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N-TRIFLUOROACETYL-L-NORVALYL-L-NORVALINE CYCLOHEXYL ESTER AS A STATIONARY PHASE AND ITS INTERACTION WITH ASYMMETRIC SOLUTES

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SUMMARY

N-Trifluoroacetyl-L-norvalyl-L-norvaline* cyclohexyl ester has been synthesized in high yield and used as a stationary phase for the gas chromatographic enantiomeric separation of N-trifluoroacetyl-D,L-amino acid isopropyl esters. Separation factors and enthalpies of solution have shown a marked increase on solute–solvent interaction to occur for α-amino acids with a four-carbon group on the asymmetric carbon, while the same factors decrease as the substituent grows from one- to three-carbon.

Mixtures of D,L-serine and D,L-leucine can now be resolved. Earlier phases have shown significant overlap between D isomers of leucine and serine as well as their L isomers, thereby precluding an accurate qualitative analysis of naturally occurring volatile amino acid derivatives.

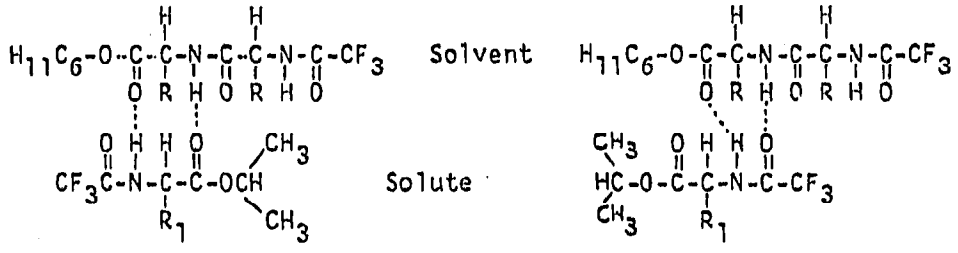
The phase has also been examined with respect to the separation of a mixture of common amino acids and their enantiomers and was found to be satisfactory.

INTRODUCTION

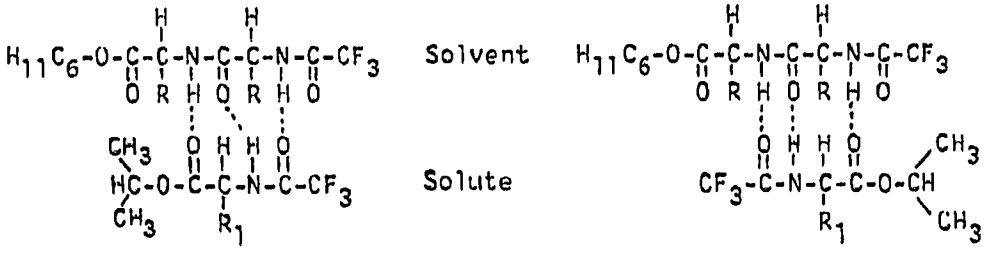
Resolution of the naturally occurring protein amino acid enantiomers by gas–liquid chromatography using capillary columns coated with optically active stationary phases was first introduced by FEIBUSH AND GIL-AV[1]. Thus far, successful experiments at enantiomeric resolution of amino acids have been conducted using N-trifluoroacetyl (TFA)-L-valyl-L-valine cyclohexyl ester[1–3], N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester[4–7], and N-TFA-L-valyl-L-leucine cyclohexyl ester[8] as stationary phases. Recently, other stationary phases based on dipeptides have been synthesized and evaluated[9]. The separation efficiency of the dipeptide phases has been explained[1] by interaction between the asymmetric solutes and solvents through formation of hydrogen-bonded diastereoisomeric association complexes A and B.

* D,L-Norvaline = D,L-α-aminovaleric acid.

