The Preparation and Properties of some 2,4-Dinitrophenyl Dipeptides and 2,4-Dinitrophenyl Amides

by

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Abstract

2,4-Dinitrophenyl glycyl-L-tryptophan, 2,4-dinitrophenyl glycyl-L-proline, 2,4-dinitrophenyl glycyl-L-phenylalanine, 2,4-dinitrophenyl glycyl-L-glutamic acid, 2,4-dinitrophenyl glycyl amide, 2,4-dinitrophenyl glycyl anilide, 2,4-dinitrophenyl glycyl-p-toluide, 2,4-dinitrophenyl-L-alanyl amide, 2,4-dinitrophenyl-L-alanyl anilide, 2,4-dinitrophenyl-L-alanyl-p-toluide were prepared in order to studies their properties. For the 2,4-dinitrophenyl dipeptides, two methods of preparation were used, each giving an identical product. (2,4-Dinitrophenyl glycyl-L-glutamic acid was difficult to recrystallize when prepared from 2,4-dinitrophenyl glycyl chloride.) The melting points of the above compounds were determined on a Gallenkamp melting point apparatus. No racemization occurred when the 2,4-dinitrophenyl dipeptides were prepared from 2,4-dinitrophenyl glycyl chloride.

Ultraviolet absorption spectra of these substances were obtained in 1 cm silica cuvettes with a Beckman DK spectrophotometer using $5X10^{-5}$ molar sodium bicarbonate solution or in 95% ethanol. Their infrared spectra were measured with a Model No. 21 Perkin-Elmer spectrophotometer (equipped with sodium chloride optics) using Nujol mulls. Molar rotations $[M]_D$ were calculated as specific rotations multiplied by the molecular weight and divided by 100. Specific rotations were obtained in a Kern Full Circle polarimeter, using a two-decimeter tube and adjusting the concentration (in 4% sodium

bicarbonate solution, 95% ethanol, acetone, or glacial acetic acid), so that the angular rotation was always more than 0.2° and usually between 0.5 to 1.0°.

The behaviour of the 2,4-dinitrophenyl dipeptides on thin-layer chromatography and their electrophoretic behaviour were studied; both methods gave good separation of the above 2,4-dinitrophenyl dipeptides.

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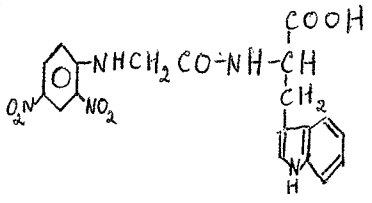
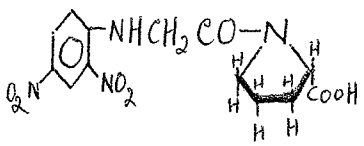
Introduction

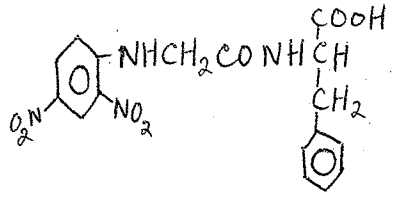
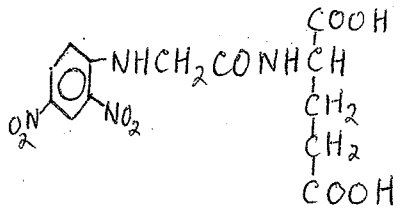
The method most widely employed for the identification of N-terminal amino acids of proteins and peptides was elaborated by Sanger(1) and is based on the reaction of N-terminal α-amino groups with 2,4-dinitrofluorobenzene(FDNB)[1].

The present study was undertaken in order to obtain more knowledge about the properties of 2,4-dinitrophenyl(DNP)[2] dipeptides. The DNP-glycyl and DNP-L-alanyl amides also have been prepared for the first time in order to obtain further means of characterising the respective DNP acid chlorides.

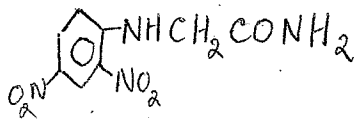
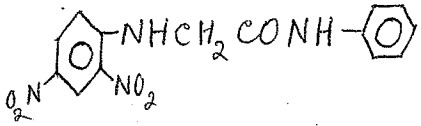
The melting points, neutral equivalents, molar rotations, paper electrophoretic behaviour, infrared and ultra-violet absorption spectra, and thin-layer chromatography behaviour were determined for four DNP-dipeptides whose structural formulae are shown below.

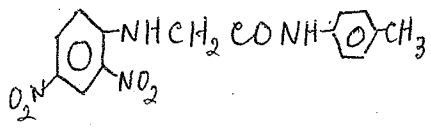
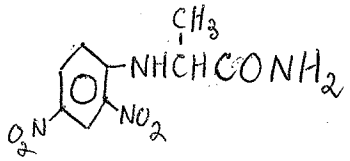
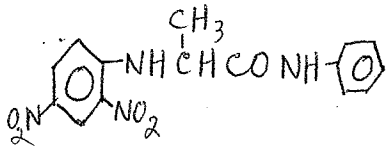
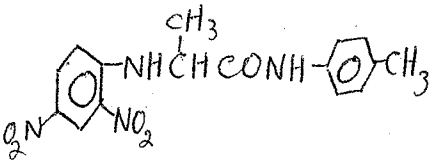
DNP-dipeptides	Structure
DNP-glycyl-L-tryptophan	
DNP-glycyl-L-proline	

(1) FDNB will be used to designate 1-fluro-2,4-dinitrobenzene.
(2) DNP will be used to designate the 2,4-dinitrophenyl radical.

DNP-dipeptide	Structure
DNP-glycyl-L-phenylalanine	
DNP-glycyl-L-glutamic acid	

In addition, the melting point, the molar rotation, infrared and ultra-violet absorption spectra were determined for all DNP-glycyl and DNP-L-alanyl amides whose structural formulae are shown below.

DNP-amide	Structure
DNP-glycyl amide	
DNP-glycyl anilide	

DNP-amide	Structure
DNP-glycyl-p-toluide	
DNP-L-alanyl amide	
DNP-L-alanyl anilide	
DNP-L-alanyl-p-toluide	

IN order to study the properties of these DNP-derivatives, it was first necessary to prepare them. For the DNP-dipeptides, two methods of preparation were used. For the others, the DNP-acid chloride was reacted with ammonia or the appropriate amine.

~~(1) FDNB will be used to designate 1-fluro-2,4-dinitrobenzene.~~

~~(2) DNP will be used to designate the 2,4-dinitrophenyl radical.~~

Literature Review

For the purpose of identification and estimation of the N-terminal amino acid residues of peptides and proteins, DNP-derivatives of amino acids have been prepared by many workers since 1910, in the field of end group and amino acid analysis.

In 1910, DNP-derivatives of glycine, DL-alanine, DL-valine, DL-leucine and L-asparagine were first prepared by Abderhalden and Blumberg,(2,3) using 1-chloro-2,4-dinitrobenzene to react with the free amino group in aqueous sodium bicarbonate solution. In 1934, Saunders(4) prepared a derivative by the action of 1-chloro-2,4-dinitrobenzene on cysteine, which he reported was difficult to crystallize, and showed that 1-chloro-2,4-dinitrobenzene reacts more readily with -SH groups than with amino groups.

Later, F. Sanger(1) found that FDNB was a more suitable reagent, capable of reacting quantitatively at room temperature with the free amino groups of proteins and peptides. The procedure comprises shaking the amino acid with a twofold excess of FDNB and an equal weight of sodium bicarbonate in 67% ethanol(by volume) for two hours at room temperature, followed by evaporation of the ethanol, dilution with water, and extraction of the excess FDNB with ether. Acidification then yields the required DNP-amino acids.

The crystalline DNP-derivatives of many L-amino acids were prepared for the first time by Krishnarau R. Rao and H. A. Sober (5) in 1953. The method used was that employed by Sanger(1).

They found that the melting points of the DNP-derivatives of optically active amino acids differed in many instances from those of the racemic form and that the molecular rotation of the optically active derivative was much larger than that of the parent amino acid and peptide.

In 1954, Anthony L. Levy and David Chung(6) found a simplified procedure for the synthesis of DNP-amino acids by modifying Sanger's original method. They found that several advantages result from using aqueous solutions at a slightly elevated temperature(40°) and from employing only an equivalent amount of FDNB. A more rapid reaction can be achieved, ethanol evaporation and extraction of excess FDNB can be eliminated and a pure product results with greater economy of reagents.

W. A. Schroeder and Joann Le Gette(7), and W. A. Schroeder and L. R. Honnen(8) in 1953 reported the preparation of a few DNP-peptides, to study their behaviour in column chromatography. Yields and other properties were not reported.

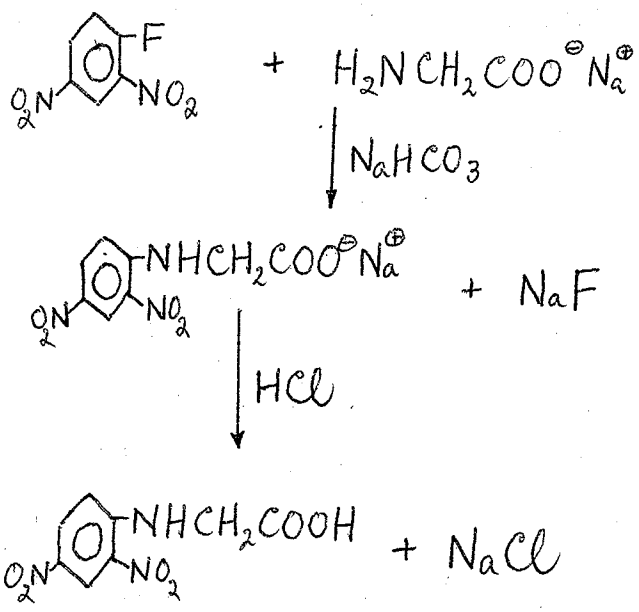
The first systematic study of the preparation and properties of DNP-peptides was undertaken by J. H. Loudfoot and J. E. Kruger (9) and reported in 1963.

Experimental and Results

Preparation of Starting Materials

2,4-Dinitrophenyl Glycine

DNP-glycine was required for the preparation of DNP-glycyl chloride. It was prepared by the method of Sanger(1), except that one equivalent FDNB was used per equivalent of amino acid, as reported by Levy and Chung(6). The equations for the overall reaction are as follows:



Materials:

Glycine	2.00gm (0.026mole)
FDNB	5.00gm (0.026mole)
Sodium bicarbonate	21.8gm (0.26 mole)
Water	180ml
Ethanol (95%)	210ml

Glycine and sodium bicarbonate were dissolved in the water

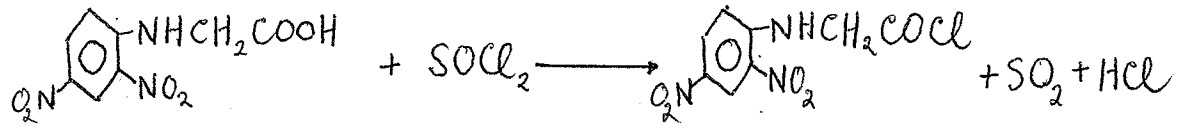
and to this was added a solution of FDNB in ethanol. The mixture was stirred mechanically for two hours at room temperature, then concentrated to remove ethanol by vacuum distillation below 40°; the residue was dissolved in water and acidified with concentrated hydrochloric acid to pH 2, which precipitated a yellow crystalline solid. The mixture was refrigerated overnight to precipitate additional product. The crystals were filtered with suction, washed with ice cold water to remove excess hydrochloric acid, stored for 24 hours in a vacuum desicator, and heated at 100° to constant weight.

The yield of product was 6.20 gm(96.2% of theory).

The melting point was 205-206° with decomposition. (literature 205°(2,9,10), 206°(12).)

2,4-Dinitrophenyl Glycyl Chloride

DNP-glycyl chloride was required as a starting material for one of the methods of preparing DNP-glycyl peptides, also for the preparation of DNP-glycyl amides. It was prepared by the method of J. H. Loudfoot and J. E. Kruger(9, 10). The overall equation is shown below:



Materials:

DNP-glycine	1.205gm (0.005 mole)
Thionyl chloride	10ml

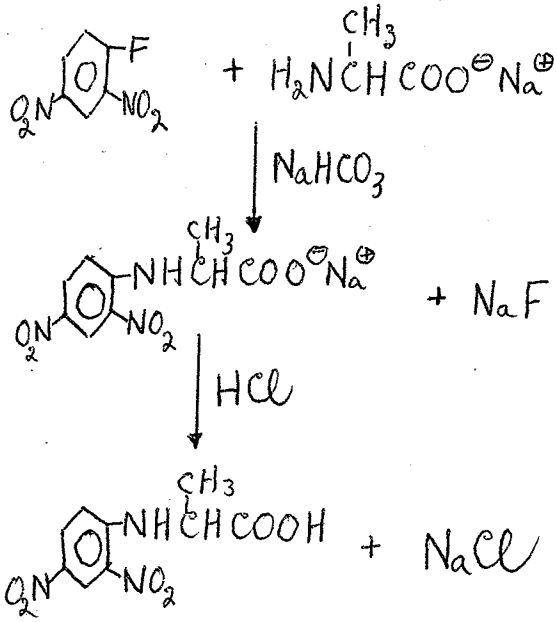
Glycine and thionyl chloride were heated under reflux on a water bath until a clear solution was obtained(20-40 minutes). The solution was then heated for an additional 30 minutes, and the excess thionyl chloride was removed by vacuum distillation using a water bath. Large yellow, needlelike crystals were formed. Throughout the preparation, a calcium chloride tube was attached to the condenser in order to exclude moisture.

