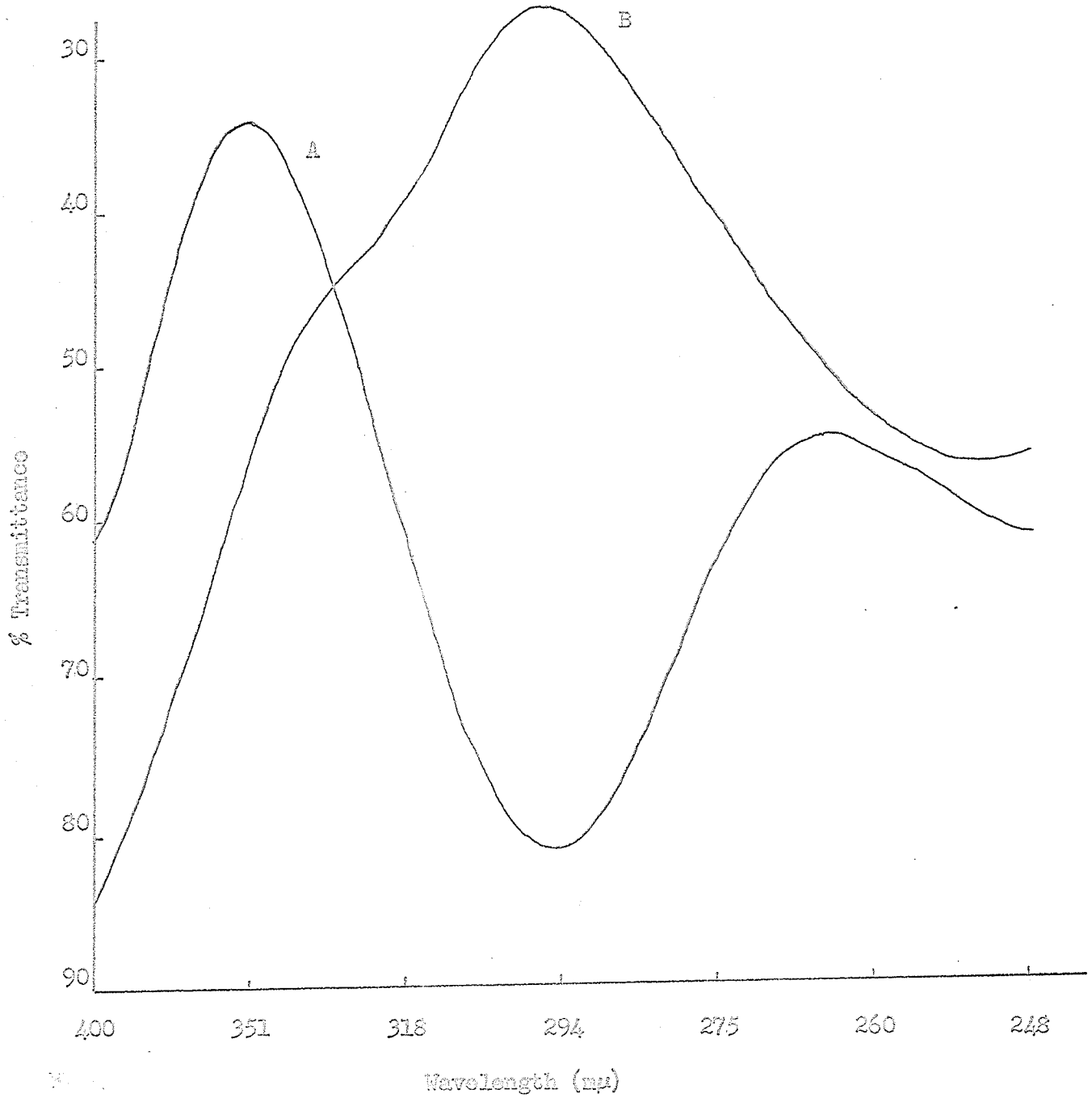



Figure 6. DNP-triglycine in sodium bicarbonate solution. A, spectrum of a freshly prepared solution; B, spectrum of a solution 3 weeks old.

Infrared Spectra

A Nujol mull of each DNP-compound was prepared, and its infrared spectrum was taken. In addition, the identity of the main benzene-soluble impurity produced in the preparation of DNP-diglycine, DNP-triglycine, and DNP-tetraglycine in sodium bicarbonate medium was established by comparing its infrared spectrum with that of 2,4-dinitrophenol. A Perkin-Elmer Model 21 Infrared Spectrophotometer, fitted with sodium chloride windows, was used to obtain all infrared spectra. The spectra are shown in Figs 7 to 14.