Complex A



Complex B



(R and R_1 are alkyl radicals) [1]

PARR AND GROHMANN[10] found that complex B (amide portion of the peptide) rather than complex A (ester portion of the peptide) is involved in the interaction between the chiral solvent and solute, as suggested earlier. The separation is clearly a function of the size of groups R and R_1 whenever the formation of the diastereoisomeric complex occurs. It is the purpose of this study to investigate the interaction between a chiral solvent, N-TFA-L-norvalyl-L-norvaline* cyclohexyl ester, and solutes of different chain lengths and different degrees of branching in the substituent R_1. This phase was selected because according to our previous results norvaline shows higher resolution factors than valine since R is a *n*-propyl group rather than an isopropyl group, which might introduce a certain steric hindrance in the phase[11]. Furthermore this new dipeptide phase should be evaluated for the separation of the naturally occurring protein amino acids.

EXPERIMENTAL

Synthesis of N-TFA-L-norvalyl-L-norvaline cyclohexyl ester

L-Norvaline cyclohexyl ester hydrogen chloride. 5 g of L-norvaline were suspended in 150 ml of freshly distilled cyclohexanol. Anhydrous HCl was bubbled through the mixture until a clear solution was obtained. The solution was then heated to 100° for 2 h and the introduction of HCl gas was discontinued. After 12 h at room temperature, excess HCl was removed with the aid of a rotary evaporator and finally cyclohexanol was removed *in vacuo*. The oily residue was triturated with petroleum ether until a solid was obtained. The precipitate was filtered off and dried *in vacuo* over KOH. Yield: 9.0 g (95%), m.p. 134–136°.

The norvaline cyclohexyl ester hydrochloride was found to be homogeneous by

thin-layer chromatography on Eastman Sheets 6061 silica gel (R_F 0.46 in acetone–acetic acid (95:5), Solvent system I; R_F 0.60 in *n*-butanol–water–ethanol–acetic acid (8:3:2:1), Solvent system II).

N-tert.-Butyloxycarbonyl (BOC)-L-norvalyl-L-norvaline cyclohexyl ester. To a solution of 2.17 g (9 mmoles) BOC-L-norvaline in 30 ml of absolute tetrahydrofuran (THF) 2.1 g (9 mmoles) of L-norvaline cyclohexyl ester hydrochloride, 1.35 g (10 mmoles) of hydroxybenzotriazole and 1.1 ml (9 mmoles) of N-ethyl morpholine were added and the mixture was cooled to 0°. A solution of 1.85 g (9 mmoles) of dicyclohexylcarbodiimide in 15 ml of ice-cold THF was added and the mixture was stirred for 1 h at 0° and for an additional hour at room temperature. The precipitate formed was filtered off, and the solvent was evaporated *in vacuo*. After addition of 250 ml of ethyl acetate the organic layer was washed once with a saturated $NaHCO_3$ solution, once with 2 *N* citric acid, again with saturated $NaHCO_3$ and then twice with water. The organic phase was dried over Na_2SO_4, and after evaporation of the solvent a clear oil was obtained, which was not further purified. Yield: 3.0 g (82%).

L-Norvalyl-L-norvaline cyclohexyl ester trifluoroacetate. The above oil was treated for 1 h with a mixture of 10 ml of methylene chloride and trifluoroacetic acid at room temperature in order to remove the BOC group. Excess methylene chloride and trifluoroacetic acid were removed *in vacuo*. Addition of anhydrous ether gave a white solid, which was filtered off and washed with ether and petroleum ether. Yield: 2.5 g (80.5%), m.p. 196–198°.

The trifluoroacetate showed a single spot when chromatographed in solvent system II with R_F 0.68.

N-TFA-L-Norvalyl-L-norvaline cyclohexyl ester. L-Norvalyl-L-norvaline cyclohexyl ester trifluoroacetate was suspended in 40 ml of methylene chloride and after cooling in an acetone–dry ice bath, 10 ml of trifluoroacetic acid anhydride were added, the solution was allowed to warm to room temperature and was shaken for another hour. The solvent and excess reagent were removed *in vacuo*, leaving a slightly yellow oily residue. The oil was dissolved in 95% ethanol, and water was added until the solution became cloudy. After standing for 12 h at 5° more water was added and the precipitate was filtered off and dried *in vacuo*. Yield: 2.3 g (95.2%), m.p. 88–90° (white needles). Elemental analysis: found: C 54.83%, H 7.33%, N 7.08%, F 14.52%; calc.: C 54.81%, H 7.41%, N 7.10%, F 14.45%.

