THE RESOLUTION OF AMINO ACID DIASTEREOMERS AND A SEARCH FOR FREE D-AMINO ACIDS IN THE HOUSE FLY, MUSCA DOMESTICA L. BY PACKED COLUMN GAS CHROMATOGRAPHY

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
GEORGE SCOTT AYERS
1972

This is to certify that the

thesis entitled

THE RESOLUTION OF AMINO ACID DIASTEREOMERS AND A SEARCH FOR FREE D-AMINO ACIDS IN THE HOUSE FLY, MUSCA DOMESTICA L. BY PACKED COLUMN GAS CHROMATOGRAPHY

presented by

George Scott Ayers

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Entomology

Major professor

Date May 10, 1972

ABSTRACT

THE RESOLUTION OF AMINO ACID DIASTEREOMERS AND A SEARCH FOR FREE D-AMINO ACIDS IN THE HOUSE FLY, MUSCA DOMESTICA L. BY PACKED COLUMN GAS CHROMATOGRAPHY

By

George Scott Ayers

The feasibility of preparative gas chromatographic resolution of small quantities of certain alcohols as the N-trifluoroacetyl-L-alanyl diastereomeric esters was demonstrated. The degree of resolution attained and the quantity resolved were strongly affected by the column length. Utilizing a 1 X 1584 cm column, both optical forms of 3,3-dimethyl-2-butanol were obtained with greater than 98% purity.

The effect of an alcohol's structure upon the resolution of its N-acylated amino acid diastereomeric ester was studied using several packed gas chromatographic columns. Using 3,3-dimethyl-2-butyl-N-trifluoroacetyl derivatives, 14 protein amino acid diastereomers could be resolved to 93% or better. Aspartic acid and proline derivatives could be resolved to 70% and 82%, respectively. Arginine, histidine and cystine derivatives were not studied.

A study of the stereo configuration of the free amino acids found in Musca domestica L. pupae was conducted using gas chromatography. The relative concentrations of the D-isomers were found to be no greater than 3% of the total amino acid concentration for each individual amino acid. The configurations of arginine, histidine and cystine were not studied.

THE RESOLUTION OF AMINO ACID DIASTEREOMERS AND A SEARCH FOR FREE D-AMINO ACIDS IN THE HOUSE FLY, *MUSCA DOMESTICA* L. BY PACKED COLUMN GAS CHROMATOGRAPHY

By

George Scott Ayers

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Entomology

1972

TABLE OF CONTENTS

Page

PART III: A SEARCH FOR FREE D-AMINO ACIDS IN PUPAE OF THE HOUSE FLY, MUSCA DOMESTICA L.

APPENDIX

LIST OF TABLES

LIST OF FIGURES

PART I: PREPARATIVE GAS CHROMATOGRAPHIC RESOLUTION OF 3,3-DIMETHYL-2-BUTANOL AND OTHER ALCOHOLS

INTRODUCTION

In instances where only small amounts (ca. 1 ml or less) of both optically active forms of a particular alcohol are required, preparative gas chromatographic resolution would offer certain advantages over the classical method of alcohol resolution (Ingersoll, 1947). Small amounts of the resolved alcohol could easily be obtained without waste and without the necessity of working with toxic alkaloids. In theory, both optical forms could be obtained in one operation rather than through numerous recrystallizations and the change of the alkaloid base often involved in obtaining both optical forms of a particular alcohol. The optical purity could also be immediately and conveniently determined by analytical gas chromatography.

Recently, correlative to the gas chromatographic resolution of amino acids, the possibility of resolving alcohols by gas chromatography has been demonstrated (Pollock and Oyama, 1966). Such work has been done predominately towards analytical goals, much of it with long capillary columns.

Motivated by the need for small amounts of both optical isomers of 3,3-dimethyl-2-butanol, the following study was initiated to investigate the possibility of resolving this alcohol, as well as others, by preparative gas chromatography.

MATERIALS AND METHODS

Instruments and equipment

The instrument used in this study was a Model 600 series Research Specialties gas chromatograph equipped with a dual hydrogen flame detector. The preparative column (Figure 1) was constructed of three main units. Each unit consisted of six vertical sections of 10 mm i.d. pyrex tubing ca. 88 cm long connected together by 4 mm i.d. horizontal crossovers. Septum sockets were mounted directly above the vertical columns on the top of the crossovers. An all glass condenser type injector head, constructed so the carrier gas would enter the column through four 0.75 mm holes just beneath the injection septum was attached to the initial vertical column of the first unit. The three main units could be connected by means of 5 mm Fischer Porter Solv Seal joints (Fischer Porter Co., Warminster, Pennsylvania.) which were held tightly together by means of a clamp consisting of steel collars (tapped and fitted with two small machine screws) on each joint socket. Thus the column could be operated at three lengths, ca. 528 cm, 1056 cm or 1584 cm.

The column was filled through the septum holders and compacted with a vibrator. A 1/8-1/4 inch plug of silanized glass wool was lightly compacted into the junctures of the crossovers and vertical columns.

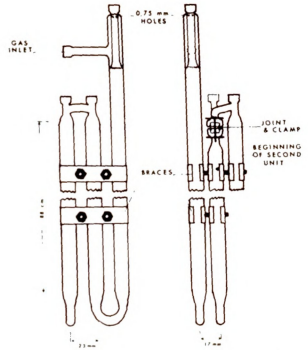

Figure 1. Diagram of first unit of preparative GLC column.

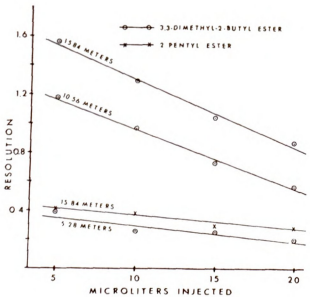

Figure 2. Effect of preparative column length on resolution of 3,3-dimethyl-2-butyl-N-trifluoroacetyl-L-alanine and 2-pentyl-N-trifluoroacetyl-L-alanine.

A splitter consisting of a stopcock and a septum socket (inserted between the terminus of the column and the stopcock) containing a septum pierced by a piece of 1/16 in stainless steel tubing which led to the hydrogen flame detector was installed in the all glass outlet. The split ratio was coarsely adjusted by the stopcock while fine adjustments were made by adjusting the hydrogen line pressure to the detector. The outlet tube was heated by means of an electrical heating tape.

Two 11 x 1 cm vacuum traps modified with $\frac{S}{J}$ balls to fit the column outlet and cooled with a dry ice-acetone bath were used to collect the material from the column.

Derivative preparation

±3,3-dimethyl-2-butyl-N-trifluoroacetyl-L-alanine: A mixture of 50 ml of ±3,3-dimethyl-2-butanol containing 1.2 milliequivalents of HCl/ml was reacted with 1 g L-alanine at 100° for 1 hr in an oil bath-magnetic stirrer. The more volatile components of the mixture were removed from the alanine and alanyl ester by fractional distillation and the alcohol fraction (118-122°C) saved and measured volumetrically. An additional 1.2 milliequivalents of HCl/ml was added to the alcohol and the mixture was again reacted with 1 g of L-alanine as before. This procedure was continued until four 1 g quantities of alanine had been used. The combined alanine-alanyl ester residues were partitioned between *ca.* equal volumes of ether and 1 M Na_2CO_3. The carbonate fraction was extracted quantitatively with ether; the ether was removed on a rotating evaporator and the residue dissolved in 10-15 ml methylene chloride. This was reacted at room temperature with *ca.* a 1.5 M excess

of trifluoroacetic anhydride. The more volatile components were removed by a rotary evaporator at 60°C and the remaining residue vacuum distilled. A distinct non-viscous fore-run occurred followed by the very pale yellow more viscous N-trifluoroacetyl alanyl ester. In this manner, 10.4 g of the N-trifluoroacetyl alanyl ester was obtained.

±2-pentyl-N-trifluoroacetyl-L-alanine: 2-pentyl-N-trifluoroacetyl-L-alanine was prepared in a manner similar to the preparation of the corresponding 3,3-dimethyl-2-butyl derivative except that more amino acid and alcohol-HCl mixture were used initially and only one esterification was carried out.