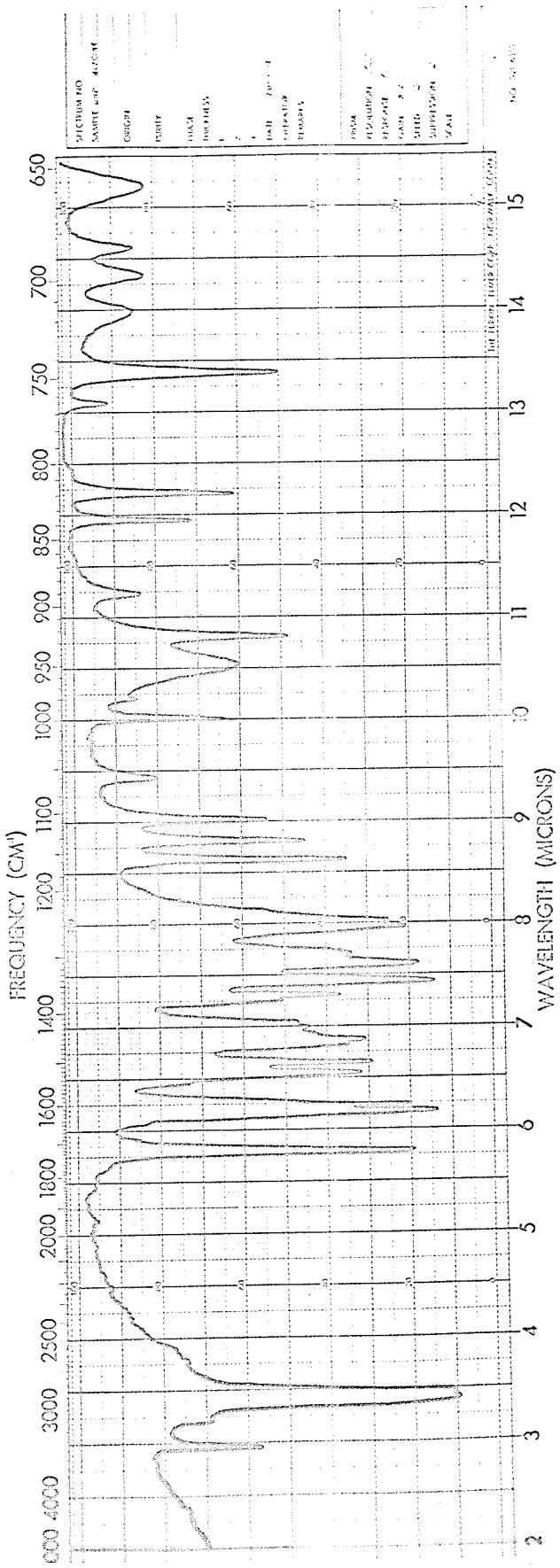



Figure 7.