Synthesis of N-TFA-amino acid isopropyl ester derivatives

All samples were prepared as reported earlier[4,5]. All derivatives were enriched in the L enantiomer in order to facilitate identification by gas chromatography (GC). For *tert.*-leucine and norvaline, however, only the racemic mixtures were available and the assignments were made by extrapolation.

Gas chromatography

A Varian Aerograph Model 1200-1 gas chromatograph equipped with attachments for capillary columns and FID detector was used to carry out these experiments. The stainless-steel capillary columns (400 ft. × 0.02 in. and 100 ft. × 0.02 in.) were cleaned prior to use with 200-ml portions of chloroform, acetone, water, conc. nitric acid, water, conc. ammonium hydroxide, water, acetone, and methylene chloride. The dried columns were coated with a solution of 1 g of dipeptide in 10 ml anhy-

drous ether as described by HORVATH[12]. The ether was blown off overnight and the columns were conditioned at 12 p.s.i. and 100° for 48 h. All retention times were recorded with an automatic integrator (Varian Model 480) and were reproducible within ± 6 sec or ± 0.3%.

RESULTS AND DISCUSSION

N-TFA-L-norvalyl-L-norvaline cyclohexyl ester has been prepared and used as a stationary liquid phase for the resolution of D,L-N-TFA-amino acid isopropyl esters by GC. The dipeptide was synthesized[13] by condensing N-*tert.*-BOC-L-norvaline with L-norvaline cyclohexyl ester via the benztriazole technique*. The desired dipeptide was obtained in high yield as white crystalline needles which gave an excellent microanalysis when the product was recrystallized twice from ethanol and water. This technique has the advantage that the tedious preparative-scale GC separation of the final product as undertaken by CORBIN *et al.*[9] is not necessary and could cause racemization due to the elevated temperatures.

Particular attention has been devoted to the quantitative effects of alkyl substituents at the α-carbon of the solute on the resolubility of the stationary phase. Additional investigations have been made concerning the qualitative separability of the commonly occurring enantiomeric amino acid derivatives by the new phase.

The side-chain of the solute can be varied in many ways by simple addition (homologous) and positional manipulation (isomeric) of alkyl groups on the α-carbon. These studies have been confined to three of the possible variations at the α-carbon, two of which deal with homologous changes in the side-chain and the third with isomeric modifications of the α-carbon substituent.

Factors chosen for comparison are separation ($r_{L/D}$)** of and differential enthalpy of solution [$\Delta(\Delta H)_{D,L}$]*** between D and L isomers. These numbers were obtained from data taken at temperatures of 100, 110, 120, and 130°.

The first variational series deals with the simple insertion of methylene groups between the initial α- and β-carbons. Derivatives of D,L-alanine (Ala, $R_1 = -CH_3$), D,L-α-amino-*n*-butyric acid (Aba, $R_1 = -CH_2-CH_3$), D,L-norvaline [nVal, $R_1 = -(CH_2)_2CH_3$] and D,L-norleucine [nLeu, $R_1 = -(CH_2)_3-CH_3$] were chosen for investigation. The lengthening of the chain from Ala to Aba is accompanied by a decrease in the separation factor and the trend continues through nVal (Fig. 1, Table I) at all temperatures except 130°, where Aba and nVal coincide. However, the addition of a third methylene group (nLeu) causes an increase in $r_{L/D}$ and this is true at each temperature. The differential enthalpies of interaction are of the order nLeu > Ala > Aba > nVal (Table III)§. The trend is the same, and it is evident that nLeu, with the largest separation factors at every temperature, shows the greatest difference in

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* All intermediates during the synthesis were checked for purity by thin-layer chromatography and found to be homogeneous.

** $r_{L/D} = \frac{\text{retention time of L enantiomer with respect to chloroform}}{\text{retention time of D enantiomer with respect to chloroform}}$.