Other amino acid derivatives: Small amounts of ±3,3-dimethyl-2-butyl esters of valine, phenylalanine and proline, were prepared in a manner similar to the preparation of the corresponding alanyl ester. Smaller quantities of alcohol and amino acid were used and only one esterification and no vacuum distillation was carried out. These esters along with small amounts of the corresponding alanyl ester were converted to their N-acyl derivatives by means of acetic anhydride, trifluoroacetic anhydride, propionyl chloride or trichloroacetyl chloride.

Small quantities of 1,1,1-trifluoro-2-propyl-N-trifluoroacetyl-L-alanine were also prepared in a similar manner.

Effect of liquid phase and particle size

To test the suitability of various liquid phases for resolving ±3,3-dimethyl-2-butanol the following liquid phases were tested in 4 mm i.d. analytical columns: cyclohexanedimethanol adipate,

tetramethylcyclobutanediol adipate, 1,2,3 tris(2-cyanoethoxy)propane, OV-1 (dimethyl silicone), OV-17 (phenyl methyl silicone) and OV-25 (phenyl methyl silicone). To test the suitability of various diastereomers for the resolution of 3,3-dimethyl-2-butanol, the N-acetyl, N-trifluoroacetyl, N-tricholoroacetyl and N-propyl derivatives of the alanyl, valyl, phenylalanyl and prolyl esters of the alcohol were chromatographed on these columns. The N-trifluoroacetyl ester chromatographed on either the OV-1 or OV-17 columns appeared to give the most favorable results. Columns (1 X 528 cm), packed with either 45/60 or 100/120 mesh DMCS treated acid washed Chromosorb W coated with either 10% OV-1 or OV-17 were used to determine the effect of particle size and of the two liquid phases on preparative scale separations. The coarser packing appeared to have a slightly higher overload capacity while OV-1 appeared to give resolutions slightly superior to those given by OV-17.

In order to appraise the effect of column length on the quality of separation, the three sections of the preparative column were packed with 10% OV-1 on 45/60 DMCS treated acid washed Chromosorb W. The column was operated at 165°C with a 230 ml/min nitrogen flow rate. Injections of 5, 10, 15, and 20 µl of ±3,3-dimethyl-2-butyl-N-trifluoroacetyl-L-alanine were made into the column. This procedure was then repeated for the two shorter lengths.

Resolution of the ±3,3-dimethyl-2-butyl-N-trifluoroacetyl-L-alanine

For the preparative resolution of the ±3,3-dimethyl-2-butyl-N-trifluoroacetyl-L-alanine diastereomers, the 1584 cm column was

utilized. The column temperature was 165°C and the nitrogen carrier flow rate was 230 ml/min. No flash heater was used. A 15 µl injection was introduced into the column every 6-7 min. Since the injected material had a retention time of approximately 30 min, approximately 5 injections were on the column at any given time after the fifth injection. Injection timing was varied slightly to facilitate concurrent injection and trapping schedules. The outlet tube to the cold trap was maintained at about 190°C. The splitter was adjusted to deliver >99% of the chromatographed material to the cold trap allowing the remainder to go to the hydrogen flame detector. The trapping schedule could thus be synchronized to the actual elution by watching the chart recorder. Trapping for the first peak was initiated after the recorder pen began to rise, and was terminated slightly before it reached the low point between the two peaks. Trapping for the second peak was initiated shortly after the pen began to rise and terminated as the pen reached base line (Figure 3).

Saponification of the resolved diastereomers

Small portions (0.1-0.3 ml) of the resolved diastereomers were placed in separate 100 X 13 mm screw cap culture tubes. A 20% (w/v) methanolic NaOH solution made of methanol-water (7:5) was added to each diastereomer until the final NaOH molarity was 3 times that of the diastereomer. Boiling chips were added and the tubes sealed with Teflon lined caps and immersed to the level of the contents in a boiling water bath for 1 min. The saponification mixture was cooled under tap water, diluted to twice its volume with water and extracted

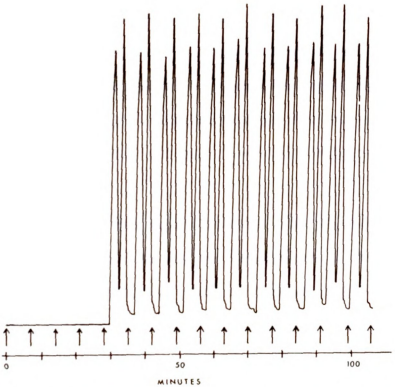

Figure 3. Preparative resolution of ± 3,3-dimethyl-2-butyl-N-trifluoroacetyl-L-alanine.[a]

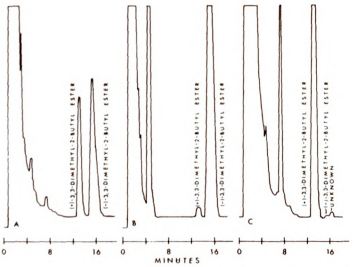

Figure 4. Gas chromatographic demonstration of optical purity of the two 3,3-dimethyl-2-butanols derived from preparative gas chromatography.[b]

---

[a] Arrows indicate 15-µl injections.

[b] Derivatives in chromatogram A are from unresolved alcohol; Chromatograms B and C are derivatives of alcohols derived from the first and second preparative peaks respectively.

quantitatively with equal volumes of ether. The pooled ether extracts were then washed with water until neutral and dried over anhydrous Na_2SO_4. The resolved 3,3-dimethyl-2-butanol was conveniently separated from the ether and methanol by preparative gas chromatography using a 1 X 528 cm column packed with 10% OV-17 on 45/60 DMCS treated acid washed Chromosorb W .

For larger amounts the saponification time may need to be increased. Trial saponifications may be conveniently monitored on unactivated silica gel G thin layer plates, chromatographed in the top phase of n-butanol-acetic acid-water (25:6:25) and developed with ninhydrin. The trifluoroacetyl moiety is removed almost immediately and the resulting ester is readily detected with ninhydrin as is alanine, one of the final hydrolysis products. The Rf value of the alanyl ester is about 4.7 times that of alanine. The disappearance of the alanyl ester may also be monitored by gas chromatography on 3 m 10% OV-1 analytical columns.

Determination of optical purity

To test the stearic purity of the resolved alcohols the (+) or (-) 3,3-dimethyl-2-butyl-N-trifluoroacetyl-D-alanyl derivative was prepared by the following microprocedure. D-alanine (50 mg) was dissolved in 1 ml of trifluoroacetic acid. To this 1 ml of trifluoroacetic anhydride was added. After 15 min at room temperature the solvent was removed under a stream of nitrogen and the N-trifluoroacetyl-D-alanine was dissolved in 1 ml of methylene chloride. To this 1 ml of thionyl chloride was added and allowed to stand 15 min at room

temperature. The solvents were again removed under a stream of nitrogen and the acid chloride dissolved in 1 ml of methylene chloride. From this two 0.1 ml aliquots were taken and reduced to 0.02 ml under a stream of nitrogen in two small conical centrifuge tubes. Separately, 5 µl of the appropriate optical form of the alcohol was added to each tube. After several minutes 1/2 ml of methylene chloride and 1/2 ml of water were added to each of the samples and the aqueous fraction was extracted quantitatively with methylene chloride. The methylene chloride fractions from each sample were dried with a small amount of anhydrous Na_2SO_4, and evaporated to 0.1 ml under a stream of nitrogen. The derivatives were chromatographed on 3 m 10% OV-17-DMCS treated acid washed Chromosorb W columns at 118°C with a nitrogen carrier gas flow rate of 90 ml/min. No flash heater was used.

