the (Leu-Ala)$_6$-hydrobromide was precipitated with 200 ml of ether. The product was filtered off and washed with ether. After drying *in vacuo*, 415 mg of a white powder was obtained (0.345 mmol = 88% with respect to the amount of alanine esterified onto the resin). A sample of this product was hydrolyzed for 20 hr in 6 *N* HCl at 110°. The amino acid analysis of the hydrolysate indicated a ratio of Ala:Leu = 1:0.97.

(Ala-Phe)$_6$. The synthesis was carried out according to the description given for (Leu-Ala)$_6$. According to amino acid analysis the ratio of Ala–Phe was 0.94:1.0. (Ala-Phe)$_6$-HBr is practically insoluble in all conventional solvents. It is soluble only in trifluoroacetic acid.

Synthesis of (Leu-Ala)$_6$ with Pellicular Resin. Boc-Ala Beads. Glass beads (150 g, 88–105μ) coated with chloromethylated polystyrene[15] (capacity 0.15 mmolar equiv/g) were put into a column (1 × 100 cm) and a solution of 1.9 g of Boc-Ala-OH (10 mmol) and 1 g (10 mmol) of triethylamine in 60 ml of ethanol soaked in the resin bed and allowed to react for 48 hr at 60°. The coated glass beads were then washed with ethanol, DMF, and CH_2Cl_2. The capacity loading on the coated beads represented 0.07 mmolar equiv of Ala/g.

Boc-(Leu-Ala)$_6$ Beads. The further synthesis of the peptide beads was carried out in a column (3 × 10 cm) with 75 g of the Boc-Ala-OCH$_2$ beads using the basic steps and washing procedures as described for (Ala-Phe)$_6$. In every coupling step, 9 mmol of Boc-L-Ala-OH and Boc-L-Leu-OH and 9 mmol of dicyclohexylcarbodiimide, respectively, were used. The product obtained from the beads after hydrolysis showed a ratio of Leu–Ala of 1.0:1.0, and was contaminated with resin.

Synthesis of (Leu-Ala)$_6$ with Acetylation. The synthesis was carried out as described above with 2.5 g of chloromethylated resin (0.7 mmolar equiv/g Bio Beads S-X2, 200–400 mesh). The total quantity of the first amino acid attached to the resin was 0.4 mmol; the amount of the free amino groups after each cleaving step of the *t*-Boc group was constant during the entire synthesis. After each coupling step, the resin was washed three times with dimethylformamide, and the acetylation was carried out with 2.5 ml of acetic anhydride in a mixture of 25 ml of dimethylformamide and 0.75 ml of triethylamine for 20 min. Afterward, the resin was washed three times with dimethylformamide. The yield of the peptide hydrobromide was 321 mg (55%). According to the amino acid analysis, the ratio of Leu–Ala was 1.0:1.0.

Gas Chromatography–Mass Spectrometry and Sephadex Chromatography. The same methods, previously described in ref 5 were used. For gas chromatographic and mass spectrometric investigations, an LKB 9000 gas chromatograph–mass spectrometer was used. All mass spectra were taken at 70 eV. For solid samples, a heated and water-cooled direct inlet was used. The separation of the trifluoroacetyldipeptide methyl esters of the partial hydrolysate was achieved on SF 96 coated steel capillary columns 500 ft × 0.03 in., isothermal at 190°: carrier gas, 15 ml He/min; detector, FID, 300°; injector 280°. For the gas chromatography–mass spectrometry combination, the total ion current detector was used.

Partial Hydrolysis of Peptides. A peptide (10–100 mg) was sealed in a glass tube with 5 ml of concentrated HCl and left for 72 hr at 37°. After evaporation of the HCl *in vacuo* (18 Torr) the peptide mixture was esterified and trifluoroacetylated for gas chromatography or directly submitted to partition chromatography. Under these conditions approximately 50% (Leu-Ala)$_6$ was hydrolyzed to dipeptides, 30% to higher peptides, and 20% to amino acids. Due to this and to the fact that random hydrolysis was not occurring, the results in Table I and II, calculated on the basis of sequences found in di- to pentapeptides, are only semiquantitative.

Esterification of Peptides. The almost neutral peptide hydrolysate was dissolved in methanol. A concentrated solution of diazomethane in diethyl ether was added until the yellow color remained. If any precipitate formed, a few drops of water and more diazomethane were added until the precipitate dissolved. The yellow solution was evaporated to dryness *in vacuo* (18 Torr).