The yield of product was 1.28gm (99%) of theory).

The melting point was 129-129.5° (Literature 129-129.5°(9, 10).)

2,4-Dinitrophenyl-L-Alanine

DNP-L-alanine was required for the preparation of DNP-L-alanyl chloride. The equations for the overall reaction are as follows:



Materials:

L-Alanine	0.89gm. (0.01 mole)
FDNB	1.86gm. (0.01 mole)
Sodium bicarbonate	8.40gm. (0.10 mole)
Water	70 ml.
Ethanol(95%)	90 ml.

The preparation was carried out in the same manner as DNP-glycine prepared from the FDNB and glycine. The yellow crystalline product was usually of such purity as not to required recrystallization. For some preparations, the crude product was recrystallized from dilute ethanol.

The yield of product was 2.44gm (95.7% of theory).

The melting point was 178-179°.(Literature 178°(2, 12), 173°(11), 177°(5).)

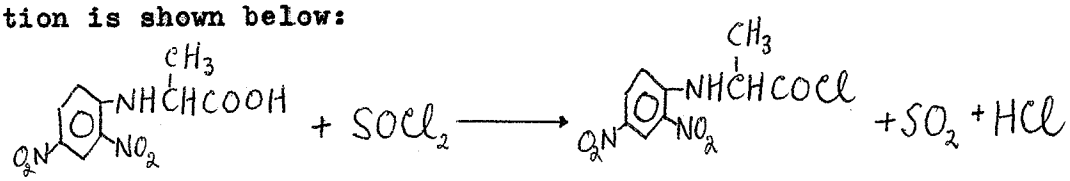
$[\alpha]_D^{22}$ = +16.4°, $[M]_D$ = +41.8° (c= 2% in 95% ethanol)

$[\alpha]_D^{22}$ = -12.5°, $[M]_D$ = +31.85° (c= 3% in acetone)

(Literature $[\alpha]_D^{20}$ = +14.3° (c= 2% in 6N HCl) (22), $[M]_D$ = +367° in N NaOH, $[M]_D$ = +39° in glacial acetic acid (5).)

2,4-Dinitrophenyl-L-Alanyl Chloride

DNP-L-alanyl chloride was required as a starting material for the preparation of DNP-L-alanyl amides. The overall equation is shown below:



Materials:

DNP-L-alanine	1.02gm (0.004 mole)
Thionyl chloride	10 ml

The preparation was carried out in the same manner as DNP-glycyl chloride from DNP-glycine. The product was a red-brown liquid. The yield was 1.08 gm(99% of theory).

Preparation of 2,4-Dinitrophenyl Dipeptides

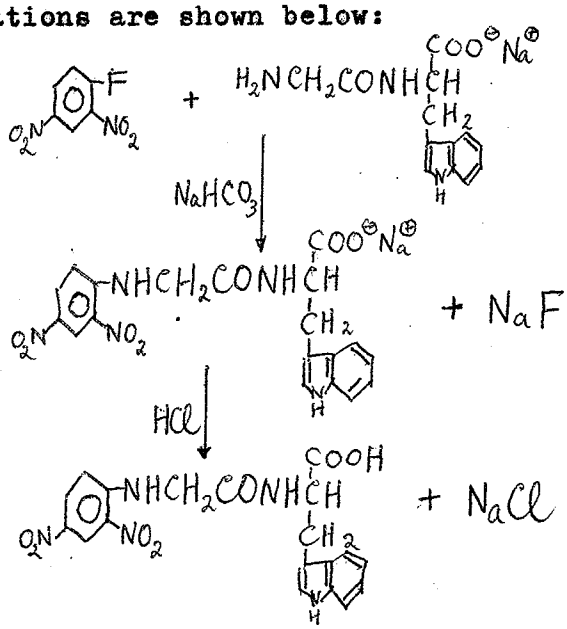
Two methods of preparing DNP-glycyl peptides are reported here as reported by J. H. Loudfoot and J. E. Kruger(9, 10).

1) By reacting FDNB with the pertinent dipeptide.
2) By reacting DNP-glycyl chloride with the pertinent amino acid.

2,4-Dinitrophenyl Glycyl-L-Tryptophan

From the Dipeptide

DNP-glycyl-L-tryptophan was prepared by the method employed by Sanger(1), except one equivalent of FDNB was used for per equivalent glycyl-L-tryptophan as reported by Levy and Chung(6). The overall equations are shown below:



Materials:

Glycyl-L-tryptophan	0.261gm (0.001 mole)
FDNB	0.186gm (0.001 Mole)
Sodium bicarbonate	0.746gm (0.009 mole)
Water	13 ml.
Ethanol	16 ml.

Glycyl-L-tryptophan and sodium bicarbonate were dissolved in the water and to this was added a solution of FDNB in ethanol. The mixture was stirred mechanically for two hours at room temperature, then concentrated to remove ethanol by vacuum distillation below 40°; the residue was dissolved in water and acidified with concentrated hydrochloric acid to pH 2, which precipitated an orange crystalline solid. The mixture was then refrigerated overnight to precipitate additional product. The crystals were filtered with suction, washed with ice cold water to remove excess hydrochloric acid, stored for 24 hours in a vacuum desicator and heated at 100° to constant weight.

The crude product was dissolved in hot ethanol, adding hot water to cause turbidity and cooling slowly. The mixture then left in the refrigerator for a few hours, and the yellow crystals were then filtered with suction and dried at 100° to constant weight.

The yield of the yellow product was 0.286gm. (67% of theory)

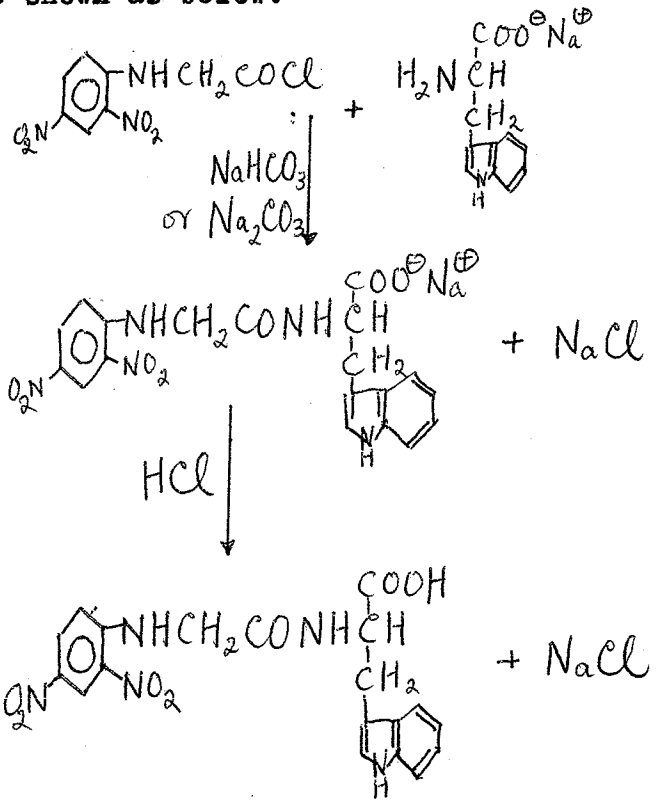
The melting point was 233-234° with decomp. and 190-227° when mixed with DNP-glycine.

$[\alpha]_D^{22} = +20°$, $[M]_D = +85.4°$ (c= 0.5% in 95% ethanol)

$[\alpha]_D^{22} = +26.2°$, $[M]_D = +112.0°$ (c= 0.5% in acetone)

From DNP-glycyl Chloride

L-Tryptophan and DNP-glycyl chloride were reacted together in sodium bicarbonate or sodium carbonate medium, followed by acidification with hydrochloric acid. The equations for the overall reaction are shown as below:



Materials:

DNP-glycyl chloride	0.519gm. (0.002 mole)
L-Tryptophan	0.408gm. (0.002 mole)
Sodium bicarbonate	1.67 gm. (0.02 mole)
Water	30 ml.
Ethanol	10 ml.

L-Tryptophan and sodium bicarbonate were dissolved in the water and to this slowly added a solution of DNP-glycyl chloride

in benzene over a two hours period with constant stirring at ice-bath temperature. Stirring at ice-bath temperature was continued a further two hours. The mixture was then transferred to a separatory funnel and allowed to stand two hours for separation of the layers. The lower aqueous layer was removed and acidified with 4 c. c. of concentrated hydrochloric acid, which precipitated an orange coloured solid. In order to precipitate the product which remained in solution, the mixture was then left overnight in the refrigerator. The crystals were then filtered with suction, and washed with ice-cold water to remove excess hydrochloric acid. The product was dried by storing overnight in a desicator and heating at 100° to constant weight.

The orange crude product melted below 130°. Recrystallization was effected by dissolving the crystals in hot ethanol, adding hot water to cause turbidity and cooling slowly. The mixture then left in the refrigerator for a few hours, and the yellow crystals were then filtered with suction and dried to constant weight.

The yield of product was 0.58 gm. (67.5% of theory).

The melting point was 233-234°, 233-234° when mixed with DNP-glycyl-L-tryptophan prepared from the dipeptide, and 190-225° when mixed with DNP-glycine.

$[\alpha]_D^{22} = +20.0^\circ$ $[M]_D = +85.4^\circ$ (c= 0.5% in 95% ethanol)

$[\alpha]_D^{22} = +26.5^\circ$ $[M]_D = +113.0^\circ$ (c= 1.5% in acetone)

$[\alpha]_D^{22} = -60.0^\circ$ $[M]_D = -256.0^\circ$ (c= 0.2% in 4% sodium bicarbonate)

The neutral equivalent was 434.38, theoretical molecualar weight was 427.

Analysis:

Based on $C_{19}H_{17}O_7N_5$ M.W. = 434.38.

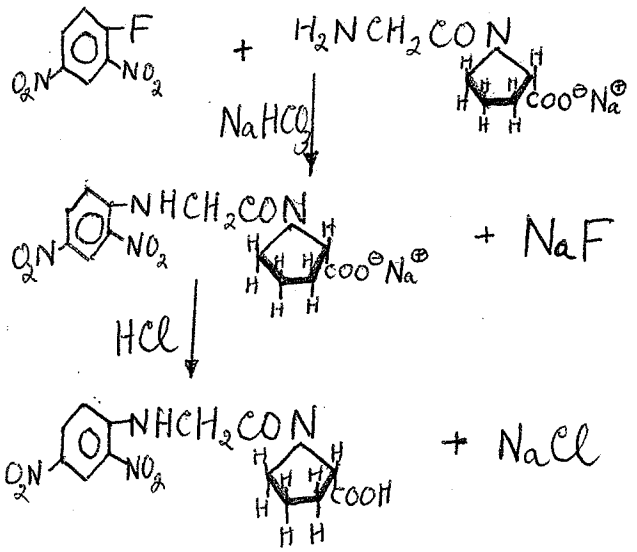
Calculated:	C= 53.40%	H=4.00%	N=16.39%
Found:	C=53.40%	H=3.89%	N=16.00%

All analyses were performed by Geller Laboratories, Charleston, West Virginia.

2,4-Dinitrophenyl Glycyl-L-Proline

From the Dipeptide

The equations for the overall reaction are as follows:



Materials:

Glycyl-L-proline	0.172 gm. (0.001 mole)
FDNB	0.186 gm. (0.001 mole)
Sodium bicarbonate	0.84 gm. (0.01 mole)
Water	10 ml.
Ethanol	14 ml.

The preparation was carried out in the same manner as DNP-glycyl-L-tryptophan prepared from the dipeptide, except that the reaction required exclusion of light. The crude product was recrystallized twice from aqueous ethanol, and then washed with ether to removed impurities.

The yield of product was 0.206 gm. (61% of theory).

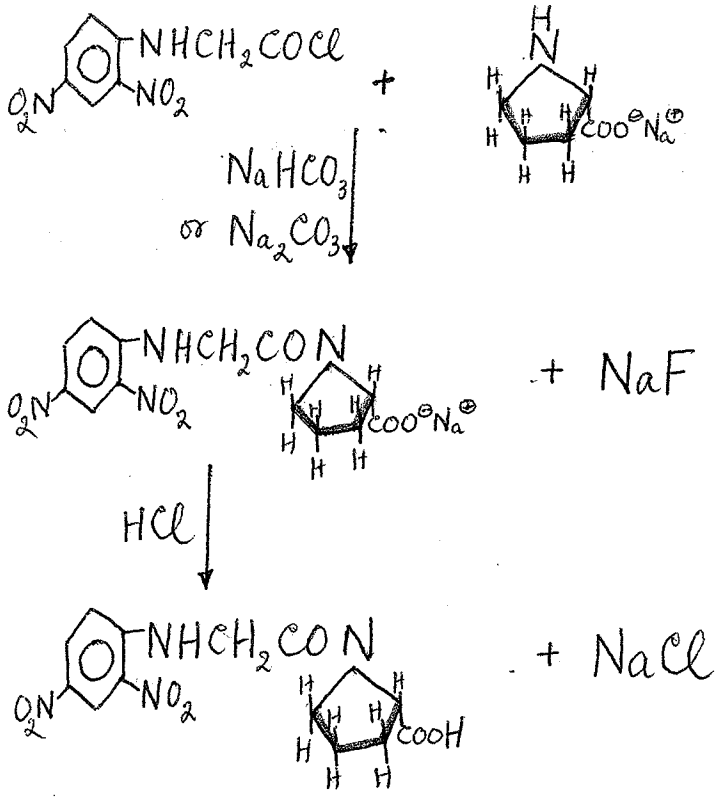
The melting point was 191.5-192.5°, and 175-200° when mixed with DNP-glycine.