RESULTS

The effect of column length on the degree of separation is quite pronounced. This is shown in Figure 2 by a plot of resolution versus the quantity injected. The number of theoretical plates, based on the first peak for 15 µl injections of unresolved material at 165°C and a carrier gas flow rate of 230 ml/min, increased with column length, ie. 553, 1345, and 2277. Figure 3 shows the separations attained with the 1584 cm column at 165°C for 15 µl injections, spaced approximately 6 minutes apart. Slightly better separations can be obtained at lower temperatures with all three column lengths, but since the peak bases are spread out more, the time interval between injections must be increased and the ratio of the amount injected/unit of time is diminished. As can be seen from Figure 2, the ability of the 1584 cm column to resolve the 2-pentyl diastereomer is much less than its ability to resolve the 3,3-dimethyl-2-butyl analogue. Slightly better separation was obtained with the 2-octyl analogue. Because of difficulties encountered in preparing large quantities of the 1,1,1-trifluoro-2-propyl derivative, only slightly larger than analytical amounts (ca. 10 µg) were tested. The degree of separation was approximately the same as that given by a similar load of the 3,3-dimethyl-2-butyl analogue.

Figure 4 shows the chromatograms of the esters formed by esterification of N-trifluoroacetyl-D-alanine with one of the resolved 3,3-dimethyl-2-butanols and thus indicates the degree of resolution achieved by the preparative column. It should be pointed out, however, that the results depicted in Figure 4 indicate the minimum degree of resolution achieved because of the possibility of small amounts of racimization of the alcohol during the saponification as well as of the alanine during the formation of the N-trifluoroacetyl acid chloride. It is also possible that the D-alanine used for the esterification was contaminated with small amounts of the L-isomer. By triangulating the peaks of the more prevalent diastereomers and comparing the peak areas of the two diastereomers, the optical purities are shown to be >98%.

Polarimetric studies showed the alcohol derived from the first peak from the preparative column to be levorotatory and from the second to be dextrorotatory. The last peak in Figure 4-C is apparently not the result of contamination by the levorotatory form and is of unknown origin, but perhaps represents a structural isomer or analogue of 3,3-dimethyl-2-butanol. The contamination due to the levorotatory form is apparently represented by the shoulder preceeding the unknown peak. The slight tailing in Figure 4-A probably is also the result of this unknown material.

DISCUSSION AND CONCLUSIONS

With the aid of automatic injection and collecting devices and improved columns, especially longer columns, preparative gas chromatographic resolution of at least certain alcohols would be quite practical. The maximum practical column length for this purpose is not known, but the results indicate that it was not surpassed in this study.

The use of an improved cold trap would also be beneficial. By using the trap described, only about 62% of the amount injected could be accounted for in the trap. Although nearly all the trapped material was condensed on the first 2 or 3 cm of the trap, quantities of smoky-appearing material issued from the cold trap outlet. The appearance of this material coincided with the elution of the two diastereomers. Some of this material could be trapped in a U-tube cooled by liquid nitrogen but even with several inches of loosely packed glass wool the smoky effluence continued from the trap outlet. A portion of what was trapped in the liquid nitrogen cooled tube was the 3,3-dimethyl-2-butyl derivative, but based on solubility studies, the smoky material also contained a more polar and unknown fraction. The smoky effluent also appeared when trapped material was rechromatographed. This phenomenon was not an artifact of the 3,3-dimethyl-2-butyl derivative alone but also occurred with the other esters tested in this study.

REFERENCES

Ingersoll, A. W. Organic Reactions. Vol. II. John Wiley. New York, N.Y. 1944. pp. 367-414.

Pollock, G. E. and V. I. Oyama. 1966. Resolution and separation of racemic amino acids by gas chromatography and the application to protein analysis. J. Gas Chrom. 4:126-131.

PART II: RESOLUTION OF AMINO ACID DIASTEREOMERS BY MEANS OF PACKED COLUMN GAS CHROMATOGRAPHY

INTRODUCTION

Almost simultaneously with the advent of the use of gas-liquid chromatography (GLC) for amino acid analysis, interest was generated for using the same technique to resolve the optical isomers of amino acids. Much of this interest was attributable to the possibility of using the technique to study optical isomerism in extraterrestrial life probes currently being planned by the United States National Aeronautics and Space Administration, but other interest has been shown in the areas of geochemistry, bacteriology and peptide synthesis.

To date most research in this area has been with capillary columns. While the results obtained with capillary columns often are quite good, the corresponding packed column technology would be beneficial in laboratories containing instrumentation not equipped for capillary columns. In some instances packed columns could also be used to resolve larger amounts of amino acids than could be done on capillary columns; Ayers et al. (1970) showed that amino acid diastereomers could be resolved on a preparative or semipreparative scale.

Motivated by a desire to use GLC as a technique to study optical isomerism of amino acids in insect hemolymph, this study was initiated to select an alcohol which, when esterified with the proper N-acylated amino acid derivatives, could be made to give suitable resolutions on conventionally packed GLC columns.

MATERIALS AND METHODS

GLC supplies

All GLC supplies except the liquid phases OV-210 and OV-225 were purchased from the Anspec Co., Ann Arbor, Mich. The latter two phases were purchased from Regis Chemical Co., Chicago, Ill. Chemicals Procurement Laboratories, College Point, N.Y. was the supplier of (+) 2-octanol; (-) 2-methyl-1-butanol was purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin; (+) 2-butanol was prepared by the method of Ingersoll (1944); and (+) 3,3-dimethyl-2-butanol was prepared by the method of Ayers et al. (1970).

Derivative formation

The amino acid esters were prepared by reacting 1-3 mg of the amino acid for 1 hr at 100°C with 1-1.5 ml of the appropriate alcohol which contained 1.2-1.5 mequiv. of HCl per ml. The alcohol was evaporated from the reaction mixture and the residue was dissolved in 1-1.5 ml methylene chloride-trifluoroacetic anhydride (4:1; V:V) and allowed to stand 15-20 min. at room temperature. This solution was then used for GLC analysis. In this manner the trifluoroacetyl derivatives of the 2-butyl, 2-pentyl, 2-octyl, 3-octyl, 4-octyl, 2-decyl, 3,3-dimethyl-2-butyl, 3-methyl-2-butyl, 2-methyl-1-butyl and 1,1,1-trifluoro-2-propyl esters of the various protein amino acids (except arginine,

histidine and cystine) were prepared. These last three amino acids appear from our own studies as well as those of others (Gehrke et al., 1968) to be special cases and no attempt was made to work with them.

The 2-butyl and 2-octyl, N-acetyl, N-propyl, and N-trichloroacetyl derivatives of alanine, valine and phenyalanine were prepared in a similar manner except that the appropriate acid chlorides were used to form the amides.

Column packings

Column packings were prepared by weighing 15.0 g of solid support into a 300 ml round-bottom flask and covering it with a suitable solvent containing the liquid phase. The solvent was evaporated on a slowly turning rotating evaporator at ca. 60°C until no more liquid was visible. During the next 15-30 min the support was maintained at the same temperature on the evaporator, and periodically rotated to expose a new surface to the vacuum. Final drying was accomplished in a shallow pan in an oven at 30-40°C.

The packings were loaded and compacted with the aid of a vacuum pump and vibrator into 4 mm ID all-glass columns having all-glass injector ports as described elsewhere (Ayers et al., 1970).

Instrumentation

Two Model 600 Series Research Specialities gas chromatographs equipped with dual hydrogen flame detectors were used during the study. The carrier gas (either nitrogen or helium) flow rate was kept at 90 ml/min. Since both resolution and peak sharpness are dependent on retention time, the chromatograph oven temperature was adjusted to

make retention times (injection to center between peaks) fall within a short time interval. In this manner it was possible to compare the abilities of several columns to resolve a given diastereomeric pair. Selected derivatives from all classes prepared were chromatographed on the following columns:

1. 6 m 1 1/2% cyclohexanedimethanol adipate on 60/80 AW Chromosorb W.
2. 3 m 10% OV-17 (phenylmethyl silicone) on 100/120 DMCS AW Chromosorb W.
3. 3 m 10% OV-1 (methyl silicone) on 100/120 DMCS AW Chromosorb W.

