of solid sodium chloride, and extracted three times with 15 ml of ethyl acetate and three times with 15 ml of peroxide-free diethyl ether. The extractions were completed within 1 hr and the organic phases were dried, first with anhydrous sodium sulfate at room temperature for 1 hr, then with anhydrous magnesium sulfate for 6 hr at −20°. Homogentisic acid was converted to the bistrimethylsilyl ether of the methyl ester as described in a previous publication.[22]

Incubation in $H_2{}^{18}O$ was carried out in a total volume of 0.25 ml and in an atmosphere of air. Otherwise the conditions were the same as for the $^{18}O_2$ experiments in the enzymic reaction.

Gas-Liquid Chromatography-Mass Spectrometry. The bis-trimethylsilyl ether of the methyl ester of homogentisic acid was analyzed on an instrument for combined gas chromatography-mass spectrometry[23] (LKB Model 9000, LKB Produkter, Stockholm, Sweden).

(22) J. Gentz, B. Lindblad, S. Lindstedt, and R. Zetterström, *J. Lab. Clin. Med.*, 74, 185 (1969).

(23) Initial work was performed on an instrument which had been designed at the Department of Medical Biochemistry, University of Gothenburg. We extend our thanks to Professor S. Stenhagen for help in this phase of the work.

Determination of D- and L-Amino Acid Residues in Peptides. Use of Tritiated Hydrochloric Acid to Correct for Racemization during Acid Hydrolysis[1]

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Received May 12, 1970

Abstract: A method for measuring the racemization of some amino acids during the acid hydrolysis of peptides is described. The peptide is hydrolyzed in tritiated HCl and the radioactivity of each amino acid is measured with a flow-cell scintillation counter attached to the amino acid analyzer. The method was tested with free L-amino acids; the samples were heated in tritiated HCl and the amount of enantiomer formed was calculated from the amount of tritium incorporated. This value was compared with the actual amount of D isomer found chromatographically after the sample was coupled with an L-amino acid *N*-carboxyanhydride; the resulting diastereoisomeric dipeptides were separated on an amino acid analyzer. The incorporation of tritium was proportional to the amount of D isomer formed within a range of 1% for alanine, valine, isoleucine, leucine, serine, threonine, lysine, arginine, methionine, and proline. The other amino acids tested (aspartic acid, glutamic acid, phenylalanine, tyrosine, and histidine) have exchangeable hydrogen atoms in the side chains and hence this technique is not applicable to them. As shown by proton nmr analysis of samples heated in deuterium chloride, the α-hydrogen atom of an α-amino acid is not removed in strong acid as readily as the α-hydrogen atom of an aliphatic carboxylic acid such as isobutyric acid. This may be a result of the absence of the positively charged conjugate acid of the α-carboxylic acid group which is energetically not favored when a protonated α-amino group is present. Analysis of synthetic L-bradykinin showed that D-serine, D-proline, and D-arginine (0.1–2.8%) present in the acid hydrolysate were formed by racemization during the hydrolysis. An acid hydrolysate of natural bacitracin A contained D-leucine (6.8%) and D-alloisoleucine (15.7%) which were formed from the corresponding L residues during the hydrolysis. In general, the amount of D-amino acid found in excess of that formed by racemization during acid hydrolysis provides a measure of the D isomer in the peptide.

Racemization of amino acids occurs during acid hydrolysis of a peptide[2] and the amount of this racemization must be determined before the configurations of the amino acids in either a synthetic or a natural peptide can be rigorously established. Treatment of the corresponding free amino acids with hot acid has been employed as a control for measurement of the racemization of amino acid residues during acid hydrolysis of a peptide.[3,4] However, this control may not always be adequate because the amino acid residues in some sequences of a peptide may be subject to increased racemization during hydrolysis; for example, in bacitracin A Ile-1 is completely epimerized during the hydrolysis,[5] and the phenylalanyl residue in L-Phe-L-Ser is racemized more than free L-phenylalanine during hydrolysis.[3]

When the stereochemical purity of a synthetic L-peptide is being evaluated by a study of an acid hydrolysate of the material, the natural L-peptide is the best control for correction for the racemization which occurs during the hydrolysis. When the natural material is not available for comparison, it has been difficult to establish unequivocally the configurations of the amino acids in the synthetic peptide because there has not been a method for measuring increased racemization of some amino acid residues during acid hydrolysis. The same consideration applies to the examination of a natural peptide for D- and L-amino acid content.