$[\alpha]_D^{22} = -71.8^\circ$ $[M]_D = -243.0^\circ$ (c= 0.2% in 95% ethanol)

$[\alpha]_D^{22} = -76.0^\circ$ $[M]_D = -257.0^\circ$ (c= 0.2% in acetone)

From DNP-glycyl Chloride

The equations for the overall reaction are shown as follows:



Materials:

DNP-glycyl chloride	0.519 gm. (0.002 mole)
L-Proline	0.230 gm. (0.002 mole)
Sodium bicarbonate	1.76 gm. (0.02 mole)
Benzene	10 ml.
Water	30 ml.

The preparation was carried out in the same manner as the DNP-glycyl-L-tryptophan from DNP-glycyl chloride, except the reaction required exclusion of light. The crude product was recrystallized twice from aqueous ethanol, then washed with ether to removed the remaining impurities.

The yield of product was 0.324 gm. (48% of theory)

The melting point was 191-192°, 191-192.5° when mixed with DNP-glycyl-L-proline prepared from the dipeptide, and 175-200° when mixed with DNP-glycine.

The neutral equivalent was 340, and theoretical molecular weight is 338.14.

$[\alpha]_D^{22}$ = -71.5° $[M]_D$ = -242.0° (c= 1% in 95% ethanol)

$[\alpha]_D^{22}$ = -76.0° $[M]_D$ = -257.0° (c= 1.6% in acetone)

$[\alpha]_D^{22}$ = -50.0° $[M]_D$ = -169.7° (c= 0.2% in 4% sodium bicarbonate)

$[\alpha]_D^{22}$ = -100.0° $[M]_D$ = -338.2° (c= 0.2% in glacial acetic acid)

Analysis:

Based on $C_{13}H_{14}O_7N_4$ [redacted]

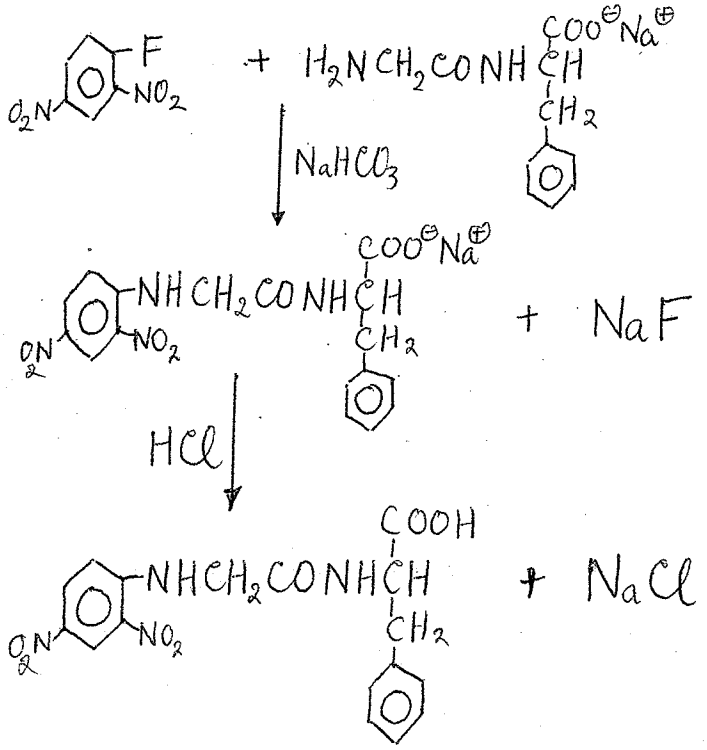
Calculated: C= 46.16% H= 4.17% N= 16.56%

Found: C= 45.90% H= 4.08% N= 16.36%

2,4-Dinitrophenyl Glycyl-L-Phenylalanine

From the dipeptide

The equation for the overall reaction are shown as follows:



Materials:

glycyl-L-phenylalanine	0.111 gm. (0.0005 mole)
FDNB	0.093 gm. (0.0005 mole)
Sodium bicarbonate	0.43 gm. (0.005 mole)
Water	5 ml.
Ethanol	8 ml.

The preparation was carried out in the same manner as the above DNP-dipeptides prepared from the dipeptides, except the solution was extracted twice with ether to remove trace impurities before acidifying. The crude product could be recrystallized from aqueous ethanol.

The yield of product was 0.120 gm. (61.6% of theory)

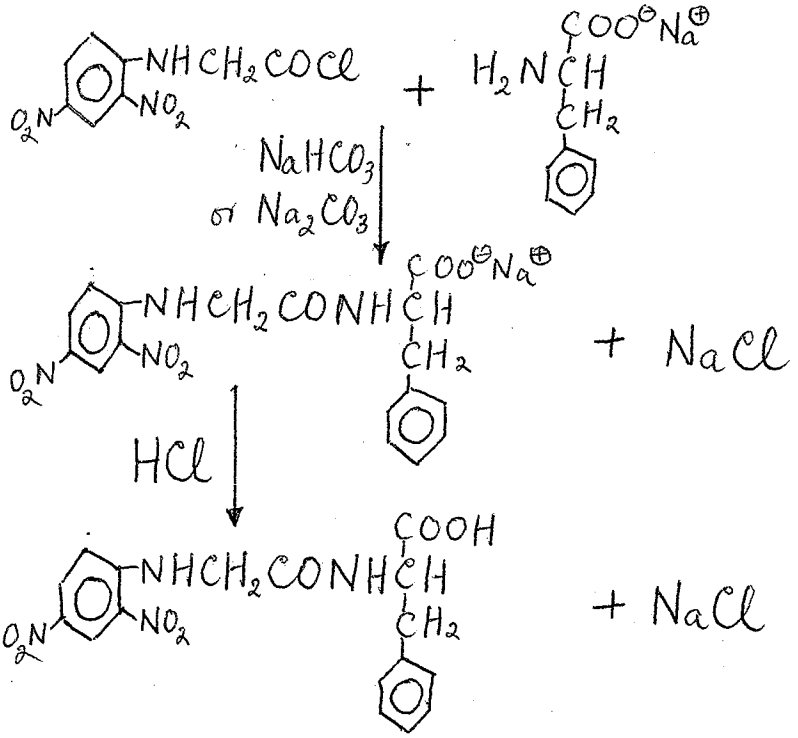
The melting point was 184-185°, and 175-200° when mixed with DNP-glycine.

$[\alpha]_D^{22} = +8.3^\circ$ $[M]_D = +32.2^\circ$ (c= 1.5% in 95% ethanol)

$[\alpha]_D^{22} = +18.2^\circ$ $[M]_D = +70.6^\circ$ (c= 1.5% in acetone)

From DNP-Glycyl Chloride

The equation for the overall reaction are shown as follows:



Materials:

L-Phenylalanine	0.330 gm. (0.002 mole)
DNP-glycyl chloride	0.519 gm. (0.002 mole)
Sodium bicarbonate	1.67 gm. (0.02 mole)
Water	25 ml.
Benzene	10 ml.

The preparation was carried out in the same manner as the above DNP-dipeptides prepared from DNP-glycyl chloride. The crude product was recrystallized twice from aqueous ethanol, then washed with a small amount of ether.

The yield of product was 0.482 gm. (62.1% of theory)

The melting point was 184-185°, 184-185° when mixed with DNP-glycyl-L-phenylalanine prepared from the dipeptide, and 175-200° when mixed with DNP-glycine.

The neutral equivalent was 400, theoretical molecular weight was 388.35.

$[\alpha]_D^{22} = +8.2°$ $[M]_D = +31.8°$ (c= 1.5%in 95% ethanol)

$[\alpha]_D^{22} = +18.2°$ $[M]_D = +70.6°$ (c= 1.5% in acetone)

$[\alpha]_D^{23} = -63.6°$ $[M]_D = -246.0°$ (c= 0.3% in sodium bicarbonate)

Analysis:

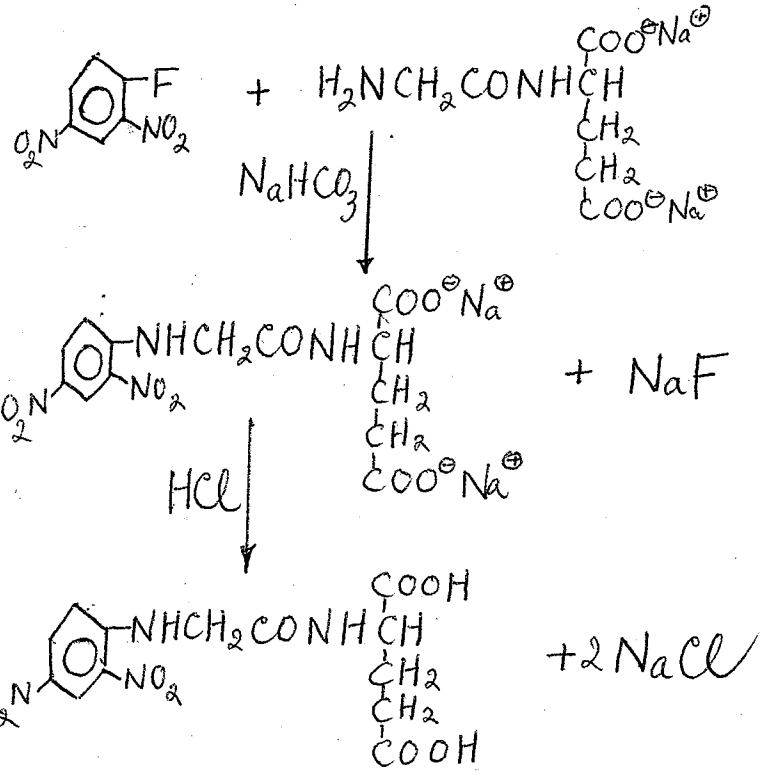
Based on $C_{17}H_{16}O_7N_4$

Calculated:	C= 52.58%	H= 4.15%	N= 14.43%
Found:	C= 52.42%	H= 4.32%	N= 13.51%

2,4-Dinitrophenyl Glycyl-L-Glutamic acid

From the Dipeptide

The equations for the overall reaction are shown as follows:



Materials:

Glycyl-L-glutamic acid	0.102 gm. (0.0005 mole)
FDNB	0.093 gm. (0.0005 mole)
Sodium bicarbonate	0.43 gm. (0.005 mole)
Water	5 ml.
Ethanol	8 ml.

Glycyl-L-glutamic acid and sodium bicarbonate were dissolved in the water and to this was added a solution of FDNB in ethanol. The mixture was stirred mechanically for 20 hours at room temperature in complete darkness, then concentrated to remove ethanol by vacuum distillation below 40°, the residue was dissolved in a small amount of water, and extracted twice with ether to remove a neutral compound which melted at 84-85°, acidified with con-

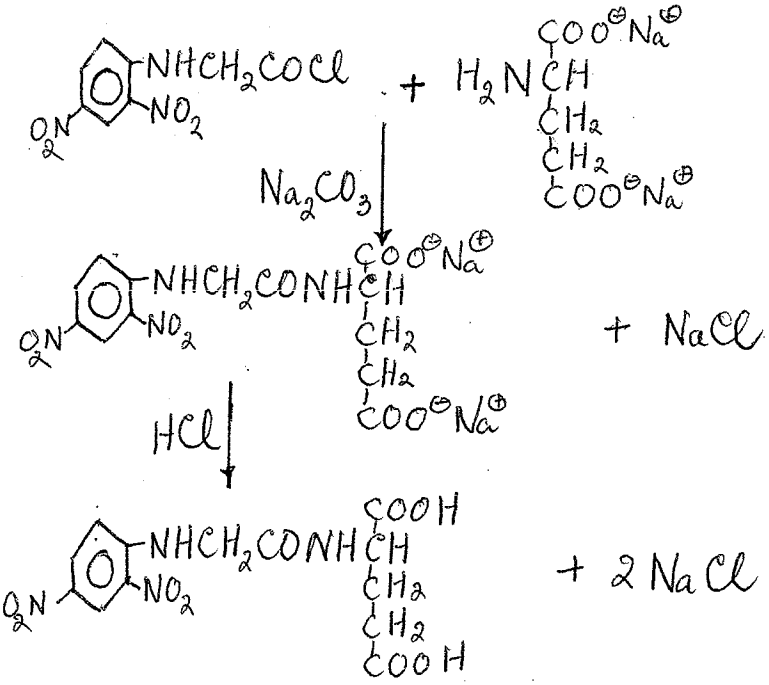
centrated hydrochloric acid to pH 2, which precipitated a yellow crystalline solid, cooled for two hours in the refrigerator and then filtered with suction. Dried in the vacuum desiccator and heated at 100° to constant weight. The crude product was recrystallized twice with aqueous ethanol.

The yield of product was 0.092 gm. (49.5%of theory)

The melting point was 194-195°, 180-202° when mixed with DNP-glycine.

From DNP-Glycyl Chloride

The equations for the overall reaction are shown as follows:



Materials:

DNP-glycyl chloride	0.519 gm. (0.002 mole)
L-Glutamic acid	0.294 gm. (0.002 mole)
Sodium bicarbonate	2.12 gm. (0.02 mole)
Water	25 ml.
Benzene	15 ml.

L-Glutamic acid and sodium carbonate were dissolved in the water and to this was slowly added a solution of DNP-glycyl chloride in benzene over a four hours periods with constant stirring at ice-bath temperature. Stirring at ice-bath temperature was continued a further four hours. The whole reaction was carried out in complete darkness. The mixture then transferred to a separatory funnel and the aqueous layer was removed and extracted twice with ether to remove the neutral compound which melted at 84-85°. The aqueous solution was acidified with concentrated hydrochloric acid to pH 2, which precipitated a yellow compound. The crystals were filtered with suction, and washed with small amount of ice-cold water to remove excess hydrochloric acid. The product was dried by storing overnight in a desicator and heated at 100° to constant weight.