The 3,3-dimethyl-2-butyl-N-trifluoroacetyl derivatives were further tested on the following columns:

1. 3 m 5% carbowax 20 M on 100/120 DMCS AW Chromosorb W.
2. 3 m 10% GE-XE-60 (cyanoethylmethyl and dimethyl silicone) on 100/120 DMCS AW Chromosorb W.
3. 3 m 5% 1,2,3,4-tetrakis-(2-cyanoethoxy)-butane on 100/120 DMCS AW Chromosorb W.
4. 3 m 5% 1,2,3-tris-(2-cyanoethoxy)-propane on 100/120 DMCS AW Chromosorb W.
5. 3 m 10% OV-225 (phenylmethylcyanopropyl silicone) on 100/120 DMCS AW Chromosorb W.
6. 3 m 10% OV-210 (methyltrifluoropropyl silicone) on 100/120 DMCS AW Chromosorb W.
7. 3 m 5% cyclohexanedimethanol adipate on 100/120 DMCS AW Chromosorb W.

8. 6 m 1 1/2% tetramethylcyclobutanediol adipate on 60/80 AW Chromosorb W.

In addition the 2-octyl and 3,3-dimethyl-2-butyl-N-trifluoroacetyl esters were tested on a 3 m 1% carbowax 20 M on 100/120 DMCS AW Chromosorb W. column. In instances where the resolution of a particular diastereomer was in question, the problem was resolved either by mass spectroscopy and/or by preparing the derivative from resolved alcohols.

RESULTS

The degree of resolution obtained with the N-trifluoroacetyl derivatives was as good as and generally better than that obtained with the N-acetyl, N-trichloroacetyl or N-propyl derivatives. Less peak tailing usually occurred with these derivatives and the retention temperatures were generally lower than for the other derivatives. Because of their superiority the remainder of this paper concerns only N-trifluoroacetyl derivatives.

The effect of the alcohol's structure upon the degree of resolution attainable with the different diastereomeric esters is quite pronounced. No resolution was achieved with 2-methyl-1-butyl esters. In the alkyl-2-ol series, 2-butyl through 2-decyl, the degree of resolution attainable increased with the size of the alcohol. In the larger alcohols of this series, when the hydroxy group occupied a more median position on the alkyl chain the degree of resolution decreased markedly (Table 1). The difference between 2-decanol and 3-decanol was similar to that found between 2-octanol and 3-octanol.

The alcohols, 3-methyl-2-butanol and 3,3-dimethyl-2-butanol, were found to be significantly superior to any of the alochols tested. Of these two alcohols, 3,3-dimethyl-2-butanol was the best as can be seen from Table 2. While complete resolution of only one or two amino acids and partial resolution of several more were obtainable with the

Table 1. Resolution of octyl-N-trifluroracetyl esters of alanine and valine on two columns.[a,b]

	Derivative					
	2-octyl		3-octyl		4-octyl	
Column	Alanine	Valine	Alanine	Valine	Alanine	Valine
1	1.14 (75.8)	0.80 (44.3)	c (c)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)
2	1.06 (79.9)	0.83 (41.7)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)

1 = 3 m 10% OV-17 on 100/120 DMCS AW Chromosorb W.

2 = 6 m 1 1/2% cyclohexanedimethanol adipate on 60/80 AW Chromosorb W.

[a]Retention times between 15-20 min. at a flow rate of 90 ml/min.

[b]Resolutions: Calculated by the method of Burchfield and Storrs (1962), and (in parentheses) by the method of Kaiser (1963).

[c]Shoulder

Table 2. Comparison of resolutions achieved with 3-methyl-2-butyl and 3,3-dimethyl-2-butyl-N-trifluoroacetyl derivatives of three amino acids on two columns.[a,b]

	Column and Derivative			
	3 m 10% OV-17[c]		3 m 5% GE XE-60[c]	
Amino acid	3-methyl-2-butyl	3,3-dimethyl-2-butyl	3-methyl-2-butyl	3,3-dimethyl-2-butyl
Alanine	1.42	2.10	0.94	1.41
	(85.0)	(95.3)	(50.7)	(91.6)
Valine	1.29	1.64	1.30	1.95
	(80.2)	(95.0)	(83.9)	(98.9)
Phenylalanine	0.74	1.20	d	1.33
	(13.9)	(73.1)	(d)	(72.5)

[a]Retention times 20 ±2 min. at a flow rate of 90 ml/min.

[b]Resolutions: Calculated by the method of Burchfield and Storrs (1962), and (in parentheses) by the method of Kaiser (1962).

[c]Packed on 100/120 DMCS acid washed Chromosorb W.

[d]Shoulder

larger 2-alkyl derivatives, resolutions of 93% or better could be obtained with 3,3-dimethyl-2-butyl derivatives for all the amino acids tested except aspartic acid and proline. Resolutions of about 70% and 82%, respectively, were obtained for these amino acids. The notable exception to this general situation occurred with proline. The larger straight-chained 2-alkyl derivatives gave resolutions superior to that of the 3,3-dimethyl-2-butyl derivatives.

The 1,1,1-trifluoro-2-propyl-N-trifluoroacetyl derivatives of alanine, valine, and phenylalanine gave excellent resolutions but esterification was found to be extremely difficult to achieve and 1,1,1-trifluoro-2-propanol was not tested further.

While the straight-chained alkyl derivatives gave essentially equal areas for the two peaks of a given diastereomeric pair, the L+ and D- peaks were greater in area than the L- and D+ pairs for 3,3-dimethyl-2-butyl derivatives. The ratio of the smaller peak to the larger peak varied from amino acid to amino acid and ranged from ca. 0.303 to 0.714. The differences of the peak areas was found to be slightly less for the 3-methyl-2-butyl derivatives than for the 3,3-dimethyl-2-butyl derivatives. Proline formed the exception to the general situation with both peaks of a given diastereomeric pair being essentially equal in area.

The liquid phases 1,2,3,4-tetrakis-(2-cyanoethoxy)-butane, 1,2,3-tris-(2-cyanoethoxy)-propane and OV-210 were generally found to be unsuitable for the resolution of the various derivatives because of lack of resolving power and/or thermal stability. The two phases tetramethylcyclobutanediol adipate and OV-1 gave at least partial

resolution of some of the derivatives but were judged inferior to the remainder of the phases tested.

Table 3 contains data pertinent to the resolution achieved by the 3,3-dimethyl-2-butyl-N-trifluoroacetyl derivatives on the remainder of the phases. When retention times were kept to between 15 and 20 min the degree of resolution was not markedly affected by the percent liquid phase on the solid support within a range of 1-5% for the non-silicone phases (1-10% for the silicone phases). The resolutions tended to be slightly better, however, at the higher phase concentrations.

Often the temperature needed to achieve a given retention time was considerably less at the lower phase levels (Table 4). At times the degree of degradation increased significantly with the higher liquid phase concentrations. This was especially significant for the di-trifluoroacetyl derivatives of the hydroxy and sulfhydryl amino acids on polar columns.

Generally slightly better resolutions were obtained when helium instead of nitrogen was used as the carrier gas (see Table 3).

The retention times and degree of resolution were found to be dependent upon several factors such as the tightness of packing and history of the column. The resolutions achieved on the 10% OV-17 column improved considerably with age (data in Table 3 are from an old OV-17 column with many hours of use). The carbowax columns tended to have a short useful life but this was only very pronounced for selected amino acids, most notably aspartic acid. The resolution of aspartic acid deteriorated in many cases after only about a weeks use, even though the column temperature was generally kept well under 180°C.

Table 3. Resolution of 3,3-dimethyl-2-butyl-N-trifluoroacetyl amino acid derivatives on several columns.