Since racemization occurs concomitant with removal of the α-hydrogen atom of an amino acid, incorporation of tritium should provide a convenient means for

(1) This study was supported in part by a grant from the National Institutes of Health. A preliminary report has been communicated (J. M. Manning and A. Marglin, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 29, 902 (1970)). The Fischer convention is used to designate the configurations of the amino acids.

(2) A. Neuberger, *Advan. Protein Chem.*, 4, 297 (1948).

(3) J. M. Manning and S. Moore, *J. Biol. Chem.*, 243, 5591 (1968).

(4) E. Bayer, E. Gil-Av, W. A. König, S. Nakaparksin, J. Oró, and W. Parr, *J. Amer. Chem. Soc.*, 92, 1738 (1970).

(5) L. C. Craig, W. Hausmann, and J. R. Weisiger, *ibid.*, 76, 2839 (1954).

measuring directly the amount of racemization that occurs during the acid hydrolysis of a peptide. Denkewalter, *et al.*,[6] utilized a similar approach to check the degree of racemization during peptide synthesis. In the present article tritiated HCl is used for the acid hydrolysis and correction for racemization is made for those amino acids whose side-chain hydrogen atoms are not exchangeable during acid hydrolysis.

The procedure described here is used in conjunction with the method previously reported,[3] which measures the total amounts of D- and L-amino acids in an acid hydrolysate of a peptide or in amino acids isolated therefrom. With that procedure diastereoisomeric dipeptides are separated on an amino acid analyzer after the sample is coupled with an L-amino acid *N*-carboxyanhydride (NCA[7]). The method was used for demonstrating the optical purity of synthetic bradykinin where the sequence Arg-Pro-Pro- had precluded the use of enzymic hydrolysis to establish the optical purity of this peptide.[8]

One of the main objects of the present study has been the development of techniques to permit examination of synthetic peptides for stereochemical purity. Methods based upon optical rotation are useful only for those cases where the natural peptide is available; their sensitivity is limited by the rotation of the peptide under study. The present approach is more definitive than one in which the synthetic product is assumed to be stereochemically pure if the coupling of two amino acid derivatives as a model proceeds without racemization. Stereochemical purity can be demonstrated in some instances by hydrolysis of the synthetic peptide with aminopeptidases[9] which are specific for L-amino acid residues. If the enzymic hydrolysis is incomplete, use of the methods described here can provide information on the nature and amount of racemic amino acid residues in the synthetic peptide.

Experimental Section

Materials. The NCA's were obtained as previously described.[3] L-Bradykinin, synthesized by the solid phase method,[10] was a gift of Dr. R. B. Merrifield; bacitracin A was kindly donated by Dr. L. C. Craig. Tritiated water (1 Ci/g) was obtained in 0.5-ml lots from New England Nuclear Corp. (Cat. No. NET-001E). Deuterium chloride (38%) in D_2O and deuterium oxide (99.7%) were products of Merck Sharp and Dohme of Canada. Deuterated chloroform was a product of Diaprep, Inc., Atlanta, Ga. β-Alanine was purchased from Merck and Company, Rahway, N. J. Isobutyric acid was obtained from Matheson Coleman and Bell.

Hydrolysis in Tritiated HCl and in DCl. Mixtures of solid L-amino acids were prepared and dissolved in concentrated HCl; each amino acid had been previously established as the pure L isomer.[3] The sample, in 100 μl of acid, was placed in a Pyrex test tube (10 × 75 mm) which had been washed with hot H_2SO_4–HNO_3 (3:1). Tritiated water (100 μl) was added and the contents were mixed; this operation and the sealing of the tubes[11] should be carried out in a hood. The tubes were not frozen or evacuated before they were sealed; hydrolysis was carried out in an oven at 110°. The evacuation of the tubes was omitted so that the concentration of tritiated HCl would be the same for each sample. Each new batch of tritiated water was standardized in terms of disintegrations per minute per milliliter with a flow-cell scintillation counter attached to the amino acid analyzer as described below.