Because a large amount of DNP-glycine and a small amount of other impurities were present in the crude product, recrystallization to yield a pure product was difficult even exhausive attempts at purification. However, DNP-glycyl-L-glutamic acid could be purified by preparative thin-layer chromatography methods; the adsorbent used was Silic AR; TLC-7FG, the layer was 2mm and the sample was applied in a narrow band, and developed in the solvent (chloroform/methanol/acetic acid; 95;5:1); five bands were present on the glass plate, the DNP-glycine was lies in the fifth band and the DNP-glycyl-L-glutamic acid lies in the second band from the bottom. The adsor-

bent containing the desired component from the glass plate was scraped, and extracted twice with ethyl acetate, the solvent was evaporated, and the solid were recrystallized from aqueous ethanol.

The yield of DNP-glycyl-L-glutamic acid from the crude product by thin-layer chromatography method was 61% (41.2% of theory).

The melting point was 194-195°, 194-195° when mixed with DNP-glycyl-L-glutamic acid prepared from the dipeptide, 180-203° when mixed with DNP-glycine.

Analyses:

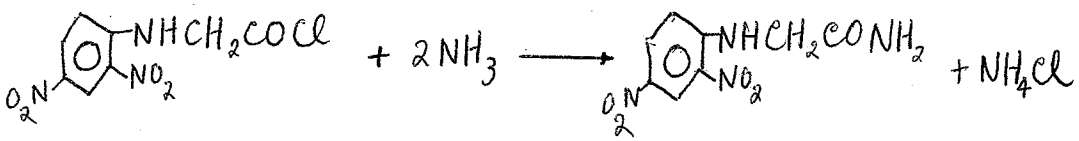
Based on $C_{13}H_{14}O_9N_4$

Calculated:	C= 42.19%	H= 3.80%	N= 15.13%
Found:	C= 41.77%	H= 4.44%	N= 15.01%

Preparation of 2,4-Dinitrophenyl Glycyl Amides

2,4-Dinitrophenyl Glycyl Amides

DNP-glycyl chloride was reacted with ammonia to convert to DNP-glycyl amide. The equation for the overall reaction is shown as follows:



Materials:

DNP-glycyl chloride	0.519 gm. (0.002 mole)
Concentrated Ammonia	10 ml.
Benzene	10 ml.

DNP-glycyl chloride was dissolved in benzene and poured into

ice-cold concentrated ammonia. The mixture was stirred at room temperature for 15 minutes. The precipitated amide was collected on a filter with suction, and purified by recrystallization from dilute ethanol.

The yield of product was 0.435 gm. (90.5% of theory)

The melting point was 230-231°.

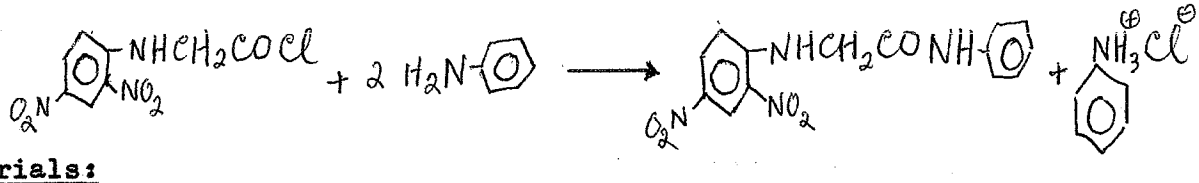
Analyses:

Based on $C_8H_8O_5N_4$

Calculated:	C= 40.00%	H= 3.36%	N=23.33%
Found:	C= 40.54%	H= 3.28%	N=23.38%

2,4-Dinitrophenyl Glycyl Anilide

DNP-glycyl chloride was reacted with aniline to convert to DNP-glycyl anilide. The equation for the overall reaction is shown as follows:



Materials:

DNP-glycyl chloride	0.519 gm. (0.002 mole)
Aniline	0.558 gm. (0.006 Mole)
Benzene	20 ml.

DNP-glycyl chloride was dissolved in 10 ml. of benzene and to this was added a solution of aniline in 10.ml. of benzene. The mixture was stirred at room temperature for 30 minutes, then the precipitated anilide was filtered with suction and purified by recrystallization from dilute ethanol.

The yield of product was 0.595 gm. (94.3% of theory)

The melting point was 261-262°.

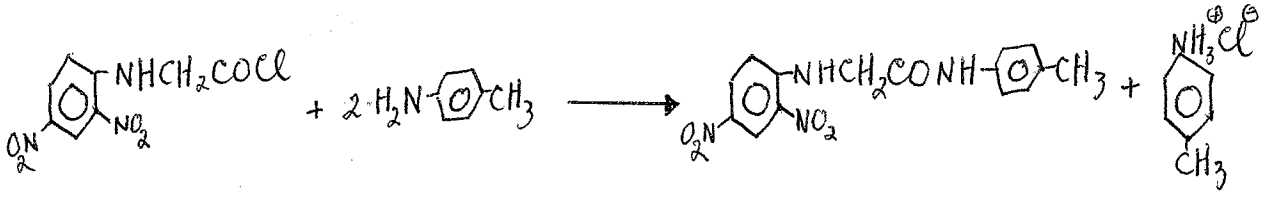
Analyses:

Based on $C_{14}H_{12}O_5N_4$

CALculated:	C= 53.17%	H=3.82%	N= 17.72%
Found:	C= 53.85%	H=3.69%	N= 17.72%

2,4-Dinitrophenyl Glycyl-p-Toluide

DNP-glycyl chloride was reacted with p-toluidine to convert to DNP-glycyl-p-toluide. The equation for the overall reaction is shown as follows:



Materials:

DNP-glycyl chloride	0.519 gm. (0.002 mole)
p-Toluidine	0.642 gm. (0.006 mole)
Benzene	20 ml.

DNP-glycyl chloride was dissolved in 10 ml. of benzene, and to this was added a solution of p-toluidine in 10 ml. of benzene. The mixture was stirred at room temperature for 30 minutes, the precipitated p-toluide was filtered, and purified by recrystallization from dilute ethanol.

The yield of product was 0.613 gm. (92.8% of theory)

The melting point was 263-264°.

Analyses:

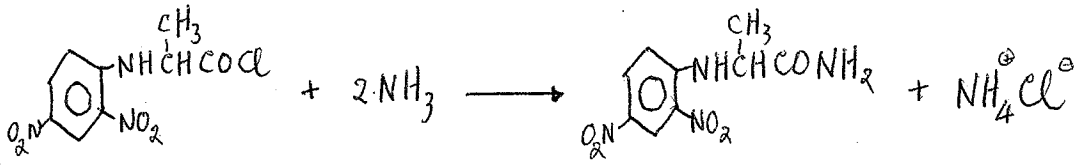
Based on $C_{15}H_{14}O_5N_4$

Calculated:	C= 54.54%	H= 4.27%	N= 16.96%
Found:	C= 54.69%	H= 4.09%	N= 16.75%

Preparation of 2,4-Dinitrophenyl-L-Alanyl Amides

2,4-Dinitrophenyl-L-Alanyl Amide

DNP-L-alanyl chloride was reacted with ammonia to convert to DNP-L-alanyl amide. The equation for the overall reaction is shown as follows:



Materials:

DNP-L-alanyl chloride	0.547 gm. (0.002 mole)
Concentrated ammonia	10 ml.
Benzene	10 ml.

DNP-L-alanyl chloride was dissolved in benzene and poured into the ice-cold concentrated ammonia, the mixture was stirred for 20 minutes at room temperature. The precipitated amide was filtered by suction, and washed with ice-cold water to remove excess ammonia. The crude product was recrystallized from dilute ethanol.

The yield of product was 0.45 gm. (90.2% of theory)

The melting point was 206-207°.

$[\alpha]_D^{22}$ = +103.2° $[M]_D$ = +261.6° (c= 0.6% in 95% ethanol)

$[\alpha]_D^{22}$ = +87.4° $[M]_D$ = +222.0° (c= 1.4% in acetone)

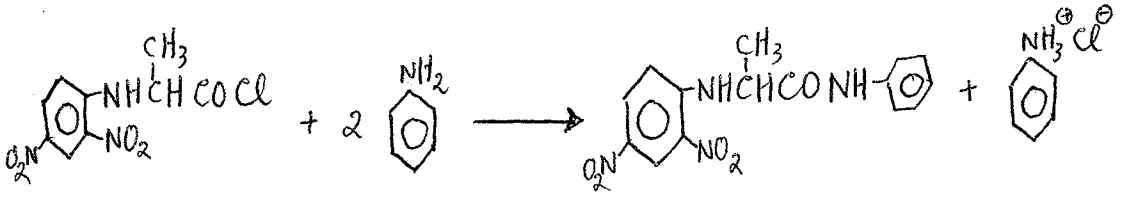
Analyses:

Based on $C_9H_{10}O_5N_4$

Calculated:	C= 42.52%	H= 3.97%	N= 22.04%
Found:	C= 42.50%	H= 4.22%	N= 21.96%

2,4-Dinitrophenyl-L-Alanyl Anilide

DNP-L-alanyl chloride was reacted with aniline to convert to DNP-L-alanyl anilide. The equation for the overall reaction is shown as follows:



Materials:

DNP-L-alanyl chloride	0.547 gm. (0.002 mole)
Aniline	0.558 gm. (0.006 mole)
Benzene	20 ml.

DNP-L-alanyl chloride was dissolved in 10 ml. of benzene and to this was added a solution of aniline in 10 ml. of benzene. The mixture was stirred for 40 minutes at room temperature, then, the precipitated anilide was filtered, dried and recrystallized from dilute ethanol.

The yield of product was 0.60 gm. (91.6% of theory)

The melting point was 203-204°.

$[\alpha]_D^{22}$= +103.3° $[M]_D$= +341.0° (c= 0.4% in 95% ethanol)

$[\alpha]_D^{22}$= +128.0° $[M]_D$= +422.5° (c= 0.4% in acetone)

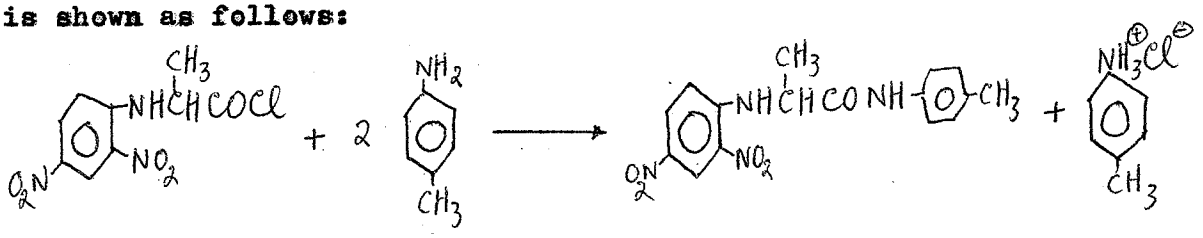
Analyses:

Based on $C_{15}H_{14}O_5N_4$

Calculated:	C= 54.54%	H= 4.27%	N= 16.96%
Found:	C= 54.12%	H= 4.22%	N= 16.78%

2,4-Dinitrophenyl-L-Alanyl-p-Toluide

DNP-L-alanyl chloride was reacted with p-toluidine to convert to DNP-L-alanyl-p-toluide. The equation for the overall reaction is shown as follows:



Materials:

DNP-L-alanyl chloride	0.547 gm. (0.002 mole)
p-Toluidine	0.642 gm. (0.006 mole)
Benzene	20 ml.

DNP-L-alanyl chloride was dissolved in 10 ml. of benzene, and to this was added a solution of p-toluidine in 10 ml. of benzene. The mixture was stirred for 40 minutes at room temperature, the precipitated p-toluide was filtered, dried, and recrystallized from dilute ethanol.

The yield of product was 0.64 gm. (92.4% of theory)

The melting point was 213-214°.

$[\alpha]_D^{22} = +96.0^\circ$ $[M]_D = +330.0^\circ$ (c= 0.3% in 95% ethanol)

$[\alpha]_D^{22} = +103.2^\circ$ $[M]_D = +355.0^\circ$ (c= 2.0% in acetone)

Analyses:

Based on $C_{16}H_{16}O_5N_4$

Calculated:	C= 55.81%	H= 4.68%	N= 16.27%
Found:	C= 55.81%	H= 4.37%	N= 15.99%

Each of the above DNP-derivatives gave a sufficient variation in ultra-violet or infrared spectra, which allowed a positive identification for the closely related DNP-derivatives, even though the spectra on the whole are more strikingly similar than they are different.

Ultra-Violet Absorption Spectra of 2,4-Dinitrophenyl Dipeptides and Derivatives

A Beckman DK spectrophotometer and cells of 1-cm path length were employed. The solvent was 0.2 molar (1.7%) of sodium bicarbonate of pH 8.43 or 95% ethanol. A concentration of $5X10^{-5}$ molar was used for all samples.

2,4-Dinitrophenyl Dipeptides

The ultra-violet absorption spectra of DNP-glycyl-L-tryptophan, DNP-glycyl-L-proline, DNP-glycyl-L-phenylalanine, DNP-glycyl-L-glutamic acid in 0.2 molar sodium bicarbonate solution are shown in Fig. 1 and Fig. 2. The wavelengths of maximum absorption varies only slightly for all the DNP-dipeptides, and is between 350-360 mμ.