	Column and Carrier Gas						
Amino acid	1 N_2	2 N_2	3 N_2	4 N_2	5 N_2	6 N_2	6 He
Alanine	1.82 (95.5)	1.69 (96.8)	1.61 (96.6)	1.48 (89.3)	1.75 (93.3)	3.39 (98.3)	3.50 (94.1)
Valine	1.39 (83.6)	1.73 (97.0)	2.00 (99.5)	1.57 (94.0)	1.70 (88.3)	1.91 (93.1)	2.23 (97.5)
Leucine	1.58 (91.1)	1.79 (90.8)	1.93 (98.3)	1.70 (92.0)	1.64 (94.2)	2.85 (98.1)	3.11 (98.5)
Isoleucine	1.43 (92.3)	1.88 (90.1)	2.21 (98.6)	1.92 (98.6)	1.94 (99.4)	2.50 (93.9)	2.78 (96.6)
Threonine	0.76 (40.0)	1.00 (64.0)	1.50 (93.3)	1.60 (90.8)	0.62 (29.2)	1.35 (92.3)	2.26 (95.9)
Proline	e (e)	0.96 (55.6)	1.03 (61.8)	0.62 (36.7)	0.36 (4.5)	1.09 (80.5)	1.07 (82.1)
Serine	2.00 (99.9)	1.16 (73.5)	1.81 (97.3)	1.65 (95.4)	1.39 (89.8)	2.56 (97.6)	2.97 (99.1)
Cysteine	1.93 (99.9)	1.60 (95.5)	2.53 (95.8)	2.35 (94.6)	2.05 (95.0)	d	d
Methionine	0.00 (0.0)	0.50 (26.7)	1.48 (87.1)	1.42 (85.8)	1.39 (84.4)	2.38 (97.7)	2.60 (98.4)
Aspartic acid	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	e (e)	0.00 (0.0)	0.92 (62.5)	1.04 (69.8)
Phenylalanine	0.88 (61.5)	1.06 (60.6)	1.86 (99.6)	1.70 (97.0)	1.83 (97.0)	2.44 (95.8)	2.67 (99.3)
Glutamic acid	0.90 (51.0)	0.91 (51.5)	b	1.17 (92.5)	1.41 (84.1)	1.77 (94.7)	1.67 (95.5)
Tyrosine	1.02 (74.0)	1.03 (62.4)	b	c	1.78 (92.6)	2.23 (93.5)	2.84 (96.7)
Lysine	0.54 (27.3)	0.36 (15.8)	b	1.04 (77.7)	1.24 (62.8)	1.58 (96.4)	1.73 (93.5)
Tryptophan	0.89 (60.8)	1.05 (70.0)	b	1.65 (91.6)	1.72 (93.4)	2.32 (97.8)	2.24 (97.5)

1. 3 m 10% OV-17 on 100/120 DMCS AW Chromosorb W.
2. 3 m 10% OV-225 on 100/120 DMCS AW Chromosorb W.
3. 3 m 10% GE-XE-60 on 100/120 DMCS AW Chromosorb W.
4. 6 m 1 1/2% Cyclohexanedimethanol adipate on 60/80 AW Chromosorb W.
5. 3 m 5% Cyclohexanedimethanol adipate on 100/120 DMCS AW Chromosorb W.
6. 3 m 1% Carbowax 20M on 100/120 DMCS AW Chromosorb W.

[a]Retention times between 15-20 min with a carrier flow rate of 90 ml/min. Resolutions: Calculated by the method of Burchfield and Storrs (1962), and (in parentheses) by the method of Kaiser (1963).

[b]Not found; excessive column bleed at these temperatures.

[c]Apparently totally degraded on column.

[d]Multiple peaks apparently due to degradation on column.

[e]Shoulder.

Table 4. Comparison of the retention temperatures of the 3,3-dimethyl-2-butyl-N-trifluoroacetyl and di-trifluoroacetyl derivatives of several amino acids on 1% and 5% carbowax 20 M columns.[a,b]

	Retention Temperature (°C)	
Amino acid	1%	5%
Threonine	74	100
Serine	95	125
Aspartic acid	128	167
Tyrosine	148	177

[a]Packed on 100/120 DMCS acid washed Chromosorb W.

[b]Retention times 20 ± 1 min. at a flow rate of 90 ml/min.

DISCUSSION

The degree of resolution attainable by an N-trifluoroacetyl amino acid ester appears to be dependent not only upon closeness of the two asymmetric centers and the size of the alcohol as reported by several authors (Cross et al., 1970 and Rose et al., 1966), but also upon the similarity of the three non-hydroxy groups on the alcohol's asymmetric carbon. This was alluded to by the esters of octanol and decanol with the hydroxy position more median than the 2-position. For example in 3-octanol the largest group off of the 2-carbon (pentyl group) would be considerably larger than the largest group off of the 2-position in either 2-butanol or 2-pentanol (ethyl and propyl groups, respectively) yet the latter two alcohols achieved better resolutions than the larger 3-octanol. The superiority of 3-methyl-2-butanol, 3,3-dimethyl-2-butanol and 1,1,1-trifluoro-2-propanol tend to support this supposition; however, these alcohols when considered alone would not discredit the hypothesis that their superiority is based upon bulk alone.

Relevant to the work reported for capillary columns, relatively non-polar phases such as OV-17 achieve surprisingly good resolutions for the various 3,3-dimethyl-2-butyl-N-trifluoroacetyl derivatives. Such phases should find general utility in resolving the di-trifluoroacetyl derivatives of serine, threonine, cysteine and tyrosine. This

would be an advantage over the additional steps suggested by Pollock et al. (1968) to circumvent degradation problems encountered when trying to resolve them on polar columns. Phases of this type may also prove useful for the resolution of arginine, histidine and cystine since such phases are needed for chromatographic studies of the n-butyl-N-trifluoroacetyl derivatives of these amino acids (Gehrke et al., 1968).

Often it was found that the di-trifluoroacetyl derivatives (with the probable exception of the cysteine derivative which apparently underwent serious degradation) could be resolved on polar phases. While there is undoubtedly some deterioration during chromatography, it is of such an order on low liquid phase packings that analysis in the µg range could conveniently be carried out on columns containing these packings. This lessened deterioration on columns of low phase concentrations may be a result of the lower retention temperatures needed on these columns. Column supports advertised to work best at low phase concentrations might prove very useful in solving this problem. It was noticed on several occasions using a 5% carbowax 20 M column and successive analyses of one of the di-trifluoroacetyl derivatives that the peak areas for a given size injection increased from one injection to the next, and eventually reached an area equal to that given by the same sized injection on a 1% carbowax 20 M column. The phenomenon appeared to be at least partly reversible if the column was not used for a period of several days. No explanation is offered for this pecularity.

In no instance was any one column capable of separating all the amino acids while at the same time resolving the optical isomers. One

or more overlaps occurred on each column. The problem could often be circumvented by using several columns since the overlap pattern is often different on different columns.

For quantitative purposes 3,3-dimethyl-2-butyl derivatives often would not be as convenient as esters having identical formation rates for each member of a given diastereomeric pair. However, if an investigator were working with traces of one amino acid isomer in much larger quantities of the other isomer, advantage could be taken of the dissimilar formation rates by choosing the proper optical form of the alcohol utilized in the esterification.

REFERENCES

Ayers, G. S., J. H. Mossholder and R. E. Monroe. 1970. Preparative gas chromatographic resolution of 3,3-dimethyl-2-butanol and other alcohols. J. Chromatog. 51:407-414.

Burchfield, H. P. and E. E. Storrs. Biochemical Applications of Gas Chromatography. Academic Press. New York, N.Y. 1962. p. 16.

Cross, J. M., B. F. Patney and J. Bernstein. 1970. Separation of diastereoisomers of 2-alkanols by glc: a cis-trans viewpoint. J. Chrom. Sci. 8:679-681.

Gehrke, C. W., R. Roach, R. W. Zumwalt, D. L. Stalling and L. L. Wall. Quantitative Gas-Liquid Chromatography of Amino Acids in Proteins and Biological Substances. Analytical Biochemical Labs. Colombia, Mo., 1968. 108 pp.

Ingersoll, A. W. Organic Reactions. Vol. II. John Wiley. New York, N.Y. 1944. pp. 376-414.

Kaiser, R. Gas Phase Chromatography. Vol. I. Butterworths, Washington, D.C. 1963. p. 39.