*** Obtained from the equation: $\Delta(\Delta H) = -2.30RT \log r_{L/D}$ by determination of the slope of $\log r_{L/D}$ plotted *versus* $1/T$ (Fig. 5).

§ The absolute values for the relative enthalpies are considered.

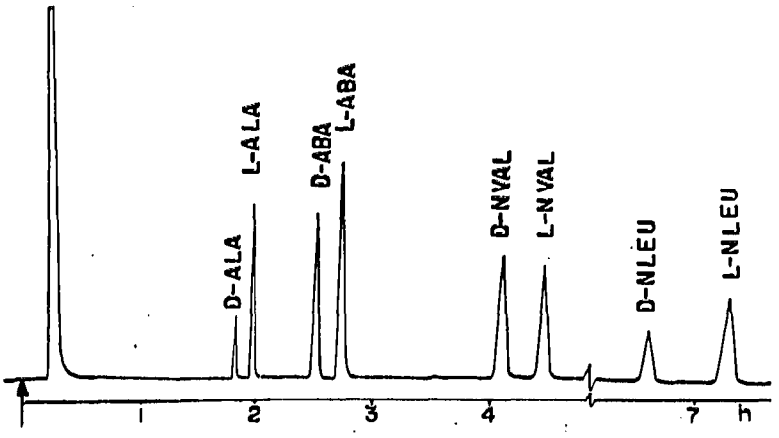

Fig. 1. Chromatogram of N-TFA-D,L-amino acid isopropyl esters on N-TFA-L-norvalyl-L-norvaline cyclohexyl ester stationary phase. Chromatographic conditions: 400 ft. × 0.02 in. stainless-steel capillary columns; 100° isothermal, injector temperature 190°; carrier gas He at 12 p.s.i.

enthalpy of solution, while nVal, with the lowest separation factors at each temperature, interacts the least.

A second comparison of homologs was made by replacing protons with methyl groups at the β-carbon. Thus Ala ($R_1 = -CH_3$), Aba ($R_1 = -CH_2-CH_3$), D,L-valine [Val, $R_1 = -CH(CH_3)_2$] and D,L-*tert.*-leucine [*t.*-Leu, $R_1 = -C(CH_3)_3$] were satisfactory homologs for this series. It is readily seen that an increase in branching is accompanied by a decrease in value for the separation factor (Fig. 2, Tables I and II).

TABLE I

EFFECTS OF INCREASING CHAIN LENGTH AT THE α-CARBON OF THE SOLUTE ON RELATIVE RETENTION TIMES AND SEPARATION FACTORS FOR N-TFA-D,L-AMINO ACID ISOPROPYL ESTERS

400 ft. × 0.02 in. stainless-steel capillary column coated with N-TFA-L-norvalyl-L-norvaline cyclohexyl ester; carrier gas He, pressure 12 p.s.i.; detector temperature 280°; injector temperature 190°.

Amino acid	*100°*		*110°*		*120°*		*130°*	
	RRT[a]	$r_{L/D}$	*RRT*	$r_{L/D}$	*RRT*	$r_{L/D}$	*RRT*	$r_{L/D}$
D-Alanine	0.231		0.253		0.273		0.299	
		1.100		1.087		1.075		1.063
L-Alanine	0.254		0.275		0.293		0.318	
D-α-Amino-butyric acid	0.330		0.354		0.362		0.396	
		1.097		1.086		1.071		1.060
L-α-Amino-butyric acid	0.362		0.384		0.388		0.420	
D-Norvaline	0.553		0.573		0.592		0.613	
		1.092		1.081		1.070		1.060
L-Norvaline	0.604		0.619		0.633		0.650	
D-Norleucine	0.903		0.920		0.924		0.935	
		1.108		1.096		1.083		1.070
L-Norleucine	1.000		1.000		1.000		1.000	

[a] RRT = relative retention time, reference compound is always N-TFA-L-norleucine cyclohexyl ester. r 100° = 426.7 min; r 110° = 259.4 min; r 120° = 163.4 min; r 130° = 101.0 min.