The wavelengths of maximum absorption and the molar absorbancies for the four DNP-dipeptides are shown as follows:

Samples	Absorption maxima (mμ)			Molar absorbancy (ε_{max})		
DNP-glycine	359;	264;	232;	17010;	9870;	9320
DNP-glycyl-L-tryptophan	353;	267;	221;	14650;	11450;	20000
DNP-glycyl-L-proline	355;	264;	213;	15030;	8103;	12020

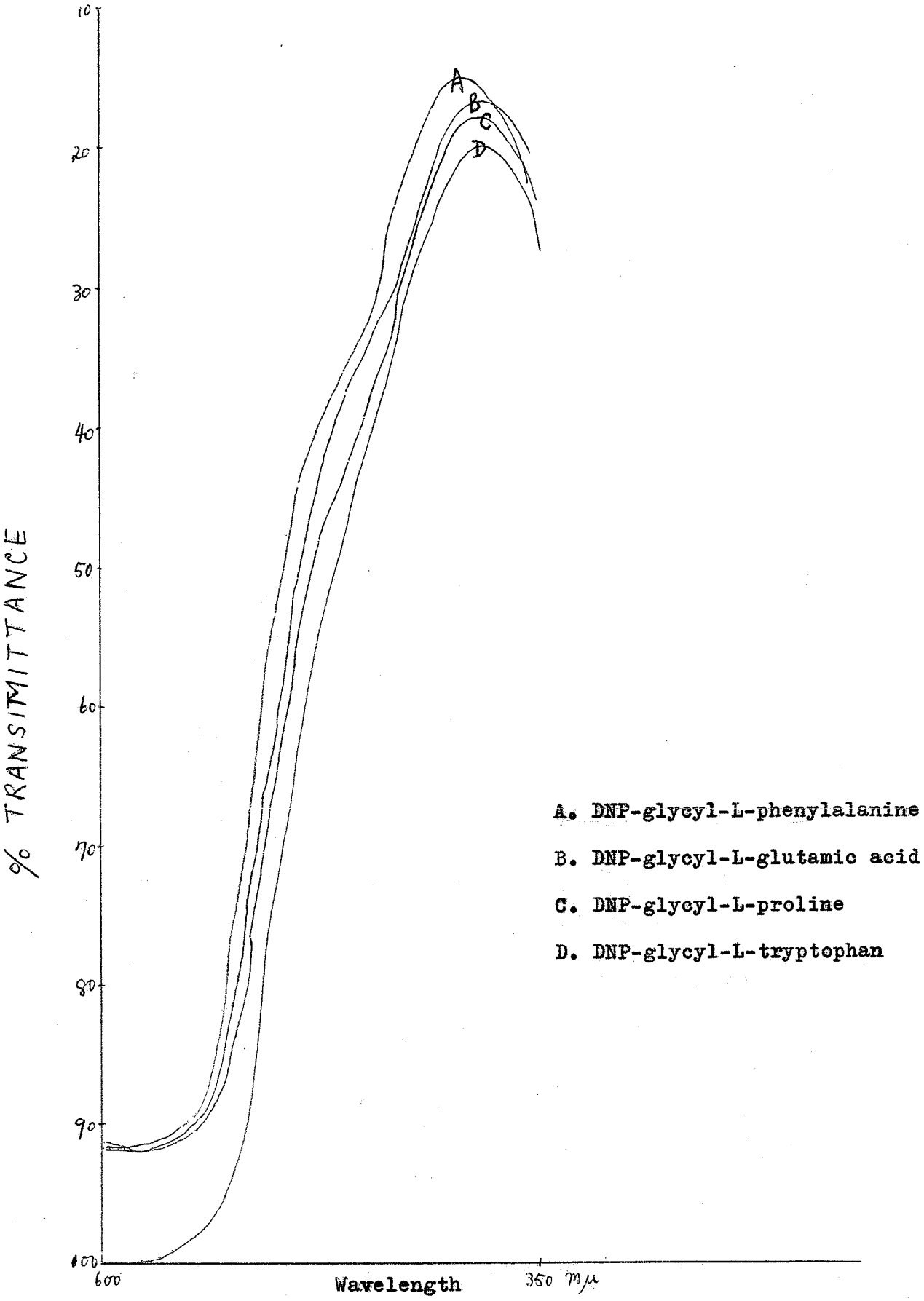


Figure 1. Absorption spectra of DNP-dipeptides. (solvent: 0.2 molar sodium bicarbonate, concentration: $5X10^{-5}$ molar)

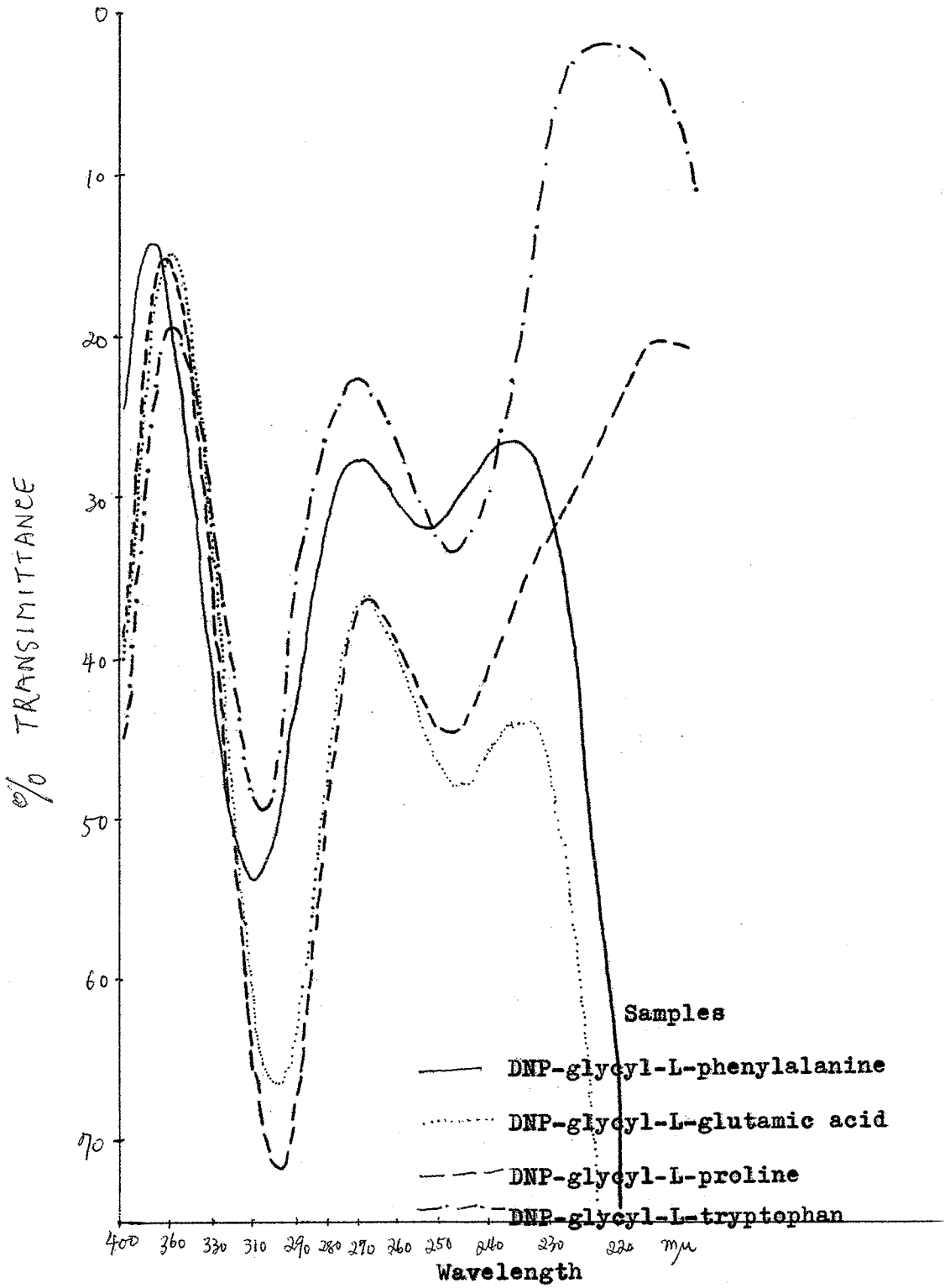


Figure 2. Absorption spectra of DNP-dipeptides.

(solvent: 0.2 molar sodium bicarbonate,

concentration: $5X10^{-5}$ molar)

Samples	Absorption Maxima (mμ)	Molar Absorbancy (ε_{max})
DNP-glycyl-L-phenylalanine	359; 264; 232;	16450; 10270; 11430
DNP-glycyl-L-glutamic acid	352; 265; 234;	15520; 9750; 8630;

DNP-glycyl-L-proline and DNP-glycyl-L-tryptophan were slightly unstable in aqueous alkaline solution; after storing for a long period, the maximum absorption of these two compounds shifted from 355 mμ to 336 mμ, and with considerably diminished intensity (ε_{max} below 10000). The differences in the absorption curves are shown in Fig. 3.

In 95% ethanol solution, all the four DNP-dipeptides have a wavelength maximum at 344 mμ, and with molar absorbancies between 17500-18000. The absorption spectra are shown in Fig. 4, and the wavelengths of maximum absorption and molar absorbancies are shown as below.

Samples	Absorption maximum (mμ)	Molar absorbancy (ε_{max})
DNP-glycine	344; 256;	17700; 10100;
DNP-glycyl-L-tryptophan	344; 256;	17400; 9240;
DNP-glycyl-L-proline	344; 256;	17850; 9400;
DNP-glycyl-L-phenylalanine	344; 264;	17580; 13240;
DNP-glycyl-L-glutamic acid	344; 256;	17340; 9400;

2,4-Dinitrophenyl Derivatives of Amides

The absorption spectra of DNP-glycyl amide, DNP-glycyl anilide, DNP-glycyl-p-toluide and DNP-L-alanyl amide, DNP-L-alanyl anilide,

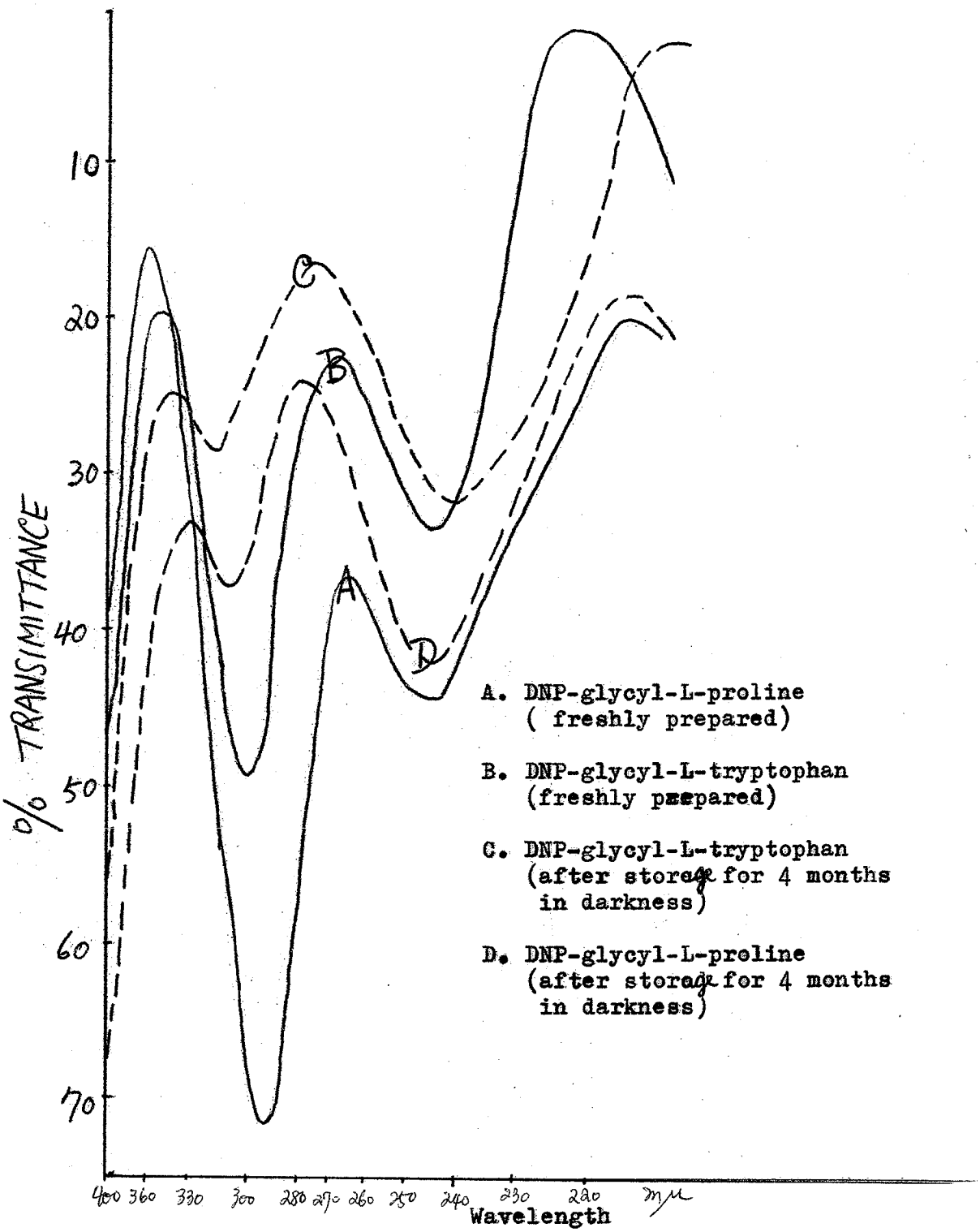


Figure 3. Absorption spectra for DNP-dipeptides.