The presence of oxygen in the hydrolysis mixture leads to oxidation of methionine during hydrolysis; 10% of the methionine is converted to the sulfoxides, which are eluted just before aspartic acid, and 5% of the methionine is oxidized to the sulfone. Methionine sulfone, which is eluted just before threonine, incorporates tritium during heating in ^{3}HCl which interferes with the determination of the small amount of radioactivity in threonine. If the peptide for analysis contains both methionine and threonine, this problem can be solved by addition to the hydrolysis mixture of 2-mercaptoethanol (1 μl), which prevents oxidation of methionine.[12]

For proton nmr analysis L-glutamic acid, L-aspartic acid, L-alanine, β-alanine (50 mg of each), and isobutyric acid (100 μl) were heated in separate tubes (18 × 150 mm) at 110° for various times with 2.0 ml of a mixture of 38% deuterium chloride and deuterium oxide (1:1).

After the heating in ^{3}HCl the amino acid solutions were transferred to 18 × 150 mm tubes and were concentrated to dryness in a rotary evaporator at 45–50° in a hood. The ^{3}HCl was collected in a Dry Ice–acetone cooled trap and transferred to a bottle for disposal. The residues were washed twice with 1–2 ml of H_2O. More than 99% of the ^{3}HCl is removed during the evaporation; the exchangeable tritium on the carboxyl and the amino groups of the amino acids is quantitatively removed during the ion exchange chromatography. The samples heated in DCl were evaporated to dryness twice in D_2O. The samples of amino acids for nmr spectroscopy were dissolved in 1–2 ml of D_2O for analysis with either a Varian 220- or a 60-MHz spectrometer. Isobutyric acid was extracted from the DCl solution with 1 ml of deuterated chloroform for nmr analysis; parts per million are expressed in terms of δ.

The samples of bradykinin and bacitracin A (1–2 μmol of each) were dried in a desiccator over NaOH and each sample was placed in a 10 × 75 mm test tube. Concentrated HCl and ^3H_2O (100 μl of each) were added and the peptides were submitted to the conditions for hydrolysis described above for the amino acids.

Determination of Incorporation of Tritium into Amino Acids. A portion of the hydrolysate (corresponding to 0.1–1.0 μmol of each amino acid) in 0.20 *M* sodium citrate, pH 2.2, was chromatographed on the amino acid analyzer designed by Spackman, Stein, and Moore.[13] The 0–∞ absorbance scale was used for this amount of amino acid. The acidic and neutral amino acids were eluted from a 0.9 × 62 cm column with 0.20 *M* sodium citrate buffers, pH 3.25 and 4.00. With the pH 4.25 buffer, which is used for routine analyses, some radioactive material was eluted at the position of the buffer breakthrough and this interfered with the determination of labeled methionine. The basic amino acids were eluted from a 0.9 × 13 cm column with 0.38 *M* sodium citrate, pH 5.26. A scintillation flow cell (Nuclear-Chicago Model 6350, 877260, 8704, 8437 Chroma/Cell Detector assembly with a 2-ml cell) was connected between the bottom of the ion-exchange column and the mixing manifold to record the amount of tritium in the effluent (0.8% counting efficiency as determined with ^3H_2O); counts were printed at 1-min intervals. Specific radioactivity is defined as disintegrations per minute (dpm) per micromole of amino acid. The large amount of tritium used in the initial mixture for hydrolysis is necessary in order to attain a minimum counting rate of 500 cpm in the flow-cell apparatus for those amino acids which are only slightly racemized during the acid hydrolysis. If a flow-cell scintillation counter is unavailable, the divider pump on most amino acid analyzers can be used to divert an aliquot of the amino acids into a fraction collector. The amount of radioactivity can then be determined separately in a liquid scintillation counter.

Determination of D- and L-Amino Acids. The total amount of D-amino acid present was determined after the amino acids in a separate hydrolysate, prepared in parallel without tritium, were coupled with either L-leucine NCA or L-glutamic acid NCA as

(6) R. G. Denkewalter, H. Schwam, R. G. Strachan, T. E. Beesley, D. F. Veber, E. F. Schoenewaldt, H. Barkemeyer, W. J. Paleveda, Jr., T. A. Jacob, and R. Hirschmann, *J. Amer. Chem. Soc.*, 88, 3163 (1966).

(7) (a) *N*-Carboxyanhydride is abbreviated NCA. The method used is a scaledown of the procedure of Hirschmann, *et al.*, *J. Org. Chem.*, **32**, 3415 (1967). (b) A method based upon the separation of diastereoisomeric tripeptides on the amino acid analyzer has been used for determination of racemization during the synthesis of model peptides with various coupling reagents (N. Izumiya and M. Muraoko, *J. Amer. Chem. Soc.*, **91**, 2391 (1969)).