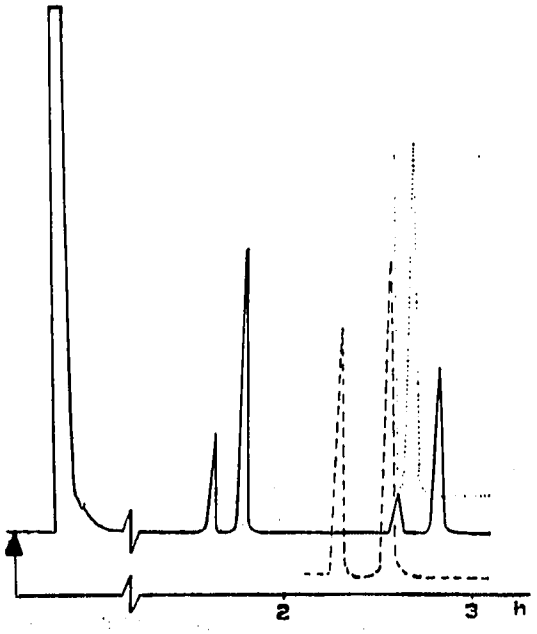

Fig. 2. Chromatogram of N-TFA-D,L-amino acid isopropyl esters on N-TFA-L-norvalyl-L-norvaline cyclohexyl ester stationary phase. Chromatographic conditions: 400 ft. × 0.02 in. stainless-steel capillary columns; 100° isothermal, injector temperature 190°; carrier gas He at 12 p.s.i. In order of increasing retention times: ———, D,L-Ala; — — —, D,L-Aba; ·····, D,L-*t*.-Leu; ———, D,L-Val.

Enthalpy changes were of the order Ala > Aba > Val ≫ *t*.-Leu (Table III). The superimposition in Fig. 2 clearly demonstrates the lack of dependency on retention times of the solution enthalpies. Aba, Val and *t*.-Leu all have similar retention times but show marked differences in differential enthalpies of solution (Table III).

TABLE II

EFFECTS OF ISOMERIZATION AND BULKINESS AT THE α-CARBON OF THE SOLUTE ON RELATIVE RETENTION TIMES AND SEPARATION FACTORS FOR N-TFA-D,L-AMINO ACID ISOPROPYL ESTERS

Gas chromatographic conditions, see Table I.

Amino acid	*100°*		*110°*		*120°*		*130°*	
	RRT[a]	$r_{L/D}$	*RRT*	$r_{L/D}$	*RRT*	$r_{L/D}$	*RRT*	$r_{L/D}$
D-*tert*.-Leucine	0.364		0.389		0.401		0.430	
		1.044		1.040		1.034		1.028
L-*tert*.-Leucine	0.381		0.404		0.414		0.442	
D-Isoleucine	0.535		0.558		0.567		0.599	
		1.108		1.093		1.079		1.068
L-Isoleucine	0.592		0.610		0.612		0.640	
D-Leucine	0.749		0.760		0.752		0.744	
		1.110		1.096		1.082		1.070
L-Leucine	0.832		0.833		0.813		0.829	
D-Norleucine	0.903		0.920		0.924		0.935	
		1.108		1.096		1.083		1.070
L-Norleucine	1.000		1.000		1.000		1.000	
D-Valine	0.364		0.386		0.409		0.435	
		1.083		1.069		1.050		1.044
L-Valine	0.397		0.416		0.437		0.459	

TABLE III

DIFFERENTIAL ENTHALPIES OF INTERACTION FOR ENANTIOMERS OF N-TFA-AMINO ACID ISOPROPYL ESTERS ON N-TFA-L-NORVALYL-L-NORVALINE CYCLOHEXYL ESTER

Amino acid	$\Delta(\Delta H)$ *(cal)* $\pm$ 5%
Ala	−342
Aba	−339
Val	−302
nVal	−293
Leu	−366
Ile	−366
nLeu	−348
t.-Leu	−156