(solvent: 0.2 molar sodium bicarbonate,

concentration: $5X10^{-5}$ molar)

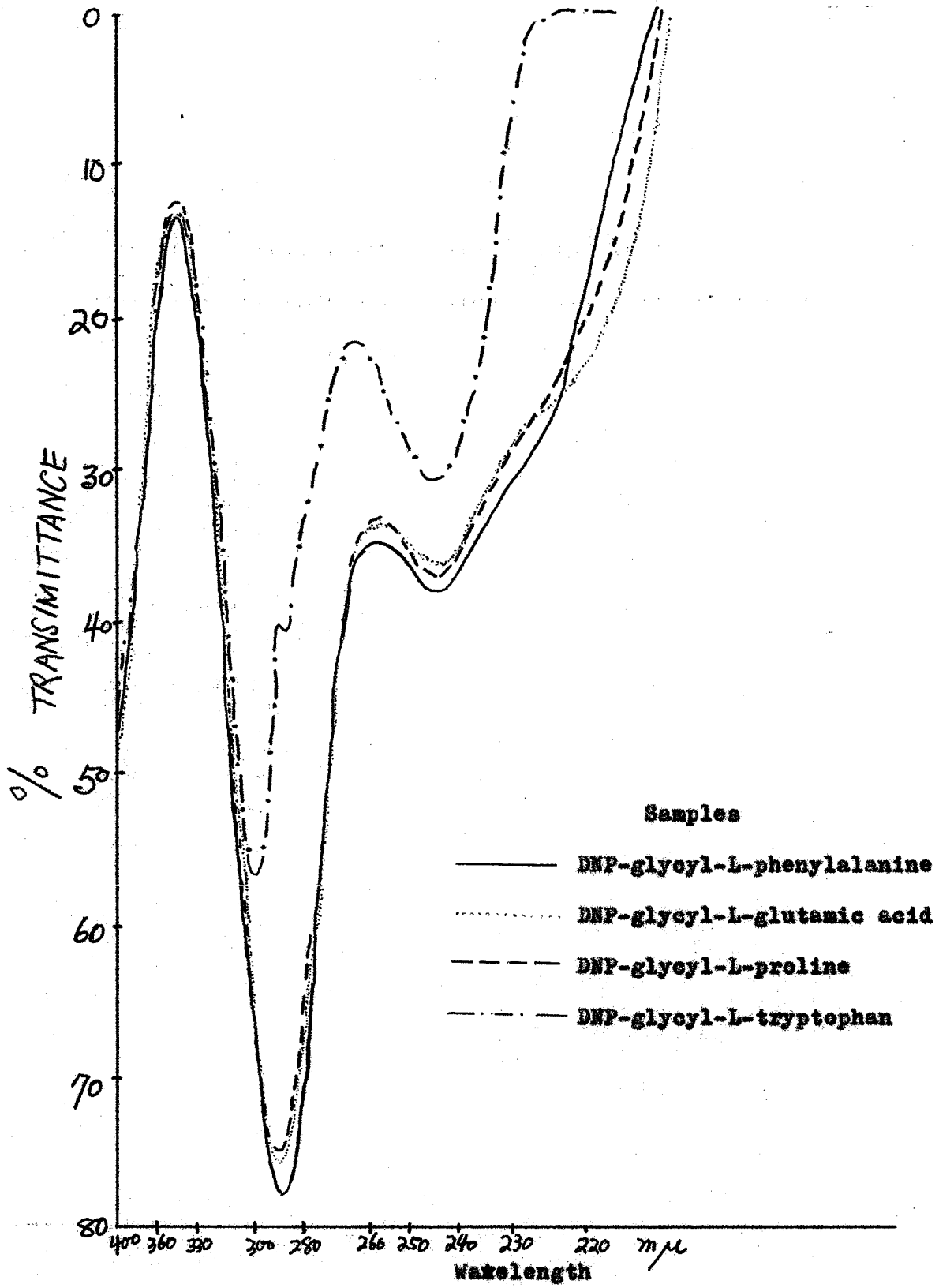


Figure 4. Absorption spectra of DNP-dipeptides.

(solvent: 95% ethanol, concentration: $5X10^{-5}$ molar)

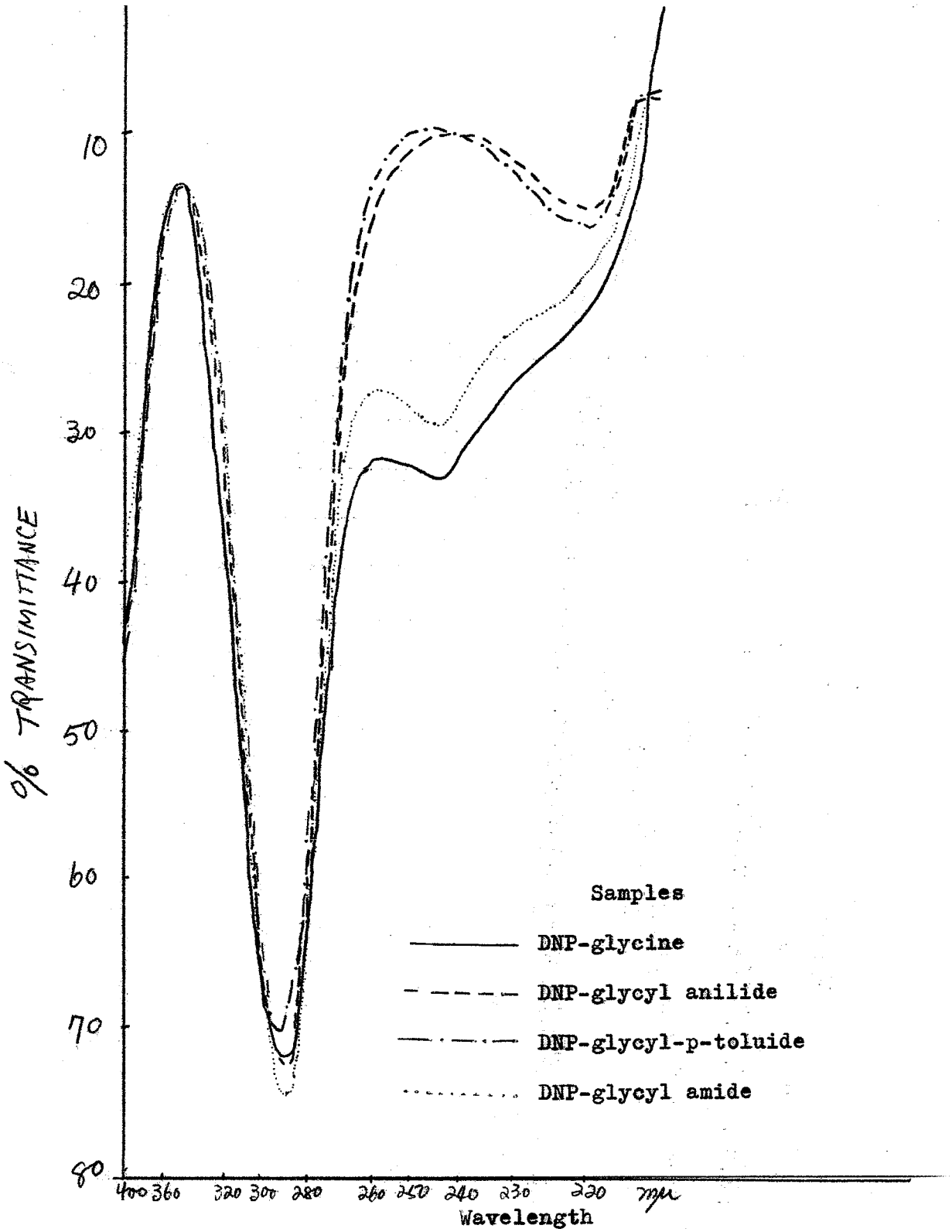


Figure 5. Absorption spectra of DNP-glycyl derivative amides.
(solvent: 95% ethanol, concentration: $5X10^{-5}$ molar)

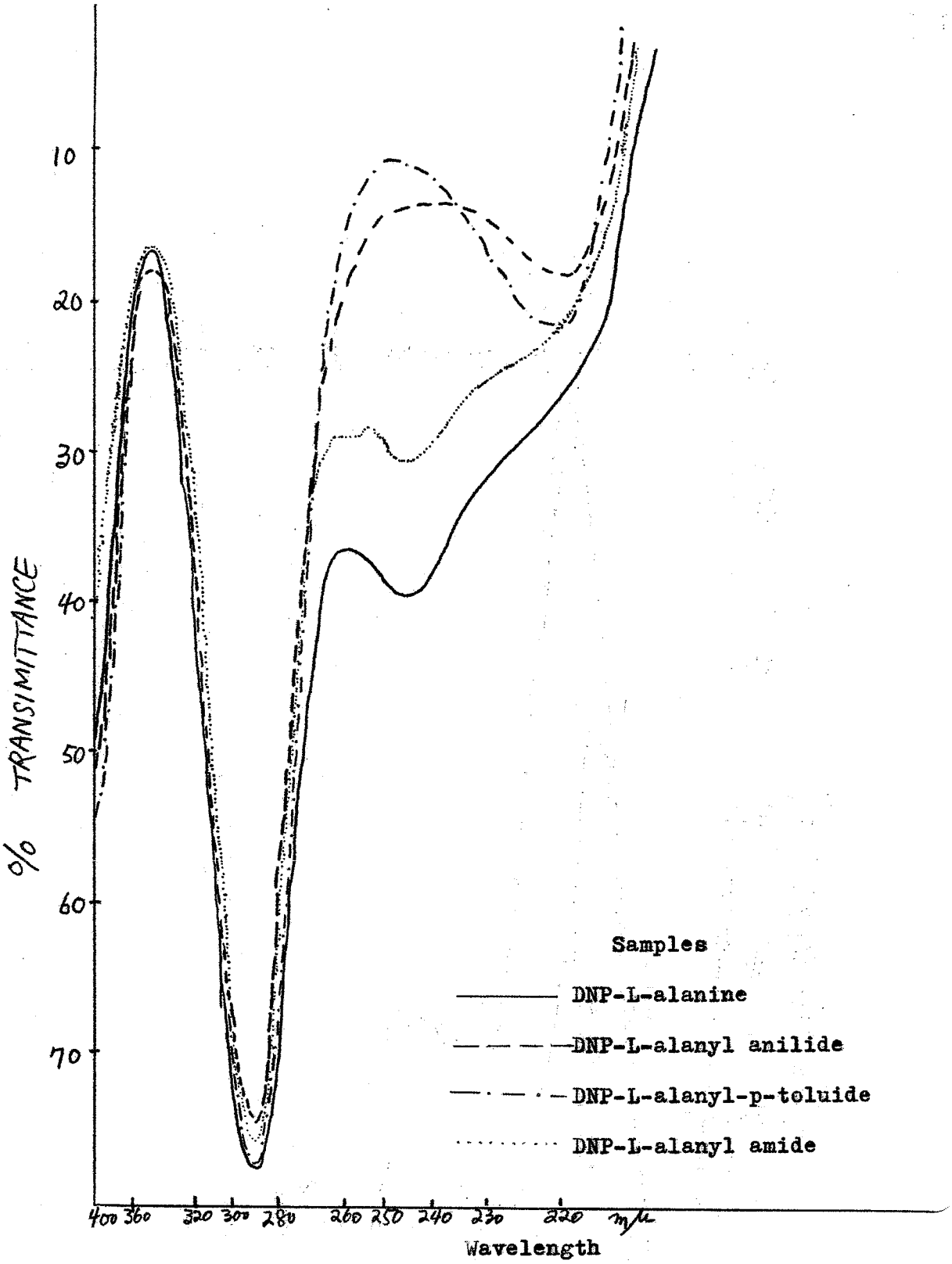


Figure. 6. Absorption spectra of DNP-L-alanyl amides.
(solvent: 95% ethanol, concentration: $5X10^{-5}$ molar)

DNP-L-alanyl-p-toluide in 95% ethanol has been studied, they all showed a similar curve, and the maximum absorption was 344 mμ for all DNP-derivatives. The absorption spectra of these compounds with DNP-glycine and DNP-L-alanine are shown in Fig. 6.

Infrared Absorption Spectra of 2,4-Dinitrophenyl Dipeptides and Derivatives

The infrared spectra of the above compounds were examined using the mull technique in the region 5000 to 625 cm^{-1}, where nujol was used as the mulling agent. The spectra of closely related DNP-glycyl peptides or DNP-amides vary sufficiently to allow positive identification.

The spectra were taken on a Model No. 21 Perkin-Elmer spectrophotometer (equipped with sodium chloride optics).

Table 1 lists and characterizes the spectral absorption bands in the 5000 to 625 cm^{-1} region. As a result of dipolar ion structure, many amino acids possess a characteristic absorption frequency at about 1587 cm^{-1} which related to the $COO^{\ominus}$ group, as well as a relatively weak absorption at about 2128 cm^{-1} which may be attributed to NH frequencies in the $-NH_3^+$ ion. The absence of this absorption peak at 2128 cm^{-1} in the case of all DNP-derivatives, where the amino group is attached to the DNP-ring, was therefore expected.