The infrared spectrum of DNP-glycine

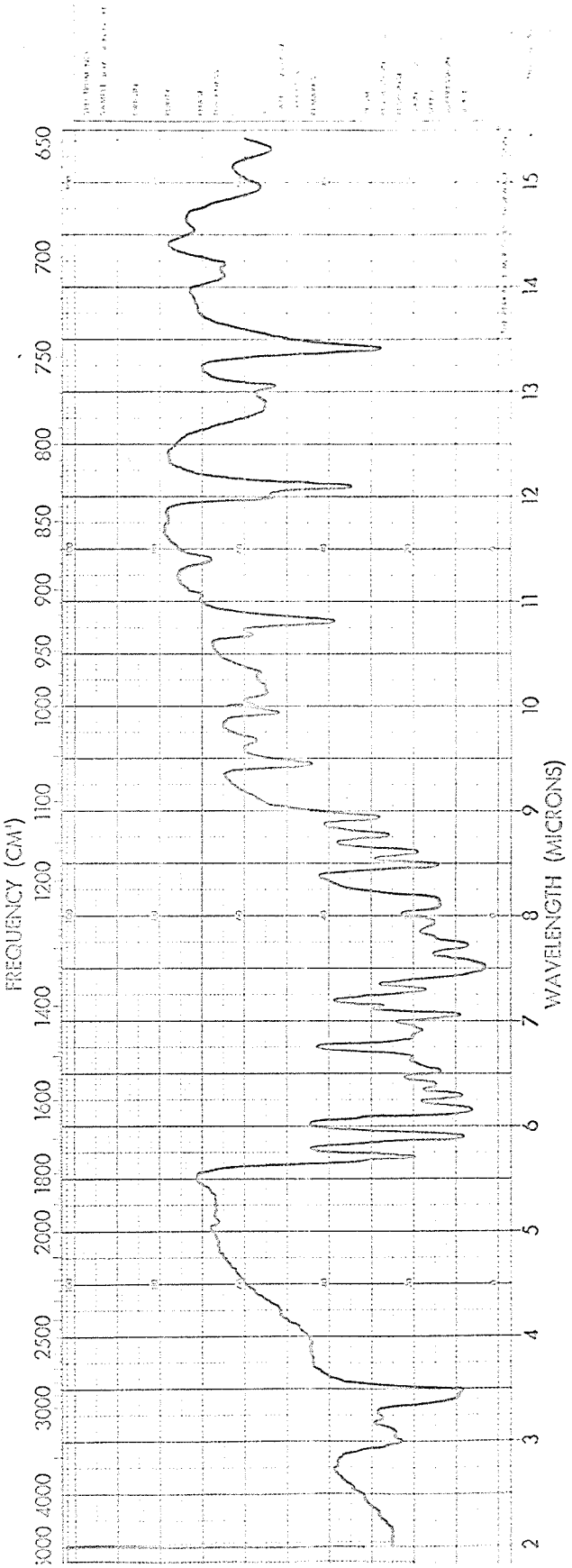



Figure 8.

The infrared spectrum of DNP-diglycine.

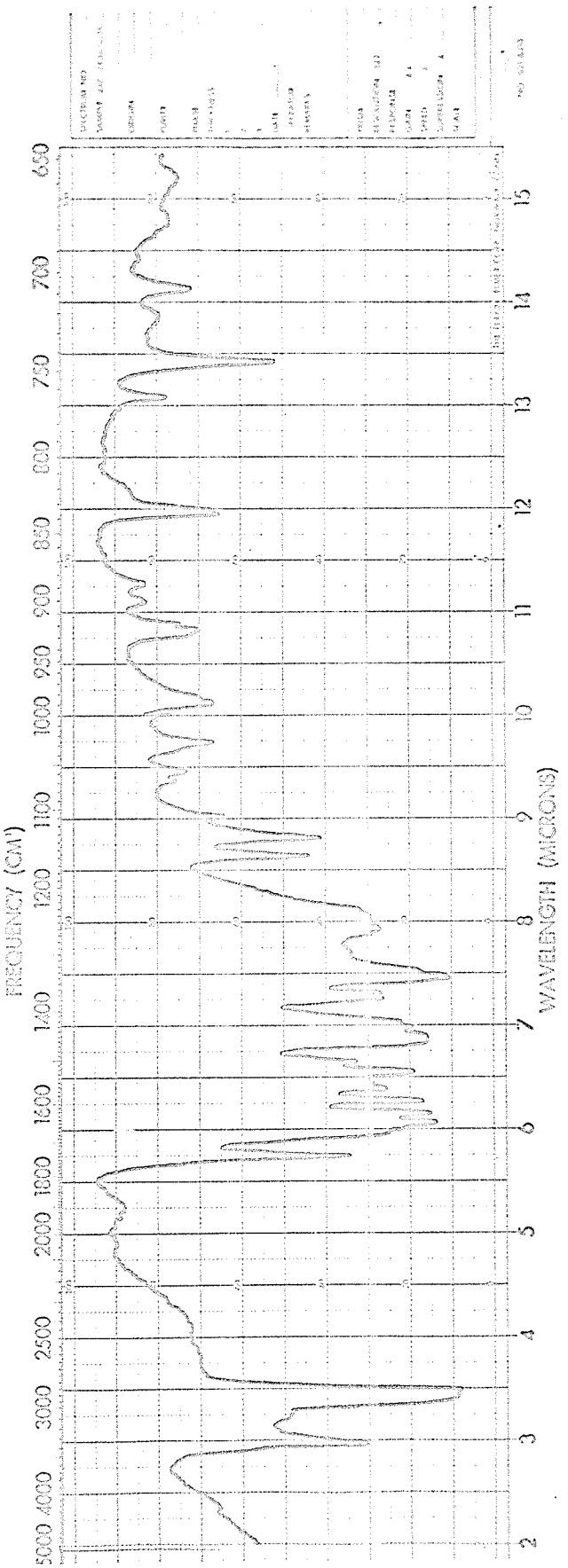



Figure 9.