A final study was made to determine the effect of isomerization within the alkyl substituent on the α-carbon. Values for nLeu [$R_1 = -(CH_2)_3CH_3$], D,L-leucine [Leu, $R_1 = -CH_2\text{-}CH(CH_3)_2$], D,L-isoleucine (Ile, $R_1 = -CHCH_3\text{–}CH_2\text{–}CH_3$) and *t.*-Leu [$R_1 = -C(CH_3)_3$] were obtained (Fig. 3, Table II). There seems to be no predictable trend here, with the separation factors for nLeu, Leu and Ile being practically the same at the different temperatures and always much greater than that of *t.*-Leu. Differential enthalpies of interaction are of the order Leu = Ile > nLeu $\gg$ *t.*-Leu (Table III).

Information provided by the preceding results might warrant further investigation, since, in some instances, it is difficult to arrive at any sound conclusions based on the findings to this point. However, it is evident that replacing hydrogens with methyl groups at the β position lowers the separability of a given sample. Furthermore, lengthening the chain attached to the α-carbon produces a similar effect up to three carbons. A four-carbon chain causes an increase in separation factors and is characterized by a larger enthalpy of interaction with the phase.

The solution energy and separability associated with the presence of a four-carbon chain seem, remarkably, to apply to all four-carbon groups, regardless of length, with the single exclusion of the bulky tertiary butyl group on *t.*-Leu. In this case, the unwieldy substituent probably prevents satisfactory hydrogen bonding in the formation of the diastereoisomeric complex with the phase.

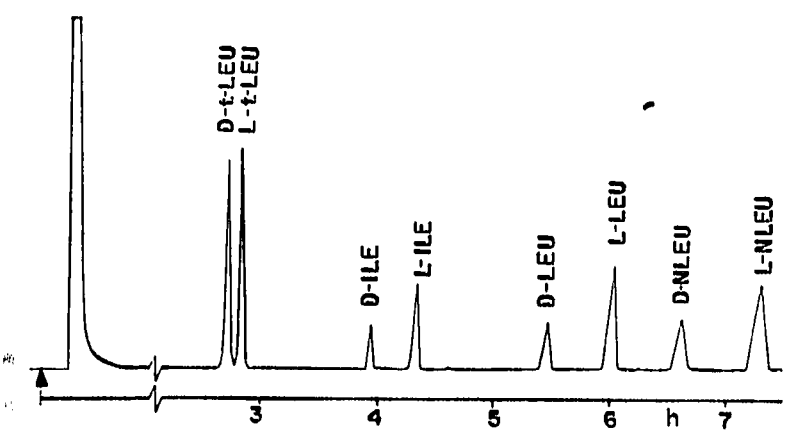

Fig. 3. Chromatogram of N-TFA-D,L-amino acid isopropyl esters on N-TFA-L-norvalyl-L-norvaline cyclohexyl ester stationary phase. Chromatographic conditions: 400 ft. × 0.02 in. stainless-steel capillary columns; 100° isothermal, injector temperature 190°; carrier gas He at 12 p.s.i.

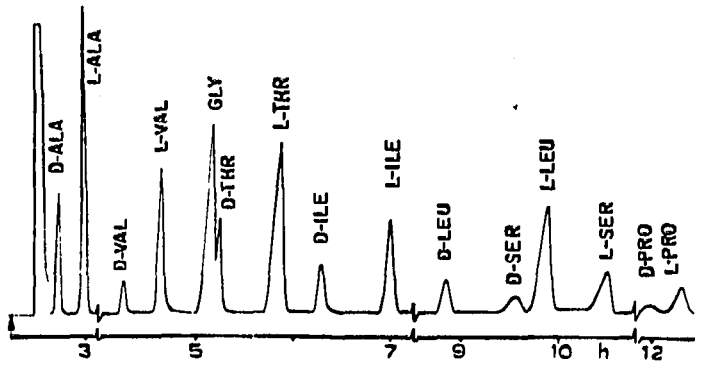

Fig. 4. Chromatogram of N-TFA-D,L-amino acid isopropyl esters on N-TFA-L-norvalyl-L-norvaline cyclohexyl ester stationary phase. Chromatographic conditions: 400 ft. × 0.02 in. stainless-steel capillary columns; 100° isothermal, injector temperature 190°; carrier gas He at 12 p.s.i.