(8) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).

(9) K. Hofmann and H. Yajima, *J. Amer. Chem. Soc.*, **83**, 2289 (1961).

(10) R. B. Merrifield and J. M. Stewart, *Nature (London)*, 207, 522 (1965).

(11) S. Moore and W. H. Stein, *Methods Enzymol.*, **6**, 819 (1963).

(12) H. T. Keutmann and J. T. Potts, Jr., *Anal. Biochem.*, **29**, 175 (1969).

(13) (a) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958); (b) D. H. Spackman, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **22**, 244 (1963).

previously described.[3] The diastereoisomeric dipeptides were separated on the amino acid analyzer at loads that permitted the determination of 0.1% of an L–D isomer. The 0–0.1 absorbance scale was used for the determination of small amounts of the L–D dipeptide present. Preliminary separation of amino acids by ion exchange would be necessary in some instances with hydrolysates containing all 18 common amino acids.[3]

Results

Measurement of Racemization of Amino Acids during Heating in Tritiated HCl. Samples of L-alanine (6.2 μmol) were heated for various times in 6 *N* ^{3}HCl. The extent of ^{3}H incorporation (disintegrations per minute per micromole) into alanine increases linearly with time and is proportional to the amount of D-alanine found (Figure 1) as determined by the relative amounts of L-Leu-D-Ala and L-Leu-L-Ala after derivatization. The same total amount of radioactivity was found in each diastereoisomer for each of the heating times. The specific radioactivity of alanine, extrapolated to 100% racemization, is lower by a factor of 3.5 than the specific radioactivity of the initial ^{3}HCl which was used for the hydrolysis; this difference is attributed to an isotope effect. Correction for this isotope effect need not be applied, however, if an empirical standard graph (Figure 1) is derived which expresses the relationship between the observed incorporation of tritium and the amount of D isomer formed. Variations in the equipment available to a user or differences in experimental conditions, particularly temperature of hydrolysis, could lead to results different from those obtained in Figure 1 in terms of the amount of radioactivity incorporated. The user should determine his own standard graph. It is particularly important to measure the specific radioactivity of each new batch of tritiated water so that the amount of tritium used for each hydrolysis may be related to that which was used in the determination of the standard graph.

The other amino acids which incorporated tritium exclusively at the α-carbon atom concomitant with racemization were determined as follows: the free L-amino acid was heated in 6 *N* ^{3}HCl as described for L-alanine in Figure 1. After chromatography on the amino acid analyzer the amount of D isomer formed was calculated from the specific radioactivity of the amino acid with Figure 1 as a standard graph. The true amount of D isomer present was determined by measurement of the relative amounts of diastereoisomeric dipeptides after derivatization with the appropriate NCA; the results of the two methods were then compared. In Table I are listed the amino acids which gave values for per cent D isomer within a range of about 1% by the two methods of analysis. Thus, hydrolysis with ^{3}HCl can be used for measurement of the racemization of these amino acids during acid hydrolysis of a peptide or a protein. Aspartic acid, glutamic acid, phenylalanine, tyrosine, and histidine incorporated more tritium than could be accounted for as the actual amount of D isomer found. This is most likely due to incorporation of tritium at positions in the molecule other than the α-carbon atom. Tritium is not completely removed from the labile positions of these amino acids when the mixture is treated with 6 *N* HCl for 24 hr at 110°.

The above findings about the exchange behavior of the side-chain hydrogens of the amino acids were confirmed for some amino acids by proton nmr analysis of samples which had been heated in 6 *N* deuterium chloride (DCl). The doublet at 3.15 ppm (J = 6 Hz) corresponding to the β-hydrogen atoms of aspartic acid (Figure 2a) was completely removed, as shown by the analysis of the amino acid which had been heated in 6 *N* DCl at 110° for 22 hr (Figure 2b). The triplet at 4.39 ppm (J = 6 Hz) due to the α-hydrogen atom was replaced by a singlet in the sample which was heated in DCl. These results indicate that the β-hydrogen atoms of aspartic acid are exchanged with the medium during acid hydrolysis. Another experiment with L-glutamic acid indicated that the γ-hydrogen atoms of this amino acid are labile, in agreement with the results of Ratner, *et al.*[14]