The infrared spectrum of DNP-triglycine.

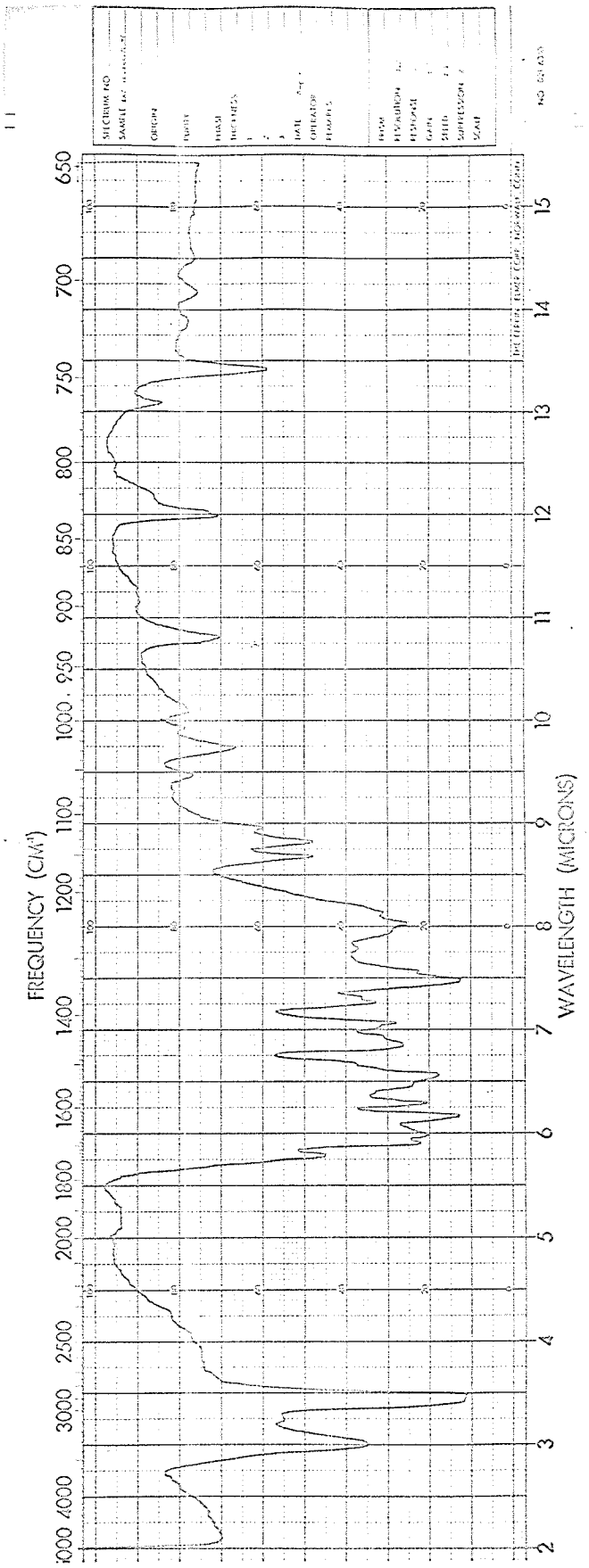



Figure 10.

The infrared spectrum of DNP-tetraglycine.