Aromatic nitro groups absorb strongly at 1530-1500 cm^{-1} and somewhat more weakly at 1370-1330 cm^{-1} (14). All of the above

DNP-derivatives examined, however, exhibit the bands at 1530 cm^{-1} and 1340 cm^{-1}, so it is believed that the band at 1530 cm^{-1} represents antisymmetrical aromatic NO stretch, even though a 1333 cm^{-1} band has been considered to be related to a CH wagging motion.

A weak band at the 3350 cm^{-1} may be interpreted as the N-H stretching vibration(14), and the band at 1625 cm^{-1} is the N-H bending vibration. A strong band at 1330-1305 cm^{-1} is due to the C-N vibration of secondary aromatic amines(14).

From the absorption spectra it shows all the above N-monosubstituted amides exists in the trans- configuration(14). Unsubstituted amides (eg. DNP-glycyl amide, DNP-L-alanyl amide) have a weak band at 3180 cm^{-1} due to the symmetric NH stretching. For monosubstituted amides (eg. DNP-glycyl anilide, DNP-glycyl-p-toluide, etc.), the CNH vibration where the nitrogen and hydrogen move in opposite directions relative to the carbon involves both NH bending and C-N stretching and absorbs strongly near 1550 cm^{-1}, and also shows a weaker band appears near 3100 cm^{-1} due to an overtone of the 1550 cm^{-1} band.

A band appears at 1730-1705 cm^{-1} shows a mono carboxylic acid or a carbonyl group which is hydrogen bonded but not dimerized.

The 1600 cm^{-1} vibration ("quadrant stretching") shows a nitro-group in substituted benzene. Substituted benzene also have two peaks at 1500-1450 cm^{-1} regions ("semicircle stretching").

Table 1

Characteristic Infrared Absorptions of DNP-Derivatives*

Group	Range (cm^{-1}) and Intensity			
DNP-glycine	3350MSh		1725SSh	
DNP-L-alanine	3350MSh	3095SB	1725SSh	1696SSh
DNP-glycyl-L-tryptophan	3350MSH		1740SSh	
DNP-glycyl-L-proline	3350MSh		1730SSh	
DNP-glycyl-L-phenylalanine	3350MSh		1725SSh	1696WSh
DNP-glycyl-L-glutamic acid	3350MSh		1743SSh 1715SSh	1696MSh
DNP-glycyl amide	3460MSh 3350MSh	3180MSh 3100MSh	1710SSh	
DNP-glycyl anilide	3350MSh	3100WSh	1710SSh	
DNP-glycyl-p-toluide	3350MSh	3100WSh	1710SSh	1682WSh
DNP-L-alanyl amide	3460MSh 3350MSh	3180MSh 3100WSh	1710SSh	1680SSh
DNP-L-alanyl anilide	3350MSh	3100WB	1710SSh	
DNP-L-alanyl-p-toluide	3350MSh	3100WSh	1710SSh	
DNP-glycine		1620SSh	1604SSh	
DNP-L-alanine	1683SSh	1633SSh		1590SSh
DNP-glycyl-L-tryptophan		1626SSh		1596SSh
DNP-glycyl-L-proline	1665WSh	1626SSh		1600SSh
DNP-glycyl-L-phenylalanine	1665WSh	1626SSh		1594SSh
DNP-glycyl-L-glutamic acid	1683SSh	1635SSh		1592SSh
DNP-glycyl amide	1665WSh	1623SSh	1614SSh	1582SSh
DNP-glycyl anilide	1665WSh	1623SSh	1614SSh	1590SSh
DNP-glycyl-p-toluide	1665WSh	1623SSh	1614SSh	1592SSh
DNP-L-alanyl amide	1660SSh	1623SSh	1614SSh	1600SSh
DNP-L-alanyl anilide	1665WSh	1623SSh	1614SSh	1588SSh 1605SSh
DNP-L-alanyl-p-toluide	1665WSh	1623SSh	1614SSh	1592SSh 1600SSh

Table 1 (Continued)

Group	Range(cm^{-1}) and Intensity			
DNP-glycine		1527SSh	1504SSh	1456 SSh
DNP-L-alanine		1530SSh	1504SSh	1465SSh
DNP-glycyl-L-tryptophan	1575SB	1528SSh	1504SSh	1465SSh
DNP-glycyl-L-proline		1528SSh	1504SSh	1456SSh
DNP-glycyl-L-phenylalanine	1565MSh 1550SSh	1526SSh	1504SSh	1456SSh
DNP-glycyl-L-glutamic acid	1550MSh	1532SSh	1504SSh	1456SSh
DNP-glycyl amide		1534SSh 1520SSh	1497SB	1456SSh
DNP-glycyl anilide	1555SSh	1538SSh 1525SSh	1494SSh	1465SSh
DNP-glycyl-p-toluide	1550SSh	1538SSh 1515SSh	1502SSh	1456SSh
DNP-L-alanyl amide		1524SSh	1492SSh	1456 SSh
DNP-L-alanyl anilide	1554SSh	1538SSh 1520SSh	1505SSh 1485SSh	1456SSh
DNP-L-alanyl-p-toluide	1550SSh	1538SSh 1513SSh	1505SSh	1456SSh
DNP-glycine	1432MB	1420MSh		1345SSh
DNP-L-alanine	1416SSh			1357SSh
DNP-glycyl-L-tryptophan		1420SSh		1345SSh
DNP-glycyl-L-proline	1446SSh			
DNP-glycyl-L-phenylalanine		1425SSh	1368SSh	1342SSh
DNP-glycyl-L-glutamic acid		1420SSh	1368SSh	1340SSh
DNP-glycyl amide		1428SSh		1341SSh
DNP-glycyl anilide	1443SSh	1422SSh		
DNP-glycyl-p-toluide		1418SSh		1341SSh
DNP-glycyl amide	1426SSh	1407SSh	1365SSh	1335SSh
DNP-L-alanyl anilide	1440SSh	1428SSh		1338SSh
DNP-L-alanyl-p-toluide	1428SSh	1402MSh	1367SSh	1335SSh

*Abbreviations: S=strong, M=medium, W=weak, B=broad, Sh=sharp,

Table 1 (Continued)

Group	Range(cm^{-1}) and Intensity			
DNP-glycine	1316SSh	1295SB	1257SSh	1247SB
DNP-L-alanine	1305SSh		1265SSh	1234SB 1217SB
DNP-glycyl-L-tryptophan	1307SSh	1300SSh	1281SSh 1254SSh	1234SSh
DNP-glycyl-L-proline	1310SB	1295SB	1274SB	1234SSh 1200SSh
DNP-glycyl-L-phenylalanine	1316SSh	1282SSh	1249SSh	1220MSh 1205MSh
DNP-glycyl-L-glutamic acid	1308SSh		1266SSh	1238SSh 1225SB
DNP-glycyl amide	1326SSh	1293SSh	1275SSh	1245SSh 1231SSh
DNP-glycyl anilide	1325SSh	1288SSh	1262SSh	1245SSh 1226SSh
DNP-glycyl-p-toluide	1316SSh	1295SSh	1271SSh 1258SSh	1248SSh 1228SSh
DNP-L-alanine amide	1307SSh		1278SSh	1242SSh
DNP-L-alanyl anilide	1324SSh 1305SSh	1298SSh	1265SSh	1230SSh 1202MSh
DNP-L-alanyl-p-toluide	1320SSh 1303SSh	1295SSh	1275SSh 1265SSh	1236SSh
DNP-glycine		1159SSh	1135SSh	1108SSh
DNP-L-alanine		1159SSh	1140SSh	1133SSh
DNP-glycyl-L-tryptophan	1194SSh	1157SSh	1140SSh	1101MSh
DNP-glycyl-L-proline	1183SB 1174SSh	1161SSh	1134SSh	1110SSh
DNP-glycyl-L-phenylalanine	1192SSh	1153SSh	1136SSh	1110SSh
DNP-glycyl-L-glutamic acid		1156SSh	1136SSh	1118MSh
DNP-glycyl amide		1157SSh	1140SSh	1112SSh
DNP-glycyl anilide	1175MSh	1150SSh	1136SSh	1110MSh
DNP-glycyl-p-toluide	1180WSh	1150SSh	1136SSh	1118SSh
DNP-L-alanyl amide	1178MSh	1155MSh	1120SSh	
DNP-L-alanyl anilide	1195SSh 1178MSh	1160SSh	1120SSh	
DNP-L-alanyl-p-toluide	1190MSh	1157SSh		1118SSh

*Abbreviations: S=strong, M=medium, W=weak, B=broad, Sh=sharp

Table 1 (Continued)

Group	Range(cm^{-1}) and Intensity			
DNP-glycine		1061WSh		982WSh
DNP-L-alanine	1082MSh	1076MSh	1058SSh	973WSh
DNP-glycyl-L-tryptophan		1058WB		
DNP-glycyl-L-proline	1091MSh	1057MSh	1044MSh	988MSh
DNP-glycyl-L-phenylalanine	1086MSh	1056WSh	1014WSh	988WSh
	1070WSh	1028WSh	1005WSh	980WB
DNP-glycyl-L-glutamic acid	1096MB	1058MSh	1014WB	
DNP-glycyl amide	1097MSh	1061MSh		991WSh
DNP-glycyl anilide	1080MSh	1060MSh	1028wSh	988WSh
DNP-glycyl-p-toluide		1065MSh	1038WB	988WSh
DNP-L-alanyl amide	1083MSh	1065MSh	1050MSh	978WSh
DNP-L-alanyl anilide	1078MSh	1057SSh	1048SSh	978WSh
DNP-L-alanyl-p-toluide		1055MSh	1050SSh	986WSh
DNP-glycine	950MB	926SSh		891WB
DNP-L-alanine	942SSh	920MSh		886SSh
DNP-glycyl-L-tryptophan	948WB	925MSh	916MSh	880WB
DNP-glycyl-L-proline		926WSh	919MSh	868MSh
DNP-glycyl-L-phenylalanine	962WB	925MSh	910MB	868MB
		918MSh	900MB	
DNP-glycyl-L-glutamic acid	952WB	929WSh		
DNP-glycyl amide	942MSh	926MSh		892WB
DNP-glycyl anilide	952MSh	927MSh	908MSh	
DNP-glycyl anilide	952MSh	927MSh	908MSh	
DNP-glycyl-p-toluide	953MSh	926MSh		
DNP-L-alanyl amide	952WB	938WSh	918WSh	
DNP-L-alanyl anilide	962WSh	924MSh	905wSh	
		918MSh	900MSh	
DNP-L-alanyl-p-toluide	956WB	920MSh	913WSh	
	941WSh	920MSh	904WSh	

*Abbreviations: S=strong, M=medium, W=weak, B=broad, Sh=sharp,

Table 1 (Continued)

Group	Range(cm^{-1}) and Intensity			
DNP-glycine	838MSh	819SSh	765WSh	747SSh
DNP-L-alanine	835MB	827SSh	766WSh	744SSh
DNP-glycyl-L-tryptophan	836MSh	816MSh	751SSh	743SSh 733MB
DNP-glycyl-L-proline	829MSh		777MSh 766WSh	742MSh
DNP-glycyl-L-phenylalanine	835MSh	818MSh	768MSh	748MB
DNP-glycyl-L-glutamic acid	836MSh	820MSh	767MSh	744MSh
DNP-glycyl amide	832SSh		782WB 766MSh	747SSh
DNP-glycyl anilide	834wSh	812WSh	770SSh	743SSh
DNP-glycyl-p-toluide	837WSh 833WSh	824MSh 813MSh	763wSh	745MSh
DNP-L-alanyl amide	835MSh 830MSh	815wSh	765MSh	744Ssh
DNP-L-alanyl anilide	842wSh 835MSh	822MSh	759SSh	745SSh
DNP-L-alanyl-p-toluide	835wSh	822SSh	763WSh	745MSh
DNP-glycine	716WB	697WB	685WB	658MB
DNP-L-alanine	721MSh	694WB	670MB	
DNP-glycyl-L-tryptophan	721WB			
DNP-glycyl-L-proline	723WB	707MSh	688WB 670WB	654MB
DNP-glycyl-L-phenylalanine	724MB	707MSh	680MB 673MB	665MSh
DNP-glycyl-L-glutamic acid	722WB	707MSh		
DNP-glycyl amide	724SSh	698WB	670WB	662MSh
DNP-glycyl anilide	722WB	697MSh	670WB	
DNP-glycyl-p-toluide		699WSh	670WB	662WB
DNP-L-alanyl amide	718MSh	693WSh	670WB	658MSh
DNP-L-alanyl anilide	717MSh	692MSh	670WB	
DNP-L-alanyl-p-toluide	714MSh	696WB	670WB	658MS

*Abbreviations: S=strong, M=medium, W=weak, B=broad, Sh=sharp,

Since its introduction in 1945 by Sanger(1), dinitrophenylation has frequently been employed as an analytical tool in studies involving proteins, peptides, amino acids and amines. Numerous techniques for identifying the DNP-derivatives have been reported, including paper chromatography, column chromatrography, thin layer chromatography, paper electrophoresis, and countercurrent distribution. The following two methods were used as the separation and identification of the four DNP-dipeptides which were prepared in this work.

Thin-Layer Chromatography

Thin-layer chromatography was carried out on a Mallinckrodt Chroma-Kit, the method described was applicable to 0.1 to 0.5 μg of material, and provided a clear separation of DNP-glycine, DNP-glycyl-L-tryptophan, DNP-glycyl-L-proline, DNP-glycyl-L-phenylalanine, DNP-glycyl-L-glutamic acid. Silic AR; TLC-7GF was used as the adsorbent in this experiment, the thickness of the layer was 0.25mm, and all plates were activated at 60° for 90 minutes. The thin-layer chromatogram was developed by placing a glass plate, containing the sample on a bound thin layer, in a vertical position in a closed, saturated system such that the bottom of the layer dips into the developing solvent*(chloroform/methanol/acetic; 95:5:1)(15,16). The time required for the

*Methanol and acetic acid were purified by distilling through a short column. Chloroform was purified by distilling through a short column.

development was 1½ to 2 hours. After the solvent ascended 10 cm, the plate was removed from the solvent, dried and the distance the spots had travelled was measured.