Pollock, G. E., and A. H. Kawauchi. 1968. Resolution of aspartic acid, tryptophan, hydroxy and sulfhydryl amino acids by gas chromatography. Anal. Chem. 40:1356-1358.

Rose, R. C., R. L. Stern and B. L. Karger. 1966. Studies on the mechanism of separation of diastereomeric esters by gas-liquid chromatography. Effect of bulk dissymmetry, and distance between optical centers. Anal. Chem. 38:469-472.

PART III: A SEARCH FOR FREE D-AMINO ACIDS IN PUPAE OF THE HOUSE FLY, *MUSCA DOMESTICA* L.

INTRODUCTION

It is well known that the great majority of amino acids found in nature are of the L-configuration. As an exception to this generalization, D-amino acids are commonly found associated with various microbes, where they are often formulated into growth inhibitors. Scattered reports have claimed the occurrence of D-amino acids within the animal kingdom, including insects, (Corrigan, 1969); however, to our knowledge none has yet been reported in the order Diptera. This paper reports on a search for D-amino acids in the pupae of house fly, Musca domestica L., using recently developed gas-liquid chromatographic techniques (Ayers et al., 1971).

MATERIALS AND METHODS

House fly larvae were reared aseptically according to the method of Monroe (1962). When a majority of the larvae had reached the migratory stage, the larvae were removed from their rearing containers and allowed to pupate in a shallow pan. Newly formed pupae were removed every 3 hrs and allowed to age so that they would fall into 3 different age groups (0-3 hrs, 48-51 hrs and 96-99 hrs). They were then frozen and stored.

Pupae (33 from each age group) were combined and homogenized with ca. 20 ml of water in a Tenbroeck homogenizer. A volume of ethanol equal to the volume of the aqueous phase was added to the homogenate which was then stirred and centrifuged. The protein thus precipitated was extracted an additional 3 times with a mixture of ethanol-water (1:1). The extracts were pooled and the ethanol removed by means of a rotating evaporator. Lipids were removed by extracting the pooled aqueous fraction twice with ether. The remaining fraction was concentrated and streaked onto two 28.5 X 40 cm Whatman No. 4 paper chromatograms. Both sides of the chromatogram were spotted with the appropriate ^{14}C-labeled amino acid(s). The chromatograms were developed in the top phase of a butanol-acetic acid-water mixture (25:6:25). The location of the ^{14}C-amino acids was determined by autoradiography and

the different amino acid portions of the chromatograms were extracted with water acidified with HCl. After evaporation by means of a rotating evaporator the residues were redissolved in a small amount of water and passed individually through Dowex 50W-X8 (100/200 mesh) columns. The amino acids were eluted with a 10 percent ammonia solution and evaporated to dryness in a small test tube by means of a stream of nitrogen and heat from a chromatogram dryer. Because threonine, tryptophan, cysteine and aspartic acid still retained interfering substances when processed in this manner, the procedure was modified slightly for these amino acids. Pupae (100 from each group) were processed as previously described up to the point of paper chromatography. The chromatograms were developed in butanol-acetone-diethylamine-water (10:10:2:5), whereupon the appropriate portions were extracted and restreaked onto a second set of chromatograms which were developed in the top phase of butanol-acetic acid-water (25:6:25). The remainder of the procedure was kept the same.

The (+) 3,3-dimethyl-2-butyl esters of all the amino acids except proline were prepared by reacting the amino acid fraction with 0.1-0.2 ml of (+) 3,3-dimethyl-2-butanol (Halpern and Westley, 1966) containing ca. 3 mequiv. dry HCl/ml at 100°C for 1.5 hrs in a sealed tube. Excess alcohol was evaporated with a stream of nitrogen and the heat from a chromatogram dryer. The residues of all the amino acid esters except tryptophan were trifluoroacetylated at room temperature for 15-30 min with an excess of trifluoreacetic anhydride-methylene chloride solution (1:1). The ester of tryptophan was trifluoroacetylated at 100°C for 5 min in a sealed tube with the same trifluoroacetylation

mixture. The (+) 2-octyl-N-trifluoroacetyl derivative of proline was prepared in a similar manner using (+) 2-octanol (Chemicals Procurement Laboratories, Inc.; College Point, N.Y.) being trifluoroacetylated at room temperature. Control authentic L-amino acids (grade A Calbiochem; Los Angeles, Cal.) were carried through all extraction, clean-up, and derivitization procedures. No attempt was made to work with the amino acids arginine, histidine, or cystine. The derivatives were chromatographed on one or more of the following columns: 3 m 1% Carbowax 20M, 3 m 10% OV-17 (phenyl methyl silicone), 3 m 5% Carbowax 20 M and 3 m 5% Carbowax 1540. Packings were prepared from 100/120 DMCS treated acid washed Chromasorb W and packed into 4 mm i.d. all glass columns. A flow rate of 90 ml/min of nitrogen or helium was used during chromatography. Model 600 Series Research Specialties gas chromatographs equipped with dual hydrogen flame detectors were used during the study.

RESULTS AND DISCUSSION

Table 5 shows the pertinent chromatographic data for each amino acid studied. The relative abundances of the D-isomers from the pupae were calculated on the assumption that the control amino acids were 100 percent L-isomer. All of the control amino acids appeared of approximately equal quality and the analysis of several of these with highly optically pure alcohol showed their optical purity to be 99 percent or better. In all cases the relative abundances of the insect D-isomers were found to be no greater than 3 percent. Very small concentrations of the D-isomer are difficult to detect since the retention times of the (D)-amino acid-(+)-alcohol ester and its enantiomer are the same. The optical purities of the (+) 3,3-dimethyl-2-butanol and (+) 2-octanol were 97-99 percent and 96-98 percent, respectively.

Table 5. Gas chromatographic data of amino acids from house fly pupae.

	Column & Gas[a]	Temp (°C)	Retention Time (min) (L-Isomer)	Resolution[b]
Alanine	4	98	12.9	2.00 (98.1)
Aspartic Acid	3	165	31.7	1.09 (81.3)
Cysteine	2	150	15.5	1.43 (94.1)
Glutamic Acid	1	152	18.2	1.47 (93.5)
Isoleucine	1	100	14.3	1.60 (91.0)
Leucine	1	100	21.6	2.00 (94.6)
Lysine	1	178	18.4	1.62 (96.4)
Methionine	1	152	8.6	1.57 (95.7)
Phenylalanine	1	150	16.5	1.52 (90.2)
Proline	1	135	17.2	1.11 (82.7)
Serine	1	101	16.4	1.72 (94.3)
Threonine	1	80	14.8	1.74 (96.4)
Tryptophan	1	180	17.5	1.54 (87.4)
Tyrosine	1	154	10.8	1.40 (83.1)
Valine	1	78	13.9	1.90 (97.8)

[a]Columns and Gas: (1) 3m 1% Carbowax 20M-nitrogen (2) 3m 10% OV-17-nitrogen (3) 3m 5% Carbowax 1540-helium (4) 3m 5% Carbowax 20M-nitrogen. All derivatives (+) 3,3-dimethyl-2-butyl-N-trifluoroacetyl derivatives except proline which is the corresponding (+) 2-octyl derivative.

[b]Resolutions calculated by method of Burchfield and Storrs (1962) and (in parentheses) by the method of Kaiser (1963).

REFERENCES

Ayers, G. S., R. E. Monroe and J. H. Mossholder. 1971. Resolution of amino acid diastereomers by means of packed column gas chromatography. J. Chromatog. 63:259-265.

Burchfield, H. P. and E. E. Storrs. Biomedical Applications of Gas Chromatography. Academic Press. New York, N.Y. 1962. p. 16.

Corrigan, J. J. 1969. D-amino acids in animals. Science 164:142-149.

Halpern, B. and J. W. Westley. 1966. Chemical resolution of secondary (·)-alcohols. Aust. J. Chem. 19:1533-1534.

Kaiser, R. Gas Phase Chromatography. Vol. I. Butterworths. Washington, D.C. 1963. p. 39.

Monroe, R. E. 1962. A method for rearing house fly larvae aseptically on a synthetic medium. Ann. Entomol. Soc. Am. 55:140.