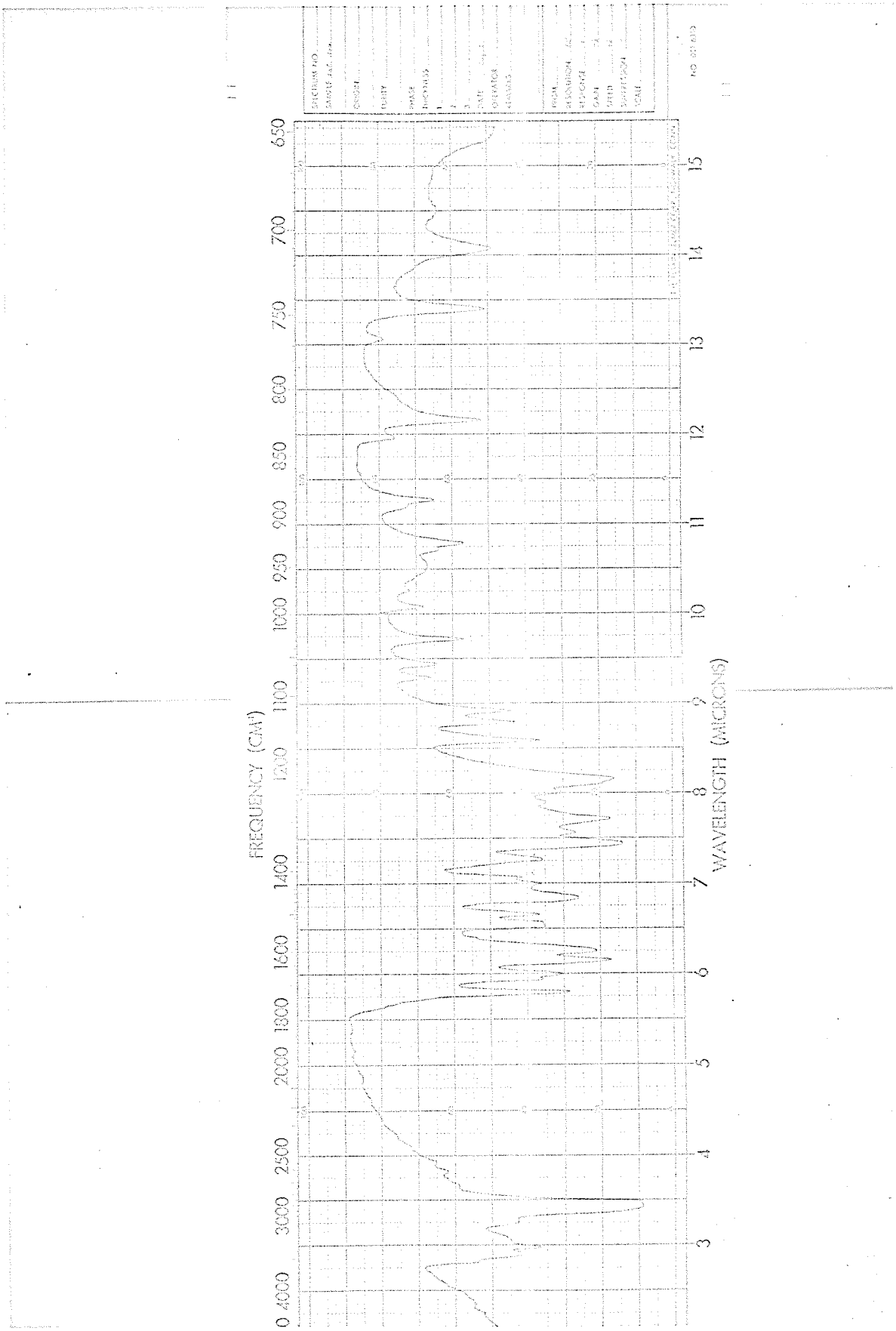



Figure 11.

The infrared spectrum of DNP-Lalanylglycylglycine.