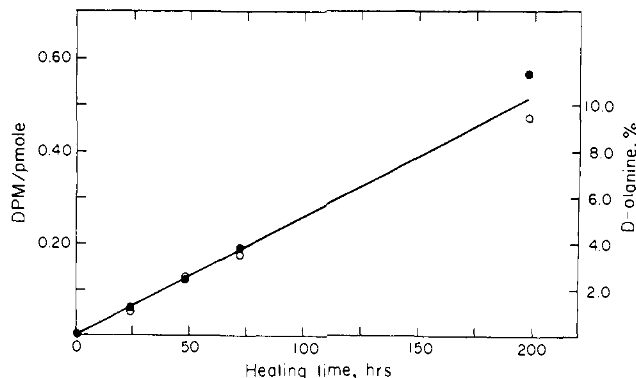

Figure 1. Correlation between the amount of tritium incorporated (●–●) and the amount of D-alanine formed (○–○) during heating of L-alanine in 6 *N* ^{3}HCl at 110°.

Table I. Racemization of Free L-Amino Acids under the Conditions of Acid Hydrolysis.[a] Comparison of the Two Methods of Analysis

L-Amino acid	% D isomer as determined by: ^{3}H incorporation[b]	L–D dipeptide[c]
Alanine	1.4	1.0
Valine	0.2	0.7
Isoleucine	0.4	1.0[d]
Leucine	0.9	1.3
Serine	0.6	0.4
Threonine	0.9	0.5
Lysine	1.8	3.0
Arginine	1.4	1.6
Methionine	2.7	2.2
Proline	2.3	2.2

[a] Hydrolysis was carried out for 22 hr at 110°. [b] The specific radioactivity of the amino acid was converted into the amount of D isomer using Figure 1 as a standard graph; average of two or three determinations, maximum deviation from mean: 0.3%. [c] Determined by the method of Manning and Moore;[3] maximum deviation from mean: 0.5%. [d] Epimerization of L-isoleucine at the α-carbon atom affords D-alloisoleucine which can be separated on the amino acid analyzer.[11]

L-Alanine incorporated about 1/100th as much tritium as did aspartic acid during hydrolysis in ^{3}HCl even though both amino acids are racemized to nearly the same extent. A sample of L-alanine was heated in 6 *N* DCl at 110° for 22 hr and was then analyzed by proton nmr spectroscopy. The quartet at 4.27 ppm (J = 7 Hz) from the α-hydrogen atom and the doublet

(14) S. Ratner, D. Rittenberg, and R. Schoenheimer, *J. Biol. Chem.*, **135**, 357 (1940).

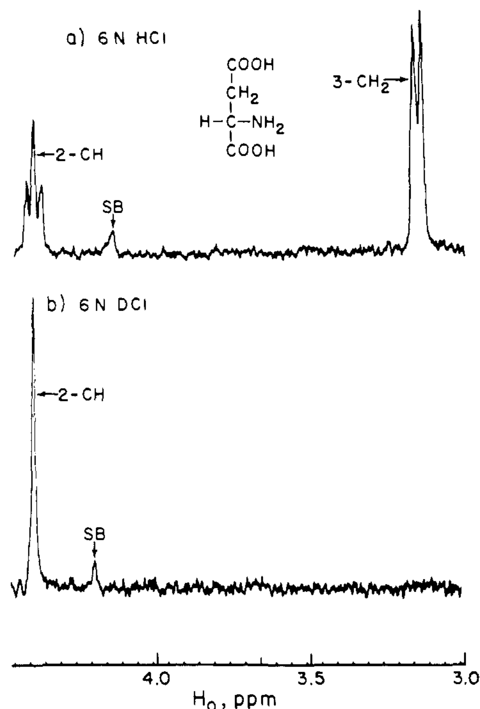

Figure 2. Proton nmr spectra (220 MHz) of L-aspartic acid in D_2O at 20°: (a) aspartic acid deuteriochloride after it was heated in 6 N HCl for 22 hr; (b) aspartic acid deuteriochloride after it was heated in 6 N DCl for 22 hr. SB refers to a side band of the HDO signal. Chemical shifts are relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DDS) as internal standard.