Trifluoroacetylation. The peptide esters were dissolved in 5 ml of dry methanol and brought to a pH of 7.5–8 by adding triethylamine. Methyl trifluoroacetate (2 ml) was added and the solution left at room temperature for 8 hr. After evaporation to dryness *in vacuo* (18 Torr), the residue was shaken with a mixture of equal amounts of ethyl acetate and water. The organic phase was evaporated *in vacuo* (18 Torr), resulting in the TFA–peptide–methyl esters.

Acknowledgment. This investigation was supported by the Robert A. Welch Foundation, Grant No. E-227, Houston, Texas, and the Dentsche Forschungsgemeinschaft.

(15) The sample of chloromethylated glass beads was provided by Dr. C. G. Horvath and S. R. Lipsky, New Haven, Conn.

Retention of Configuration in the Solid Phase Synthesis of Peptides

E. Bayer, E. Gil-Av,[1] W. A. König, S. Nakaparksin, J. Oró, and W. Parr

Contribution from the Department of Chemistry and Biophysical Sciences, University of Houston, Houston, Texas. *Received August 2, 1969*

Abstract: Amino acid enantiomers can be resolved by the use of optically active stationary phases in a gas chromatographic system. This technique has allowed the study of racemization in the solid phase synthesis of polypeptides. The results indicate that almost complete retention of configuration is obtained.

Peptide synthesis by the solid phase method is assumed to proceed without racemization. Merrifield,[2] in his first publication on the subject has reported that the L-leucyl-L-alanyl-glycyl-L-valine prepared by his method was completely digested by leucine aminopeptidase. Furthermore, the high biological activity (450 units/mg, potency on the isolated rat uterus), coupled with high synthetic yield (79%) of the oxytocin prepared by Bayer and Hagenmaier[3] indicates that racemization did not occur to any appreciable extent. Nevertheless, this aspect of solid phase synthesis of peptides has not as yet been thoroughly investigated and the question has been raised whether racemization is completely excluded in all cases. With the advent of new, sensitive gas chromatographic methods for the resolution of enantiomeric amino acids[4–6]

(1) On sabbatical leave from the Weizmann Institute of Science, Rehovoth, Israel.

(2) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963).

(3) E. Bayer and H. Hagenmaier, *Tetrahedron Lett.*, 2037 (1968).

(4) E. Gil-Av, R. Charles-Sigler, G. Fischer, and D. Nurok, *J. Gas Chromatog.*, 4, 51 (1966).

(5) J. W. Westley and B. Halpern in "Gas Chromatography 1968," S. L. A. Harbourn, Ed., Institute of Petroleum, London, 1969, p 119.

(6) E. Gil-Av, B. Feibush, and R. Charles-Sigler, *Tetrahedron Lett.*,

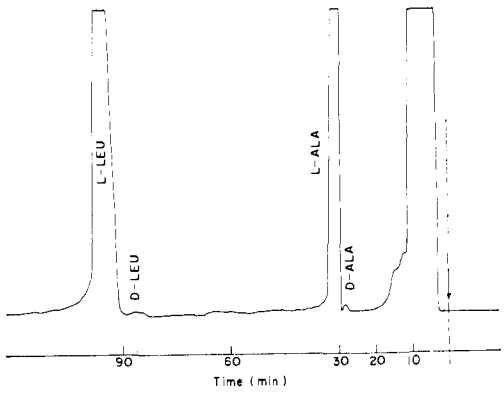

Figure 1. Gas chromatogram of the N-TFA-amino acid isopropyl esters of the $(Leu\text{-}Ala)_6$ hydrolysate. Column: 500 ft × 0.02 in. stainless steel capillary column, coated with N-TFA-L-valyl-L-valine cyclohexyl ester, temperature 110°; injector, 180°; detector FID, 280°; carrier gas He, pressure 20 psi.

procedures became available for an investigation of this problem.