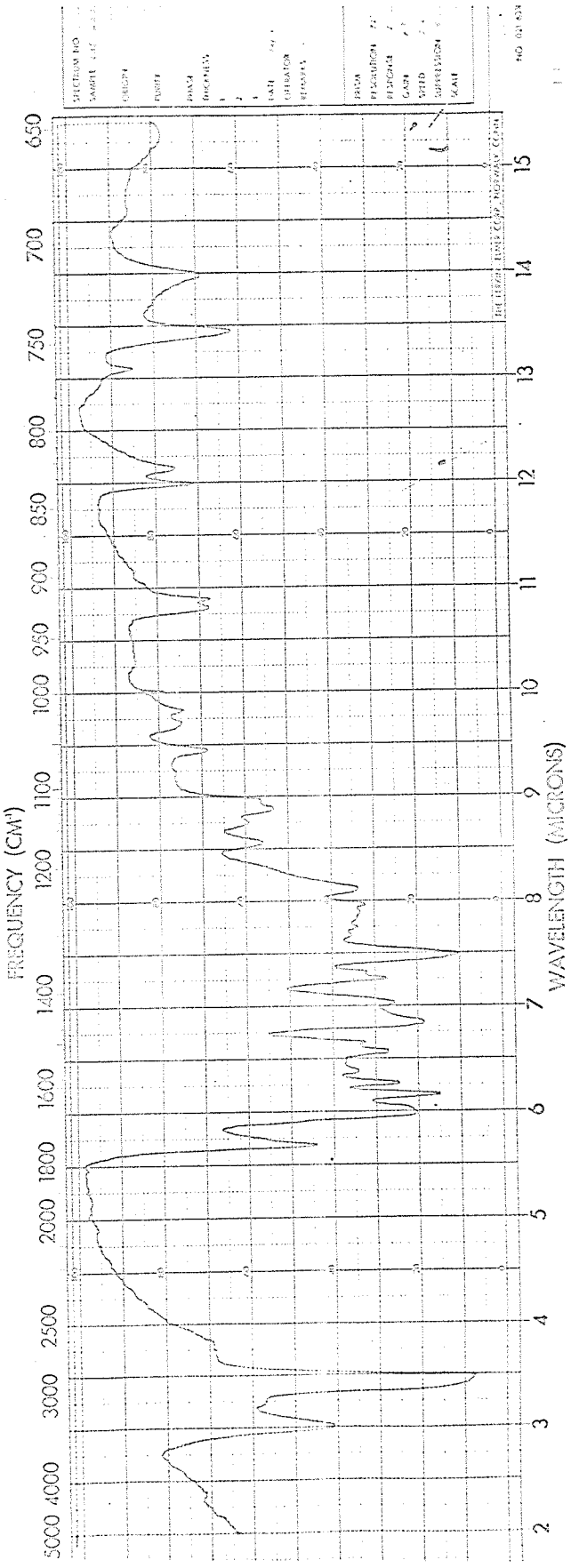



Figure 12.

The infrared spectrum of DNP-L-leucylglycylglycine.

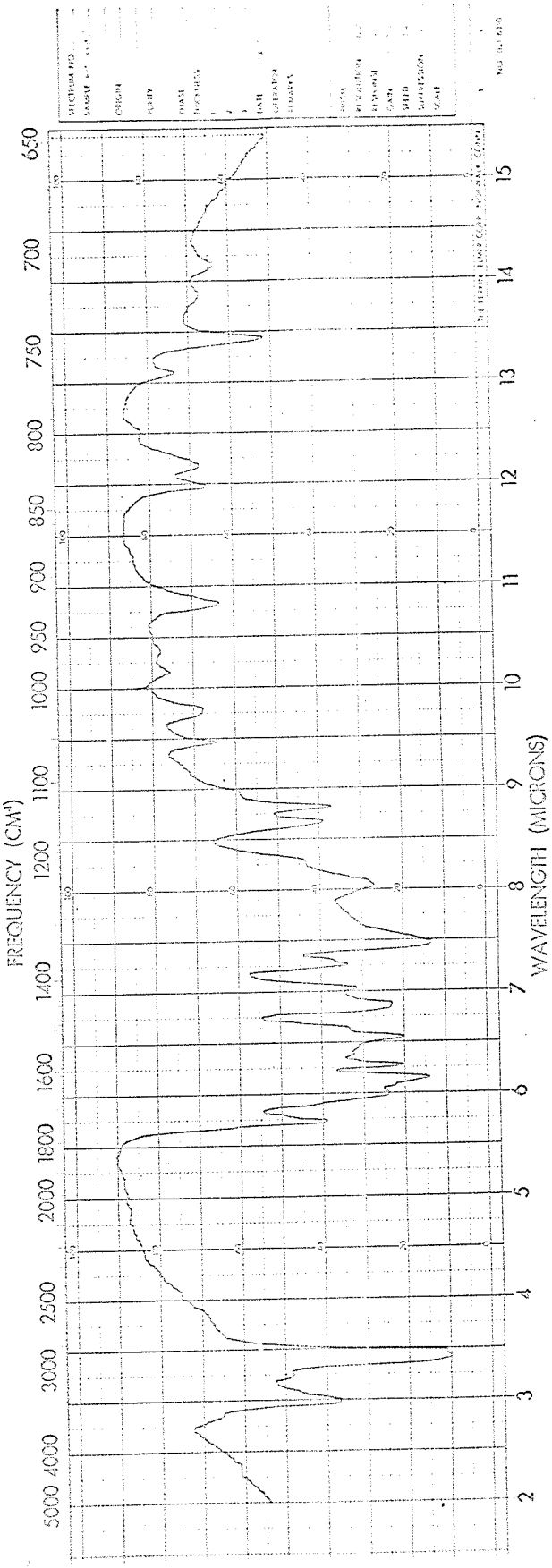



Figure 13.

The infrared spectrum of DNP-glycylglycyl-L-alanine.