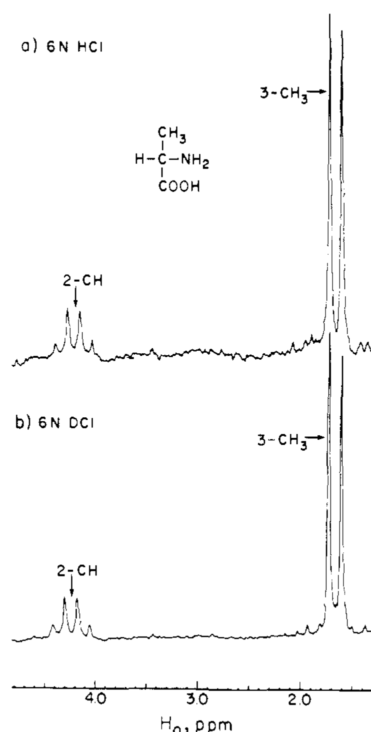

Figure 3. Proton nmr spectra (60 MHz) of L-alanine in D_2O at 20°: (a) alanine deuteriochloride after it was heated in 6 N HCl for 22 hr; (b) alanine deuteriochloride after it was heated in 6 N DCl for 22 hr. Chemical shifts are relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DDS) as internal standard.

at 1.67 ppm (J = 7 Hz) corresponding to the methyl hydrogen atoms were present both in the sample heated in 6 N HCl (Figure 3a) and the sample heated in 6 N DCl (Figure 3b). Thus, the methyl hydrogen atoms of alanine are not exchanged with the medium during acid hydrolysis. These results confirm the finding that incorporation of tritium into alanine is proportional to the racemization which occurs during acid hydrolysis.

Application to the Analysis of Synthetic and Natural Peptides. As a practical test for correction of racemization during acid hydrolysis, synthetic L-bradykinin, which was synthesized by the solid phase method,[10] was chosen for study. The amounts of D-amino acids in the acid hydrolysate of this peptide had been determined previously.[3] The synthetic peptide (1.4 μmol) was heated for 22 hr at 110° in 200 μl of 6 N ^{3}HCl. A portion of the hydrolysate was chromatographed on the amino acid analyzer equipped with the flow-cell scintillation counter. Results for D-serine, D-proline, and D-arginine were calculated from the specific radioactivity of each amino acid (Table II) with Figure 1 as the standard graph; the amounts of the D isomers thus found were formed during the acid hydrolysis. Since the values obtained are the same as those for the total amount of each D-amino acid in the hydrolysate, as determined by the amount of L–D dipeptide, we conclude that these residues in the original synthetic peptide were optically pure. The amount of D-phenylalanine that was formed during acid hydrolysis could not be determined from the tritium incorporation because there was labeling in the side chain of the molecule. However, indirect evidence for the optical purity of the phenylalanyl residues of synthetic bradykinin has been previously provided.[3]

Another use of this method is for detecting labile hydrogen atoms which are a result of interactions between amino acid residues in a peptide. In natural bacitracin A, one isoleucine residue, Ile-1, forms a

Table II. Correction for Racemization Occurring during Acid Hydrolysis of Peptides[a]

Peptide	Amino acid	% D isomer as determined by: ^{3}H incorporation[b]	L–D dipeptide[c]
Synthetic L-bradykinin	Serine	0.1	0.3[d]
	Proline	2.8	2.3[d]
	Arginine	1.7	1.6[d]
Natural bacitracin A	Isoleucine	17.4[e]	15.7[f]
	Leucine	5.8	6.8
	Ornithine[g] + lysine	2.2	3.0[h]

[a] Hydrolysis was carried out for 22 hr at 110°. [b] The specific radioactivity of the amino acid was converted into the per cent D isomer using Figure 1 as a standard graph; maximum deviation from mean: 0.3%. [c] Determined by the method of Manning and Moore;[3] maximum deviation from mean: 0.5%. [d] Previously reported.[3] [e] Epimerization of L-isoleucine affords D-alloisoleucine. [f] Determined as alloisoleucine on the amino acid analyzer. [g] Present as the D isomer in the peptide. [h] Value for lysine.