Enantiomeric amino acids can be separated by gas chromatography using two different approaches. The mixtures of enantiomers to be analyzed can be transformed into diastereoisomers by reaction with an asymmetric reagent, such as (+)-2-butanol[4] or N-trifluoroacetyl-L-prolyl chloride,[5] or, alternatively, separation is achieved by chromatography on an optically active stationary phase.[6]

In the present study the second approach was used since it is more suitable for the detection of small amounts of antipodes. In fact, the method based on separation of diastereoisomers requires a derivatizing agent of high optical purity which cannot always be readily attained. The antipodal impurity in the reagent gives rise to a second peak which has to be corrected for in the calculation of the results, and this may introduce a large error when only small amounts of antipodes are present in the sample. Furthermore, one of the diastereoisomers can be formed preferentially so that the correction is not necessarily simply proportional to the amount of antipodal impurity in the reagent. In the second approach, on the other hand, symmetric reagents are used for derivatization and the only correction to be made is for the amount of racemized product formed during the hydrolysis of the peptide.

Results and Discussion

Retention of configuration in the dodecapeptide $(Leu\text{-}Ala)_5\text{-}Leu\text{-}Ala$ and in desamidosecretin, a heptacosapeptide, both synthesized by the solid phase method, was examined. The compounds were hydrolyzed in 6 *N* HCl at 100° for 20 hr, and the amino acids obtained transformed into trifluoroacetyl-O-methyl esters and/or trifluoroacetyl-O-isopropyl esters which were then chromatographed on capillary columns coated with N-TFA-L-valyl-L-valine cyclohexyl ester.[7]

As can be seen in Figure 1, the dodecapeptide showed only traces of D isomers, determined as N-TFA-O-isopropyl esters, in amounts below 0.1%. The sensitivity of the gas chromatographic method is between 0.05 and 0.1% according to the position of the peak in the chromatogram.

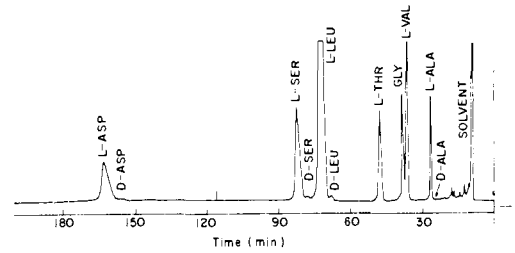

Figure 2. Gas chromatogram of the N-TFA-amino acid methyl esters of the desamidosecretin hydrolysate. Conditions as in Figure 1.

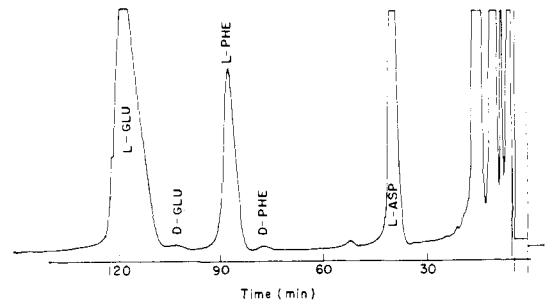

Figure 3. Gas chromatogram of the N-TFA-amino acid isopropyl esters of the desamidosecretin hydrolysate. Column: 100 ft × 0.02 in. stainless steel capillary, coated with N-TFA-L-valyl-L-valine cyclohexyl ester, temperature 110°; injector, 180°; detector FID, 280°; carrier gas He, pressure 5 psi.

Desamidosecretin contains the following amino acids: alanine 1, valine 1, glycine 2, threonine 2, leucine 6, serine 4, aspartic acid 2, phenylalanine 1, glutamic acid 3, histidine 1, and arginine 4. The enantiomeric analysis of this hydrolysate is understandably somewhat less simple than in the first case. Histidine and arginine do not at present come within the scope of our gas chromatographic method of enantiomer separation. Alanine, valine, threonine, leucine, serine, and aspartic acid were analyzed for antipodal impurities in the form of their methyl esters on a 500-ft column (Figure 2). For leucine, 0.81% of D isomer was found, for alanine 0.76%, for serine 1.1%, and for aspartic acid 2.2%, whereas for valine and threonine the amount was below the detection limit of the gas chromatographic method of (0.1%).

Finally phenylalanine and glutamic acid were analyzed for the presence of D enantiomers by chromatographing their N-TFA-O-isopropyl esters on a 100-ft capillary column (Figure 3). For phenylalanine 0.39% D isomer was detected and for glutamic acid 0.73%.

The small amounts of D isomers found in the hydrolysate of $(Leu\text{-}Ala)_6$ are not caused by optical impurities in the Boc-amino acid derivatives used for the synthesis, as a control reaction showed less than 0.05% of the D isomers. Therefore they must be accounted for by racemization of the liberated amino acids in acid solution. In fact it has been shown on a series of neutral amino acids[8] that slow inversion occurs upon heating L-amino acids under the conditions of peptide hydrolysis. Thus, about 0.2% of D-valine, 0.5% of

1009 (1966); "Gas Chromatography 1966," A. B. Littlewood, Ed., Institute of Petroleum, London, 1967, p 227.