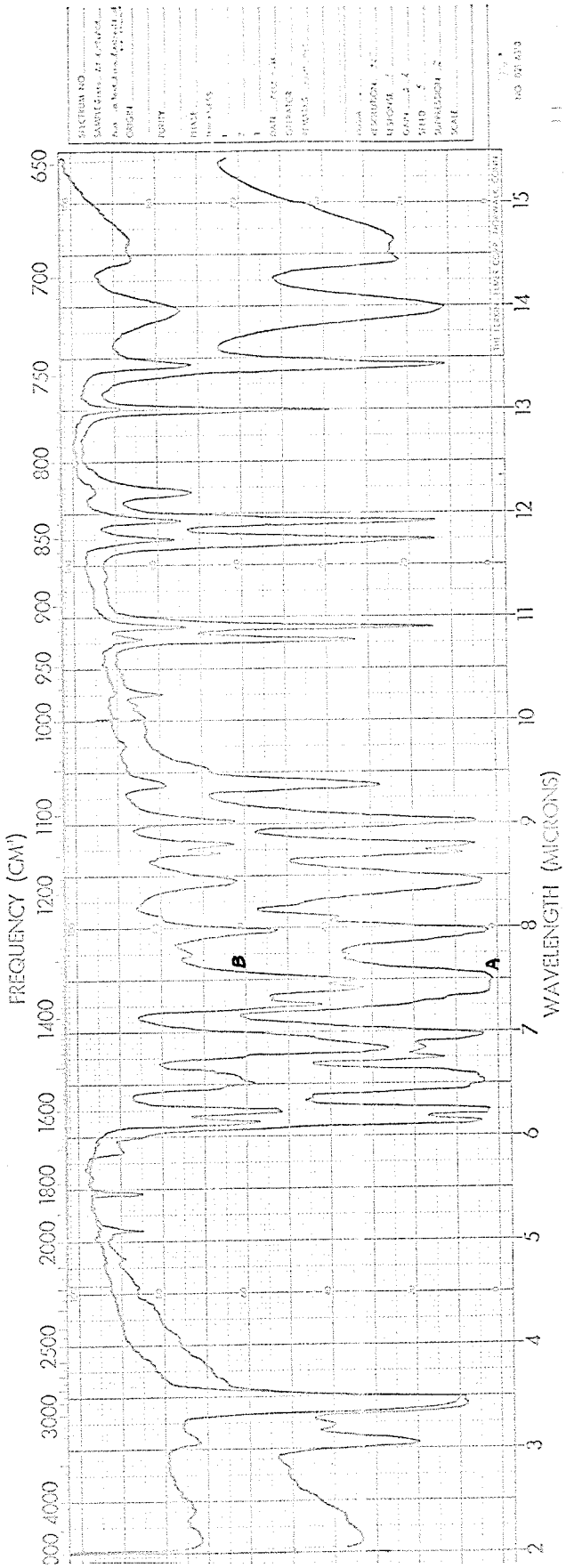



Figure 14.

A, the infrared spectrum of 2,4-dinitrophenol; B, the infrared

## DISCUSSION OF RESULTS

DNP-glycine, DNP-diglycine, DNP-triglycine, and DNP-tetraglycine were prepared by condensing the amino acid or peptide with FDNB in a sodium bicarbonate medium. From studies on these compounds, it is apparent that the use of slightly elevated temperatures during the reaction period is beneficial, since a much shorter period of time is required to obtain a given yield of the compound if the reaction mixture is warmed than if it is allowed to remain at room temperature. Long reaction periods at elevated temperatures are to be avoided, however, since the melting point of the crude product decreases, and additional recrystallizations may be necessary to obtain the pure compound.

Trimethylamine has been used as a condensing agent (25, 26), but its use has been confined primarily to producing the DNP-compounds in microgram quantities. The use of triethylamine, a slightly more basic compound than trimethylamine, was applied to the synthesis of the DNP-peptides containing a varying number of glycyl residues. DNP-diglycine was most satisfactorily prepared by using two or three equivalents of base per equivalent of peptide, and reacting the mixture for three hours at room temperature. The optimum yields of DNP-triglycine were obtained by reacting FDNB and the peptide at room temperature in a medium containing three equivalents of base per equivalent of peptide. By employing a slightly elevated temperature and two equivalents of base, a slightly purer product could be separated from the reaction mixture, but the yields were somewhat lower. DNP-tetraglycine was most satisfactorily prepared in a medium containing two equivalents of base per equivalent of peptide and maintained at 40°. The use of a larger amount of base resulted in considerably decreased yields. For all three peptides,

the use of triethylamine as condensing agent improved the yields of the DNP-compounds over those obtained by using sodium bicarbonate as the condensing agent, the improvement increasing with the increasing length of the peptide chain.