thiazoline ring with the adjacent cysteine residue, Cys-2. This ring formation results in the removal of the α-hydrogen atom of this particular isoleucine residue during acid hydrolysis to give 0.5 equiv of alloisoleucine.[5] Since the peptide contains three L-isoleucine residues, the complete epimerization of one of them should give

rise to 50% of 33.3% (16.7%) of the D-allo isomer in the hydrolysate; this is close to the value found (Table II). The result from tritium incorporation into isoleucine is also consistent with this course for the hydrolysis. However, in contrast to the findings with alanine, it was observed that alloisoleucine and isoleucine contained different amounts of total radioactivity (5.2×10^5 and 6.3×10^5 dpm, respectively). Heating of free isoleucine in ^{3}HCl for 22 hr leads to 1.4 times more incorporation into isoleucine than into alloisoleucine. This difference indicates that the asymmetric β-carbon atom of the anionic intermediate induces stereoselective protonation of the adjacent α-carbon atom during epimerization. Since isoleucine is formed more rapidly than alloisoleucine, according to Ingold's rule[15] isoleucine is predicted to be thermodynamically the less stable isomer. The finding[16] that complete epimerization of isoleucine leads to an equilibrium ratio for alloisoleucine–isoleucine = 1.3:1 indicates that Ingold's rule is applicable in this case.

The single leucine residue of bacitracin A (Leu-3) is of the L configuration.[17] The amount of D-leucine in the hydrolysate, determined as the diastereoisomeric dipeptide, is 5–6 times higher than that expected from the racemization of free L-leucine during acid hydrolysis (Table I). The amount of D-leucine agrees with that calculated from the amount of tritium incorporated and indicates that the increased racemization of leucine occurred during the hydrolysis. Craig, *et al.*,[17] noted the low optical rotation of the leucine isolated from a hydrolysate of bacitracin A. The increased racemization of Leu-3 during acid hydrolysis may be due to some interaction with Cys-2.

In the chromatographic system used in this study ornithine and lysine were eluted together; the data indicate that these residues in bacitracin A are not racemized any more than the corresponding free amino acids during acid hydrolysis. The present method could not be applied to the other amino acid residues in bacitracin A since they contained exchangeable hydrogen atoms in the side chains.

Exchange Studies with β-Alanine and with Isobutyric Acid. The exchange behavior of a compound in which the amino group is further separated from the carboxyl group was studied. β-Alanine was heated in 6 *N* DCl at 110° for 22 hr. The triplet at 2.84 ppm corresponding to the α-hydrogen atom was completely removed in the DCl-treated sample and the triplet at 3.30 ppm from the β-hydrogen atoms was converted to a singlet.

Isobutyric acid was tested since it bears a structural resemblance to alanine except that a methyl group replaces the amino group. Isobutyric acid was heated in 6 *N* DCl at 110° for 67 hr. As shown by the loss of the multiplet at 2.56 ppm (J = 7 Hz) (Figure 4a), the α-hydrogen atom was almost completely exchanged (Figure 4b) during this treatment. Simultaneously, the doublet at 1.20 ppm (J = 7 Hz) corresponding to the β-methyl protons was almost completely converted to a triplet (J = 1 Hz). This triplet is the result of coupling between the methyl hydrogen atoms and the α-deuterium atom.[18] The remaining doublet is due to the unexchanged α-hydrogen atoms.

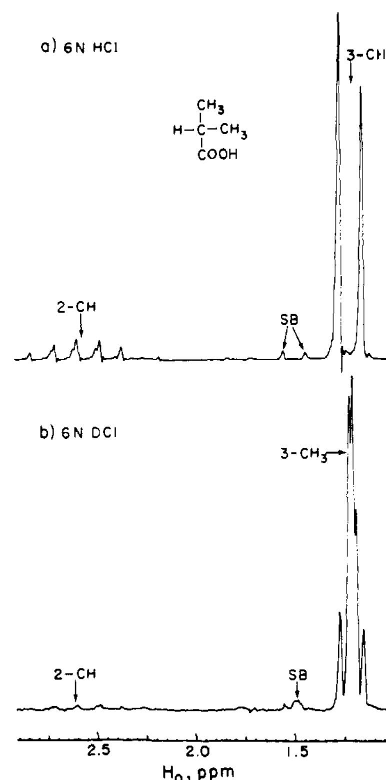

Figure 4. Proton nmr spectra (60 MHz) of isobutyric acid in $CDCl_3$ at 20°: (a) isobutyric acid after it was heated in 6 *N* HCl for 67 hr; (b) isobutyric acid after it was heated in 6 *N* DCl for 67 hr. Chemical shifts are relative to tetramethylsilane (TMS) as internal standard.