(7) P. Birrell, E. Gil-Av, S. Nakaparksin, and J. Oró, presented at the Southwest Regional Meeting of the American Chemical Society, Dec 4–6, 1968.

(8) S. Nakaparksin, E. Gil-Av, and J. Oró, *Anal. Biochem.*, in press.

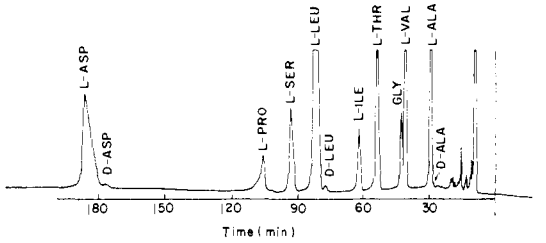

Figure 4. Gas chromatogram of the N-TFA-amino acid methyl esters of the bovine albumin hydrolysate. Conditions as in Figure 1.

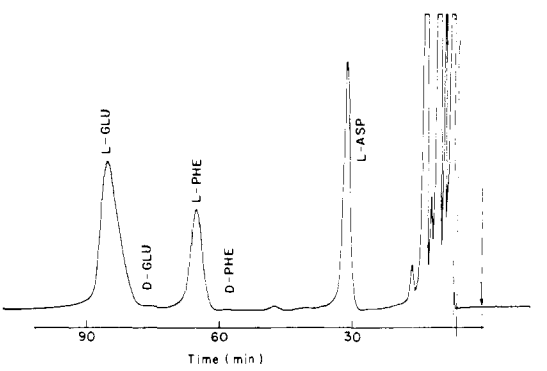

Figure 5. Gas chromatogram of the N-TFA-amino acid isopropyl esters of the albumin hydrolysate. Conditions as in Figure 3.

D-alloisoleucine, and 1.0% D-alanine were detected, when the corresponding L-amino acids were heated at 105° for 24 hr; that is, under somewhat more drastic conditions than those used in the present investigations.

The higher amounts of D-isomers found desaminosecretin, as compared with $(Ala\text{-}Leu)_6$, can in part also be explained by racemization of the liberated amino acids. In particular, it should be mentioned that aspartic acid has been reported[9] to racemize in 18 hr to the extent of 3.7% in 6 *N* HCl at 110°. In addition, however, inversion seems to occur, when opening certain peptide bonds, to a degree depending on the nature of the neighboring amino acid. Thus bovine albumin, which being a natural peptide, should not contain any D-isomer, gave by the identical procedure even larger amounts of some D-amino acids, than desaminosecretin, namely 1.26% D-leucine, 0.95% D-alanine, 0.9% D-glutamic acid, 1.6% D-aspartic acid, 0.8% serine, and 0.46% phenylalanine (Figures 4 and 5). Further evidence is given in the literature.[9] In conclusion it would appear that the D-amino acids found in the hydrolysates of the two synthetic peptides are essentially artifacts.

As far as can be judged by the present analyses, the solid phase synthesis proceeds with practically complete retention of configuration under the conditions specified.

Experimental Section

Desamidosecretin. The synthesis was carried out in a manner similar to that described by Bayer, *et al.*[3,10,11] *t*-Butyloxycarbonyl amino acids were prepared from *t*-butylcarbazate and the appropriate amino acid according to the pH-stat method of Schnabel.[12] The N-terminal amino acid L-histidine was used as the bisbenzoxycarbonyl-L-histidine-*p*-nitrophenyl ester,[13] because all the protected groups could then simultaneously be removed by catalytic hydrogenation.