APPENDIX

LITERATURE REVIEW

Because of its potential sensitivity, accuracy, and speed, the possibility of gas chromatography of amino acids has received interest for some time. More recently, additional interest has been generated by the fact that during gas chromatography, an amino acid whose identity is unknown, may be directly subjected to mass spectrometry.

Gehrke and Stalling (1967) reviewed the amino acid derivatives which were tried in early attempts at amino acid gas chromatography. The main disadvantage of these early derivatives lay in the fact that no single derivatizing process rendered all the protein amino acids gas chromatographable. Gehrke et al. (1965) introduced the n-butyl-N-trifluoroacetyl (TFA) derivative by which all protein amino acids could be gas chromatographed. Since 1965, Gehrke and coworkers have made many improvements in their original methods, many of which can be found in a single reference (Gehrke et al., 1971).

Different researchers have tried various combinations of N-acyl-amino acid-alkyl esters since Gehrke's review of the early derivative literature. The same problems pointed out by Gehrke in 1968 have, for the most part, not been resolved. The N-acetyl and higher N-acyl amino acid esters demonstrate serious chromatographic problems associated with arginine, histidine, and cystine. In the N-TFA-amino acid ester series, serious quantitative difficulties arise with the lower molecular

weight esters (especially methyl), due to their high volatility, while chromatographic problems result from the low volatility of the high molecular weight esters. The higher N-perfluoroacyl amino acid ester derivatives, however, appear very promising. Moss et al. (1971) separated 20 protein amino acids as the N-heptafluoro-butyryl-n-propyl derivatives on a single column.

The possibility of gas chromatographing amino acids as the trimethylsilyl (TMS) derivatives is an appealing approach to the subject since only a single derivatizing step is required (Smith et al., 1969, Stalling et al., 1968; Gehrke et al., 1969; Gehrke and Leimer, 1970, and Gehrke and Leimer, 1971). While all the protein amino acids could be chromatographed as TMS derivatives, several amino acids, depending on the derivatizing conditions, were subject to multiple peaks. The TMS derivatives were also quite labile to hydrolysis.

Because of its applicability to the Edman sequential degradation scheme, the gas chromatography of amino acids as their phenylthiohydantoins has received some interest. While some phenylthiohydantoins could be chromatographed directly, others must be derivatized further. The additional derivatization procedures tried have been silylation (Pisano and Bronzert, 1969), and trifluoroacetylation (Roda and Zamorani, 1970). With silylation, all of the protein amino acids except arginine could be gas chromatographed as the phenylthiohydantoins. Tschesche et al. (1970) claimed all of the protein amino acids except arginine and histidine could be gas chromatographed as the methylthiohydantoins and that after silylation histidine could be gas chromatographed.

Several attempts have been made to find gas chromatographable derivatives which would be more stable than either the N-TFA or TMS derivatives. Davis and Furst (1968) reported on several N-alkyl and N-alkylidine amino acid methyl esters, and Pettitt and Stouffer (1970) reported on N-isopropyl amino acid isopropyl esters. Arginine could not be chromatographed in either study.

Almost simultaneously with the advent of the use of gas chromatography for amino acid analysis, interest was generated for using the same technique for the resolution of amino acid optical isomers. Two techniques were developed to achieve this goal. One technique relied on forming suitable derivatives with non-optically active reagents and resolving the enantiomers on optically active phases, while the other technique relies on forming suitable diastereomers with optically active reagents and resolving these on non-optically active liquid phases. Both methods have relied almost exclusively upon capillary columns.

The only optically active phases found to be successful in resolving amino acid enantiomers have been derivatives of amino acids or peptides. Feibush and Gil-Av (1967) showed that primary amines could be resolved on the ureide of L-valine isopropyl ester. Corbin and Rogers (1970a) found that the ureide worked best in one of its solid states and extended its application to the resolution of several amino acids (Corbin and Rogers, 1970b). Gil-Av et al. (1966) successfully resolved several N-TFA amino acid ester derivatives on N-TFA-L-isoleucine lauryl ester. Gil-Av and Feibush (1967) introduced N-TFA-L-valyl-L-valine cyclohexyl ester which gave better resolutions than the

non-peptide phases. Nakaparksin et al. (1970) investigated the behavior of various N-TFA-amino acid esters on the valyl-valine phase. Feibush and Gil-Av (1970) systematically investigated the relationship between chromatographic behavior and the structures of both the N-acyl amino acid esters being chromatographed and the N-acyl-L-valyl-L-valine esters upon which they were chromatographed. In an attempt to improve upon the temperature stability of the valyl-valine phases, Koenig et al. (1970) reported on the chromatography of N-TFA amino acid isopropyl esters on N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester. Parr et al. (1970) investigated the behavior of various acyl-amino acid esters on the phenylalanyl-leucine phase. Corbin et al. (1971) found that it was apparently the amide end of the dipeptide derivatives that was responsible for effecting the major portion of the resolution achieved on these phases.

The main difficulty encountered when chromatographing amino acid enantiomers on optically active phases has been the phases' relatively high volatility. Parr et al. (1970) investigated the possibility of increasing the volatility of the amino acid derivatives by using higher N-perfluoroacyl derivatives than the TFA compounds. They found the N-pentafluoropropionyl (PFP) derivatives to have favorable volatility properties and also to give good resolutions on the peptide phases. Parr et al. (1971) were able to resolve 17 amino acid pairs as the N-PFP amino acid isopropyl esters.

Two approaches to the resolution of amino acids on non-optically active phases have been reported. In one approach the diastereomer is formed by placing the moiety containing the second asymmetric center

on the α-amino nitrogen while in the other approach, it is placed on the carbonyl atom. By using the N-(N-TFA-L-prolyl) amino acid methyl esters, Halpern and Westley (1966) were able to partially resolve six aliphatic amino acids. After silylation of the derivative and converting the methionyl derivative to the corresponding thiazolidine-4-carboxylic acid, several polyfunctional amino acids could be resolved as the N-(N-TFA-L-prolyl) peptide derivatives (Halpern and Westley, 1966). In an attempt to find more volatile derivatives, Halpern and Westley (1965) investigated the N-α-chloroisovaleryl amino acid methyl esters. Seven amino acids were resolved via this derivative. Conversely, eight amino acids could be converted to the corresponding α-chloro derivatives and then be resolved as the valine methyl ester.

The more common and rewarding approach to the resolution of amino acids as diastereomers has been by forming the diastereomers by esterfication with an alcohol containing an asymmetric carbon. Vitt et al. (1965) resolved several amino acids as the N-TFA-L-menthyl esters. Gil-Av et al. (1965) studied the chromatographic behavior of the N-TFA-2-butyl and 2-octyl esters of six amino acids. Gil-Av et al. (1965) studied the resolving properties of α-alkanoyloxypropioates of 10 2-n-alkanols and resolved 10 amino acids as either their N-TFA-2-butyl or 2-octyl esters. Pollock et al. (1965) studied the gas chromatographic behavior of 10 N-TFA-2-butyl amino acid derivatives on 12 liquid phases. Pollock and Oyama (1966) extended the use of the N-TFA-2-butyl derivatives to the resolution (at least partial) of 21 amino acids, 11 of them protein amino acids. Pollock and Kawauchi (1968) succeeded in resolving several of the polyfunctional amino acids.

Threonine, serine, and cysteine were resolved as the N-TFA O-acetyl and S-acetyl-2-butyl esters; aspartic acid as the N-TFA-di-3,3-dimethyl-2-butyl ester, and tryptophan as the N,N-di-TFA-2-butyl ester.

Several authors have made systematic changes in diastereomeric structures containing amino acids and related oxygen analogues to study the mechanism of resolution (Rose et al., 1966, Stern et al., 1969, Westley et al., 1968, Cross et al., 1970, and Feibush, 1971). There was general agreement that two of the major factors involved were distance between asymmetric centers and bulk dissymmetry.