The successful use of triethylamine as a condensing agent in the reaction of FDNB with the above-mentioned peptides prompted its use in the synthesis of DNP-L-alanylglycylglycine, DNP-L-leucylglycylglycine, and DNP-glycyl-glycyl-L-alanine. The most favorable yields of DNP-L-alanylglycylglycine were obtained by using three equivalents of base per equivalent of peptide, and reacting the reagents at room temperature. DNP-L-leucylglycylglycine was prepared by employing three equivalents of base per equivalent of peptide, and maintaining the reaction mixture either at room temperature or at 40°. Preliminary studies with DNP-glycylglycyl-L-alanine indicate that three equivalents of base will give the most satisfactory yields of the product. The temperature may be elevated, although this is not necessary. For all three compounds, the use of excess triethylamine, that is, more than three equivalents per equivalent of peptide, was detrimental to the quality of the crude compound, and therefore to the final yield.

DNP-triglycine and DNP-tetraglycine have been prepared and purified, and their molar absorptivities in 0.2 M sodium bicarbonate solution have been determined at the wavelength of maximum absorption. Contrary to the findings of Kruger (2), the molar absorptivity of DNP-tetraglycine is not significantly different from that of DNP-triglycine. The values obtained in the present investigation are both appreciably greater than those obtained by Kruger. The samples employed in the previous investigation were impure,

the melting point of DNP-triglycine being slightly lower, and that of DNP-tetraglycine being appreciably lower, than those obtained in the present work. The fact that the molar absorptivities obtained previously were lower than those presently obtained suggests that the solutions may have been allowed to stand for some time before their molar absorptivities were measured. It was observed that the absorbance at 350 mμ decreased if the solutions were allowed to stand for any length of time.

Thin layer chromatography of the DNP-compounds prepared indicates that, under the conditions employed, DNP-diglycine had an $R_f$ value very similar to that of DNP-L-alanylglycylglycine and to that of DNP-glycylglycyl-L-alanine. Furthermore, the $R_f$ value of DNP-L-leucylglycylglycine was greater than that of DNP-diglycine. Therefore, there is no strict relationship between the number of amino acid residues in the compound and the $R_f$ value obtained.

# BIBLIOGRAPHY


1. Sanger, F. Biochem. J. 39 507 (1945).

2. Kruger, J. M. Sc. Thesis, Univ. of Man. 1963.

3. Sanger, F. Advances in Protein Chem. 7, 1 (1952).

4. Fischer, E., and Abderhalden, E. Ber. 40, 3544 (1907).

5. Abderhalden, E., and Blumberg, P. Hoppe-Seyl. Z. 65, 318 (1910).

6. Abderhalden, E., and Stix, W. Hoppe-Seyl. Z. 129, 143 (1923).

7. Schroeder, W. J. Am. Chem. Soc. 74, 5118 (1952).

8. Green, F., and Schroeder, W. J. Am. Chem. Soc. 73, 1385 (1951).

9. Blackburn, S. Biochem. J. 45, 579 (1949).

10. Partridge, S., and Swain, T. Nature 166, 272 (1950).

11. Mills, G. Nature 165, 403 (1950).

12. Lowther, A. Nature 167, 767 (1951).

13. Green, F., and Kay, L. Anal. Chem. 24, 726 (1952).

14. Schroeder, W., and Honnen, L. J. Am. Chem. Soc. 75, 4615 (1953).

15. Blackburn, S., and Lowther, A. Biochem. J. 48, 126 (1951).

16. Ramachandran, L. Anal. Chem. 33, 1074 (1961).

17. Ramachandran, L., and Sastry, L. Biochem. 1, 75 (1962).

18. Thompson, A. Nature 168, 390 (1951).

19. Dickmans, S., and Asplund, R. J. Am. Chem. Soc. 74, 5208 (1952).

20. Isherwood, F., and Cruickshank, D. Nature 174, 123 (1954).

21. Heikens, D., Hermans, P., and van Velden, P. Nature 174, 1187 (1954).

22. Tonge, D. Nature 195 491 (1962).

23. Schroeder, W., and LeGette, J. J. Am. Chem. Soc. 75, 4612 (1953).

24. Levy, A., and Chung, D. J. Am. Chem. Soc. 77, 2899 (1955).

25. Sanger, F., and Thompson, E. Biochem. J. 53, 353 (1953).