t-Boc-L-valine (1.52 g, 7 mmol) was treated with 5 g of chloromethylated polymer (Bio-Beads S-X2 200–400 mesh, Bio-Rad Laboratories). The amino acid analysis of a dried sample showed that the Boc-L-valyl polymer contained 0.11 mmol of L-valine/g. The following cycles of reactions were used to introduce each new amino acid: cleavage of the Boc groups with 30 ml of *N* HCl in glacial acetic acid for 30 min; washing three times with 30-ml portions of glacial acetic acid, absolute ethanol, and N,N-dimethylformamide (DMF); neutralization of the resulting hydrochloride with 30 ml of triethylamine solution (10% triethylamine in DMF) for 10 min; washing three times with 30-ml portions of DMF and methylene chloride; and coupling of the new amino acid (2.2 mmol) with the free amino groups on the polymer with 2.2 mmol of dicyclohexylcarbodiimide (DCC) as condensation agent in methylene chloride. For the *t*-BOC-nitro-L-arginine cycles DMF was substituted for methylene chloride. His, Asp, Gln, and Gly were introduced as *p*-nitrophenyl esters of the Boc-amino acids in DMF as solvent. Boc-amino acids and Boc-amino acid *p*-nitrophenyl esters were used in 4-fold excess. The reaction time was 2 hr in the case of the DCC method and 8 hr in the case of the *p*-nitrophenyl esters. Excess reagents were removed by washing successively with methylene chloride (DMF in the case of *t*-Boc-nitro-L-arginine and *t*-Boc-amino acid *p*-nitrophenyl esters), ethanol, glacial acetic acid, ethanol, and methylene chloride.

The fully protected peptide, esterified to the resin, was suspended in 30 ml of trifluoroacetic acid, and a slow stream of anhydrous hydrogen bromide was bubbled through a fritted disk into the suspension for 90 min. The resin was filtered off and washed three times with 20 ml of trifluoroacetic acid. The combined filtrates were evaporated under reduced pressure. The resulting oil was dissolved in glacial acetic acid and lyophilized: yield 1.6 g.

A portion of the protected heptacosapeptide (750 mg) and 750 mg of 10% palladium on charcoal were hydrogenated in 50 ml of 90% acetic acid at 40 psi. After 24 hr the hydrogenation was stopped and 250 mg of additional catalyst was added and the mixture rehydrogenated for an additional 24 hr. Then, the catalyst was removed by filtration and the filtrate freeze dried: yield 500 mg. The amino acid analysis of this product was as follows: His 0.8, Ser 3.8, Asp 1.9, Gly 2.0, Thr 1.8, Phe 0.9, Ala 1.0, Glu 3.0, Leu 5.9, Arg 3.4, and Val 1.2. To detect racemization the peptide was hydrolyzed without further purification.

$(Leu\text{-}Ala)_6$ was synthesized as described in ref 13.

Bovine albumin was purchased from Mann Research Laboratories.

N-TFA-L-valyl-L-valine cyclohexyl ester was purchased from the Research Products Division, Miles Laboratories Ltd.

Hydrolysis of Peptides. The peptide (10 mg) was hydrolyzed in 5 ml of 6 *N* HCl at 100° for 20 hr in a sealed glass tube.

Esterification and Trifluoroacetylation of the Amino Acids. After evaporation of the hydrolysate, 3 ml of the desired alcohol, 1.25 *N* in HCl, was added per 5 mg of amino acid, and the mixture heated in a sealed tube at 100° for 3 hr. The product was evaporated *in vacuo*, and 3 ml of methylene chloride added per 5 mg of amino acid in a flask provided with a drying tube. The flask was then cooled to −20° in an acetone Dry Ice mixture, and 3 ml of trifluoroacetic anhydride added per 5 mg of amino acid. The mixture was allowed to heat up to room temperature and after 1 hr, was evaporated to dryness and dissolved in approximately 1 ml of chloroform.

Gas Chromatographic Analysis. The gas chromatographic conditions are described in Figures 1 and 3. Peak areas were measured with an Infotronics integrator. Since optical isomers have identical detector response, calibration factors are not required for the determination of the ratio of antipodes.

Acknowledgments. This investigation was supported by the Robert A. Welch Foundation (Grant No. E-227) and by NASA (Grant No. NGR-44-005-002, NGR-44-005-020, and NASA Contract NAS-9-8012).

(9) J. Manning and S. Moore, *J. Biol. Chem.*, 243, 3591 (1968).
(10) E. Bayer, G. Jung, and H. Hagenmaier, *Tetrahedron*, 24, 4853 (1968).
(11) E. Bayer, H. Eckstein, K. Hägele, W. A. König, W. Brüning, H. Hagenmaier, and W. Parr, submitted for publication.
(12) E. Schnabel, *Liebigs Ann. Chem.*, 702, 188 (1967).
(13) K. Inoue, *J. Org. Chem.*, 30, 1151 (1965).