The moot facts at this point are the high enthalpies of solution associated with three of the four leucine isomers compared to steadily decreasing values with increasing size up to four carbons. Further investigations of these findings are currently in progress.

The general utility of N-TFA-L-norvalyl-L-norvaline cyclohexyl ester (nVal-nVal) as a means for the separation of enantiomers of commonly occurring amino acids has also been determined. TFA-isopropyl ester derivatives of D,L-alanine (Ala), D,L-valine (Val), D,L-threonine (Thr), glycine (Gly), D,L-isoleucine (Ile), D,L-leucine (Leu), D,L-serine (Ser) and D,L-proline (Pro) were chosen for investigation. Enantiomers of the less volatile common amino acids were not considered, since N-TFA-D,L-isopropyl esters of aspartic acid, glutamic acid, phenylalanine, methionine,

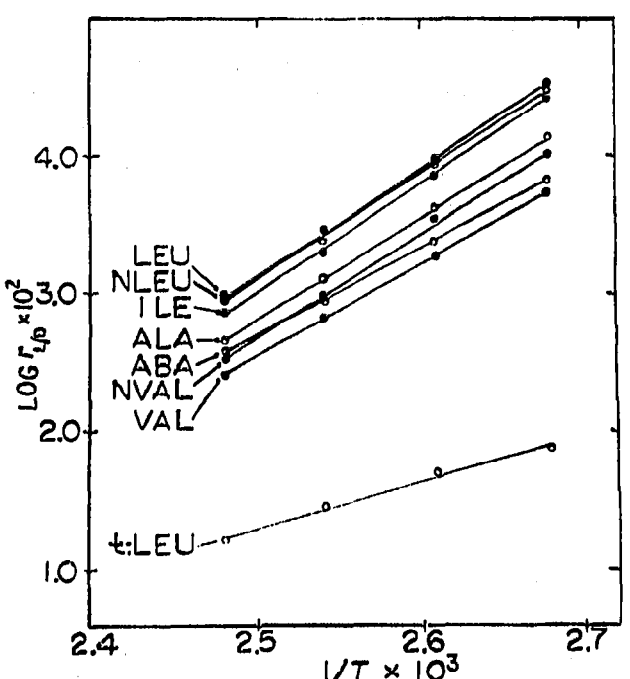

Fig. 5. Plot of the logarithms of the separation factors ($r_{L/D}$) *versus* the inverse of the absolute temperature for N-TFA-amino acid isopropyl esters.

tyrosine and lysine have been well resolved on N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester[4].

Enantiomers of Ser and Thr were of particular interest since these hydroxyl amino acids have shown changes in their retention times relative to the other amino acids on earlier dipeptide phases[8]. There have been some difficulties in separating the Thr isomers from Gly and extreme difficulties with overlap between enantiomers of Leu and Ser. In fact, there has ,as yet, been no report of the complete differentiation between the TFA-Leu and Ser isopropyl ester enantiomers by GC; nor have analogous derivatives of D, L-Thr and Gly been well resolved. It might also be pointed out that until injection on nVal-nVal, these same derivatives of D,L-*l*.-Leu had not been completely resolved (Fig. 3, Table II).

A 400-ft. × 0.02 in. column coated with nVal-nVal showed acceptable distinction between Gly and Thr and complete separation for the enantiomeric pairs of Leu and Ser as well as for enantiomers of Ala, Val, Ile and Pro (Fig. 4).

The quality of nVal-nVal as an excellent stationary phase for the separation of derivatized enantiomers of amino acids has been demonstrated by the work reported here. Fig. 5, from which the differential enthalpies of solution for the amino acid enantiomers were determined, shows the linear relation of the separation factor logarithm to temperature, which is characteristic of an acceptable stationary phase.

ACKNOWLEDGEMENTS

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