REFERENCES

Corbin, J. A., and L. B. Rogers. 1970a. Improved gas chromatographic separation of enantiomeric secondary amine derivatives. Anal. Chem. 42:974-979.

Corbin, J. A. and L. B. Rogers. 1970b. Separations of enantiomeric derivatives of amines and amino acids by absorption chromatography. Anal. Chem. 42:1786-1789.

Corbin, J. A., J. E. Rhoad and L. B. Rogers. 1971. Effects of structure of peptide stationary phases on gas chromatographic separations of amino acid enantiomers. Anal. Chem. 43:327-331.

Cross, J. M., B. F. Putney and J. Bernstein. 1970. Separation of diastereoisomers of 2-alkanols by glc:a cis-trans viewpoint. J. Chrom. Sci. 8:679-681.

Davis, J. W. and A. Furst. 1968. Preparation of alkylidine and alkyl amino acid esters for gas chromatographic analysis of amino acids. Anal. Chem. 40:1910-1912.

Feibush, B. 1971. Interpretation and correlation of bulkiness chirality and separation coefficients in resolution of diastereoisomers by gas-liquid partition chromatography. Anal. Chem. 43:1098-1100.

Feibush, B. and E. Gil-Av. 1967. Gas chromatography with optically active stationary phases. Resolution of primary amines. J. Gas Chrom. 5:257-260.

Feibush, B. and E. Gil-Av. 1970. Interaction between asymmetric solutes and solvents. Peptide derivatives as stationary phases in gas liquid partition chromatography. Tetrahedron 26:1361-1368.

Gehrke, C. W., W. M. Lamkin, D. L. Stalling and F. Shahrokhi. 1965. Quantitative gas chromatography of amino acids. Biochem and Biophys. Res. Comm. 19:328-334.

Gehrke, C. W. and K. Leimer. 1970. Trimethylsilylation of amino acids. Effect of solvents on derivatization using bis(trimethylsilyl) trifluoroacetamide. J. Chromatog. 53:201-208.

Gehrke, C. W. and K. Leimer. 1971. Trimethylsilylation of amino acids. Derivatization and chromatography. J. Chromatog. 57:219-238.

Gehrke, C. W., H. Nakamoto and R. W. Zumwalt. 1969. Gas-liquid chromatography of protein amino acid trimethylsilyl derivatives. J. Chromatog. 45:24-51.

Gehrke, C. W., D. Roach, R. W. Zumwalt, D. L. Stalling and L. L. Wall. Quantitative Gas-Liquid Chromatography of Amino Acids in Proteins and Biological Substances. Analytical Biochemical Labs. Columbia, Mo. 1968, 108 pp.

Gehrke, C. W. and D. L. Stalling. 1967. Quantitative analysis of twenty natural protein amino acids by gas liquid chromatography. Separation Sci. 2:101-138.

Gehrke, C. W., R. W. Zumwalt and K. Kuo. 1971. Quantitative amino acid analysis by gas-liquid chromatography. J. Agr. Food Chem. 19:605-618.

Gil-Av, E., R. Charles and G. Fischer. 1965. Resolution of amino acids by gas chromatography. J. Chromatog. 17:408-410.

Gil-Av, E., R. Charles-Sigler, G. Fischer and D. Nurok. 1966. Resolution of optical isomers by gas liquid partition chromatography. J. Gas Chrom. 4:51-60.

Gil-Av, E. and B. Feibush. 1967. Resolution of enantiomers by gas liquid chromatography with optically active stationary phases. Separation on packed columns. Tetrahedron Letters 35:3345-3347.

Gil-Av, E., B. Feibush and R. Charles-Sigler. 1966. Separation of enantiomers by gas liquid chromatography with an optically active stationary phase. Tetrahedron Letters 10:1009-1015.

Halpern, B. and J. W. Westley. 1965a. High sensitivity optical resolution of DL-amino-acids by gas chromatography. Chem Comm. 12:246-247.

Halpern, B., and J. W. Westley. 1965. High sensitivity optical resolution of D,L-amino acids by gas chromatography. Biochem Biophys. Res. Comm. 19:361-363.

Halpern, B. and J. W. Westley. 1966. High sensitivity optical resolution of poly-functional amino acids by gas liquid chromatography. Tetrahedron Letters. 21:2283-2286.

Koenig, W. A., W. Parr, H. A. Lichtenstein, E. Bayer and J. Oro. 1970. Gas chromatographic separation of amino acids and their enantiomers: Non-polar stationary phases and a new optically active phase. J. Chrom Sci. 8:183-186.

Moss, C. W., M. A. Lambert and F. J. Diaz. 1971. Gas-liquid chromatography of twenty protein amino acids on a single column. J. Chromatog. 60:134-136.

Nakaparksin, S., P. Birrell, E. Gil-Av and J. Oro. 1970. Gas chromatography with optically active stationary phases: Resolution of amino acids. J. Chrom. Sci. 8:177-182.

Parr, W., J. Pleterske, C. Yang and E. Bayer. 1971. Resolution of racemic amino acids by gas chromatography on optically active stationary phases. J. Chrom. Sci. 9:141-147.

Parr, W., C. Yang, E. Bayer and E. Gil-Av. 1970. Interaction between asymmetric solutes and solvents: N-trifluoroacetyl-L-phenylalanyl-L-leucine cyclohexyl ester as solvent. J. Chrom. Sci. 8:591-595.

Parr, W., C. Yang, J. Pleterski and E. Bayer. 1970. Rapid gas chromatographic separation of amino acid enantiomers using N-perfluoroacyl esters. J. Chromatog. 50:510-512.

Pettitt, B. C. and J. E. Stouffer. 1970. A new approach to the gc analysis of amino acids. J. Chrom. Sci. 8:735-737.

Pisano, J. J. and T. J. Bronzert. 1969. Analysis of amino acid phenylthiohydantoins by gas chromatography. J. Biol. Chem. 244:5597-5608.

Pollock, G. E. and A. H. Kawauchi. 1968. Resolution of aspartic acid, tryptophan, hydroxy and sulfhydryl amino acids by gas chromatography. Anal. Chem. 40:1356-1358.

Pollock, G. E. and V. E. Oyama. 1966. Resolution and separation of racemic amino acids by gas chromatography and the application to protein analysis. J. Gas Chrom. 4:126-131.

Pollock, G. E., V. I. Oyama and R. D. Johnson. 1965. Resolution of racemic amino acids by gas chromatography. J. Gas. Chrom. 3:174-176.

Roda, G. and A. Zamorani. 1970. Gas chromatographic analysis of amino acids as trifluoroacetylated phenylthiohydantoins. J. Chromatog. 46:315-316.

Rose, R. C., R. L. Stern and B. L. Karger. 1966. Studies on the mechanism of separation of diastereomeric esters by gas-liquid chromatography. Effect of bulk dissymmetry and distance between optical centers. Anal. Chem. 38:469-472.

Smith, E. D. and K. L. Shewbart. 1969. A quantitative comparison of trimethylsilylating reagents for protein amino acids. J. Chrom. Sci. 7:704-707.

Stalling, D. L., C. W. Gherke and R. W. Zumwalt. 1968. A new silylation reagent for amino acids. Bis(trimethylsilyl) trifluoroacetamide (BSTFA). Biochem and Biophys Res. Comm. 31:616-622.

Stern, B. L., B. L. Karger, W. J. Keane and H. C. Rose. 1969. Studies on the gas-liquid chromatographic separation of diastereo-isomeric esters. J. Chromatog. 39:17-32.

Tschesche, H., R. Obermeier and S. Kupfer. 1970. Gas chromatographic identification of thiohydantoins of degradation products of peptides and proteins. Agnew. Chem. Internat. Edit. 9:893-894.

Vitt, S. V., B. Saporovskaya, I. P. Gudkova and V. M. Belikov. 1965. The termination of an optical purity by v.p.c. Tetrahedron Letters. 30:2575-2580.

Westley, J. W., B. Halpern and B. L. Karger. 1968. Effect of solute structure on separation of diastereoisomeric esters and amides by gas-liquid chromatography. Anal. Chem. 40:2046-2049.