Discussion

The method described here can be used to measure the racemization of those amino acids whose side-chain hydrogen atoms are not exchangeable under the conditions of acid hydrolysis (Table I). Hill and Leach[19] studied the exchange behavior of amino acids in strong acid, but they did not identify the positions of the exchangeable hydrogens. With phenylalanine the β-hydrogen atoms are exchanged very slowly probably due to the inductive effect of the adjacent phenyl group; Levene and Steiger[20] showed that phenylglycine is racemized more than phenylalanine during heating in strong acid. The hydrogen atoms ortho to the phenolic hydroxyl group of tyrosine[21] and the C-2 hydrogen atoms of histidine, which are exchangeable at neutral pH,[22] are probably the ones which are exchanged in strong acid. Cystine incorporates considerable tritium during heating in ^{3}HCl concomitant with its almost complete racemization during this treatment.[23]

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Those amino acid residues which cannot be analyzed by this method can be studied in another way. When one of these residues is suspected of undergoing increased racemization during hydrolysis, the segment of the peptide chain in which it occurs can be synthesized and the amount of racemization which takes place during the hydrolysis can be studied in a separate experiment. This approach is tedious, but it was useful in the study of the amount of racemization of Phe-5 during hydrolysis of bradykinin.[3]

Mechanism of Acid-Catalyzed Racemization of Amino Acids. Complete exchange of the β-hydrogens of aspartic acid and the γ-hydrogens of glutamic acid probably proceeds by enolization mediated by the adjacent carboxylic acid group, which exists as the conjugate acid, $-^{+}COOH_2$, in strong acid solution. There is considerably less exchange of the α-hydrogen atom of most amino acids under the same conditions as evidenced by the low amount of racemization (Table I). A possible explanation for these findings is that the α-carboxylic acid group of an α-amino acid is not readily protonated in the presence of a protonated α-amino group because the presence of two positively charged functional groups on the same carbon atom is probably unfavorable. Lactic acid is not measurably racemized during treatment with hot acid;[24] formation of the positively charged conjugate acid of the α-carboxylic acid group may be prevented when the α-hydroxyl group, a stronger base,[25] is protonated ($-^{+}OH_2$). The experiment with β-alanine was done to test this proposal. The results indicate that if the amino group is one carbon atom removed from the α-carboxylic acid group, formation of the conjugate acid of the latter is more favorable. With isobutyric acid, which is not prevented from forming the positively charged conjugate acid, relatively facile replacement of the α-hydrogen atom occurs (Figure 4); about 75% is exchanged compared with about 5% of the α-hydrogen atoms of alanine under the same conditions. These results are consistent with the above proposal.

The racemization of an α-amino acid which does occur in strong acid may be due either to the inductive effect of the α-amino group or to a small amount of the conjugate acid of the α-carboxylic acid group. When glycine is heated in acid, 15% of the α-hydrogen atoms are exchanged in 22 hr, whereas only 2% of the α-hydrogen atoms of alanine are exchanged under the same conditions. Rittenberg, *et al.*,[21] observed exchange of the α-hydrogen atoms of glycine for deuterium. Thus, the inductive effect of the β-carbon atom of an amino acid plays a role, although probably a minor one, in stabilizing the α-C–H bond.

Some amino acid residues in peptides are racemized to the same extent as the corresponding free L-amino acids (*cf.* Tables I and II and ref 3). This may indicate that protonation of the peptide bond, which is favored over formation of the conjugate acid of the free carboxylic acid group,[25] results first in hydrolysis followed by racemization of the free amino acids which are released. Increased racemization of amino acid residues compared with that observed with free amino acids may be due to an interaction between adjacent residues, as in bacitracin A, or to an increased ease of protonation of the α-COOH group of a peptide caused by the decreased tendency of protonation of the adjacent peptide group. The method described in this study can be used to measure the racemization in either case.

Acknowledgments. The author expresses appreciation to Dr. Stanford Moore and Dr. William H. Stein in whose laboratory this work was carried out for their generous support and timely advice, to Dr. R. Bruce Merrifield for suggesting the experimental approach, to Dr. William C. Agosta and Dr. Arnold Stern for helpful discussions and for their suggestions with the nmr studies, and to Miss Wanda Jones for expert assistance in the laboratory.

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