The DNP-dipeptides are yellow, as little as 0.1 μg giving spots that are easily visible by transmitted daylight, therefore, no sprayed reagent was used for detection.

The R_f values of the four DNP-dipeptides were determined, which compared with DNP-glycine were shown as follows:

Compounds	R_f
DNP-glycyl-L-glutamic acid	0.10
DNP-glycyl-L-tryptophan	0.25
DNP-glycyl-L-phenylalanine	0.35
DNP-glycyl-L-proline	0.38
DNP-glycine	0.60

The distance travelled by the individual DNP-dipeptides and DNP-glycine, and in the mixture is shown in Fig. 7.

Paper Electrophoresis

Paper electrophoresis was carried out on a Reco Model E-800-2 electrophoresis unit. The apparatus was used as an horizontal open strips electrophoresis. Whatman No. 1 paper strips of dimensions 3.8X60 cm, and 0.02 molar sodium borate with PH 9.21 were used in all experiments.

All experiments were done in complete darkness to prevent photolyses. As reported by J. H. Loudfoot and J.E. Kruger(9, 10), a lower field strength of 300 volts (current of 3-4.5 milliamperes) and an electrophoretic time (16-22 hours) were used.

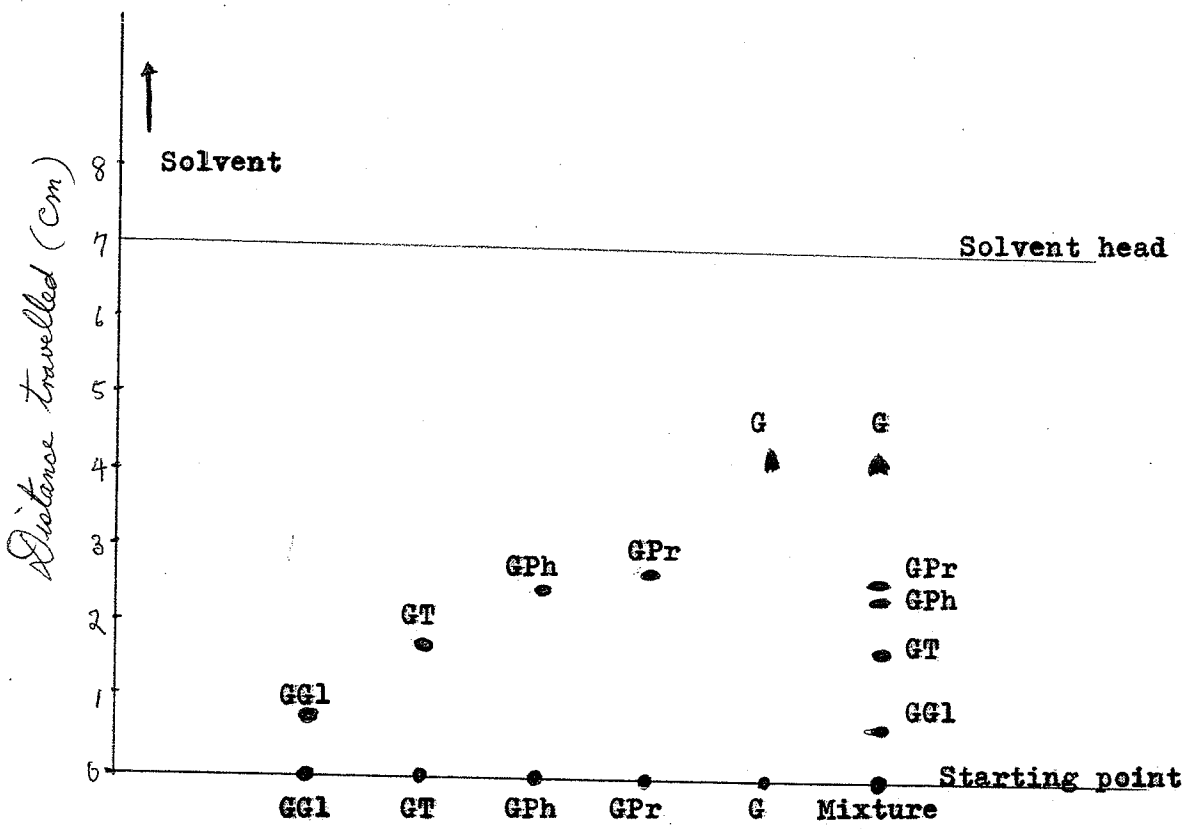


Figure 7. One-dimensional Chromatogram of DNP-glycine and DNP-dipeptides individually and in admixture.
(solvent: chloroform/methanol/acetic acid; 95:5:1)
Where G=DNP-glycine
GPr=DNP-glycyl-L-proline
GPh=DNP-glycyl-L-phenylalanine
GT=DNP-glycyl-L-tryptophan
GGl=DNP-glycyl-L-glutamic acid

The four DNP-dipeptides prepared in this work were now studied electrophoretically under these conditions. The electrophoretic time was 16½ hours and each of the four DNP-dipeptides travelled a different distance individually which compared with DNP-glycine are shown as follows:

Compounds	Distance Travelled(cm)
DNP-glycyl-L-tryptophan	18.0
DNP-glycyl-L-phenylalanine	20.8
DNP-glycyl-L-proline	21.6
DNP-glycyl-L-glutamic acid	33.5
DNP-glycine	25.0

A mixture of all four DNP-dipeptides and DNP-glycine after electrophoresis resulted in a fairly good separation except DNP-glycyl-L-proline and DNP-glycyl-L-phenylalanine. The distances travelled by these two compounds very close together, therefore, an elongated spot was shown on the paper strip.

An electrophoretic pattern illustrating this is shown in Fig. 8.

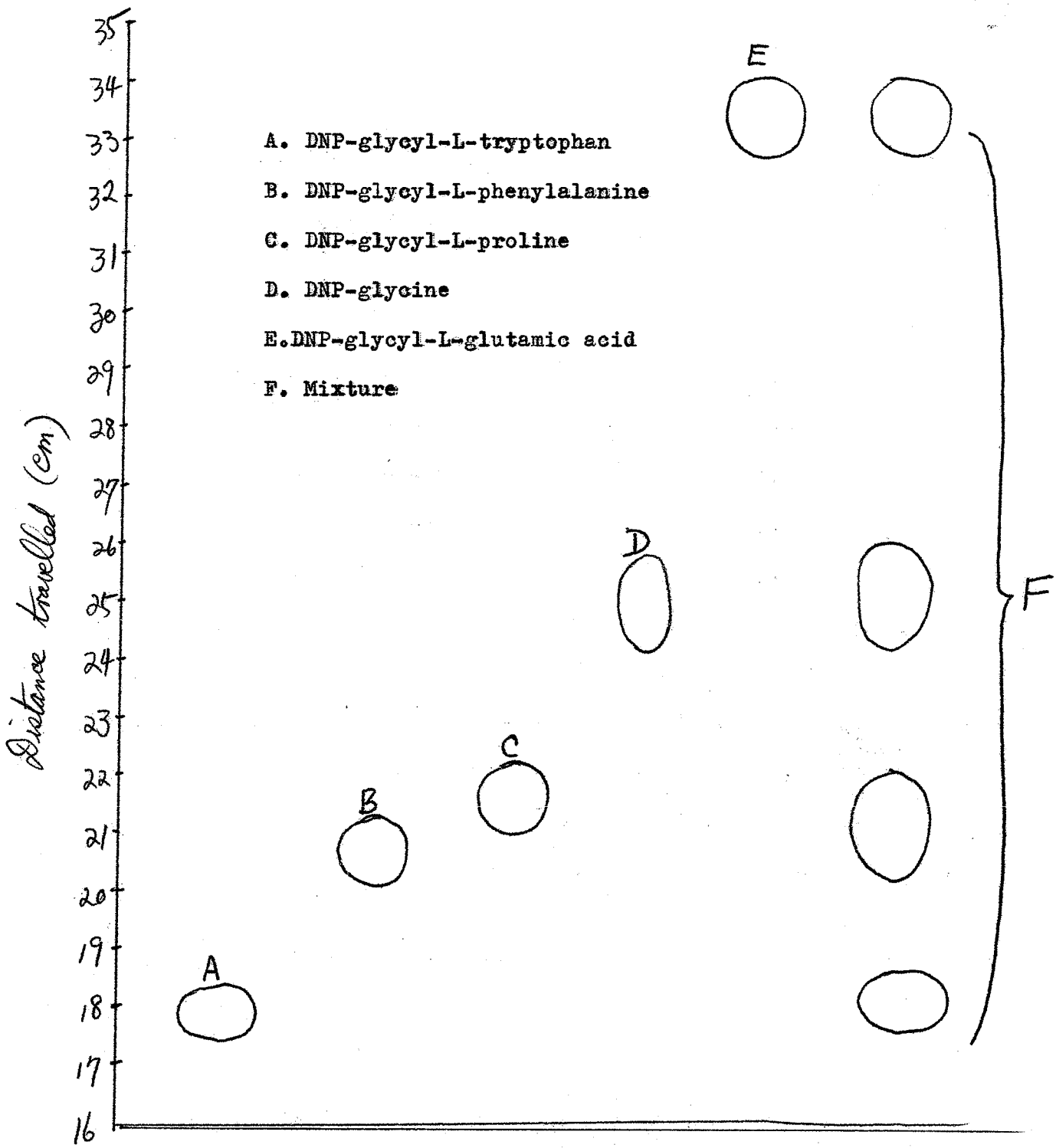


Figure 8. Electrophoretic pattern of DNP-glycine and DNP-dipeptides individually and in admixture. (solvent: 0.02 molar sodium borate with pH 9.21, electrophoretic time 16½ hours.)

Discussion of Results

Each of the DNP-derivatives in this work was obtained in solid form. It was found no racemization occurs when the acid chloride method is used, the DNP-dipeptide prepared from both methods gave an identical product in all properties. (eg. Specific rotation, melting point, ultra-violet and infrared spectra etc.) In comparing the two methods, the DNP-dipeptides prepared from dipeptides always gave a better yield and good quality, but some dipeptides are more expensive and less readily available than amino acids, therefore, the acid chloride method is still the better way to prepare large quantities of DNP-dipeptides.

As G. L. Mills(17) reported that the dicarboxylic amino acids reacted more slowly with FDNB than was expected, similarly in the work reported here, preparation of DNP-glycyl-L-glutamic acid from the dipeptide required a reaction time of 20 hours at room temperature in order to obtained a reasonable yield. DNP-glycyl-L-glutamic acid prepared from acid chloride failed to yield product even after exhausive attempts at purification, however, when preparative thin-layer chromatography was used, a very good separation and reasonable yield was obtained; the product was identical with that prepared from the dipeptide.

In dilute sodium bicarbonate solution, the absorption maximum in ultra-violet light at 350-360 mμ is as expected for the DNP-dipeptides(13). A shift of the maximum to the

shorter wavelengths (344 mμ) in 95% ethanol solution was shown by all DNP-derivatives. DNP-glycyl-L-phenylalanine, DNP-glycine and DNP-L-alanine in 0.2 molar sodium bicarbonate solution showed little or no change in the spectral absorption curves with time, indicating that the compounds were stable in alkaline solution under these conditions (4 months at room temperature in complete darkness). However, solutions of DNP-glycyl-L-proline and DNP-glycyl-L-tryptophan showed significant decreases in ultraviolet absorption at maxima when kept for four months at room temperature.

It would be surprising if the infrared spectra of closely related compounds did not reveal some differences by which pure, individual samples of such compounds could be differentiated from each other. A review of the table suggests that among the DNP-dipeptides and DNP-amides studied, the spectra on the whole are more strikingly similar than they are different, and that the use of the spectral tool for the purposes of identification is much weaker and less reliable than such procedures as chromatography. If, however, some DNP-dipeptide should be difficult to identify by chromatography, spectral analysis after purification would still be of value. Even the spectra of structurally very similar DNP-derivatives show sufficient variation to allow positive identification.

The optical rotation of most of the compounds would be obtained in 95% ethanol and in acetone, the molar rotation of the DNP-dipeptides and the DNP-amides was in general higher than that

of the corresponding dipeptides or the amides. The high optical rotation of DNP-dipeptides may be useful as an aid to characterization of dipeptides present individually or in combination in natural products.

It was found both paper electrophoresis and thin-layer chromatography made a good separation for the four DNP-dipeptides in mixture. Electrophoretic studies have shown that the distances travelled by DNP-dipeptides which have the same charge was dependent on the size of molecule, the larger the size of the molecule, the slower its travels. DNP-glycyl-L-glutamic acid has two negative charge in the molecule, therefore, it travelled much farther than the other DNP-dipeptides. Thin-layer chromatography of the DNP-dipeptides is clearly preferable to paper electrophoresis(saving of time, greater sensitivity, better separation.).

As reported by a number of workers(18,19,20,21), in alkaline or neutral solution, DNP-amino acids and DNP-peptides tended to decompose if exposed for long periods to light. It has been found necessary during the course of this work to protect the DNP-dipeptides(specially DNP-glycyl-L-proline, and DNP-glycyl-L-glutamic acid) from light at all stages of their preparation and separation. This particularly applied to solutions in alkali and in chloroform.

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