Hydrogen Exchange at Carbon-Hydrogen Sites during Acid or Alkaline Treatment of Proteins

JULIE HILL* AND S. J. LEACH†

From the Division of Protein Chemistry, C.S.I.R.O., Wool Research Laboratories, Parkville, Melbourne, Victoria, Australia

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When proteins were treated with alkali or hot acid, exchange was promoted between hydrogen atoms at CH sites and hydrogen atoms in the aqueous medium. The extent of such exchange was estimated by incorporating tritiated water in the solutions during treatment, removing the labile tritium atoms bound at NH and OH sites, and estimating the residual tritium in the protein by liquid-scintillation counting. Under the conditions normally used for the acid hydrolysis of proteins prior to amino acid analysis, 30–42 g-atoms of hydrogen were exchanged at CH sites per 10,000 g of insulin, lysozyme, ribonuclease, and wool keratin. Examination of the individual amino acids separated by chromatography showed that their susceptibilities to such exchange were closely similar among different proteins. Aspartyl and glutamyl residues were the most readily labeled (1–2 g-atoms of hydrogen/residue), followed by tyrosyl (approximately 1 g-atom of hydrogen/residue). These three acids were also the most readily labeled when a synthetic mixture of amino acids was treated with acid under the same conditions. Optical-rotation measurements showed that the exchange produced very little racemization and probably occurred at CH sites in the β or γ position relative to the peptide bond. Hydrogen exchange at CH sites in proteins occurred also at *p*H 12.5 at room temperature, but the residues affected in this case were glycyl, seryl, threonyl, aspartyl, and cystyl. The extent of exchange in alkaline solution varied much more between different proteins than in acid solution. Like racemization, it did not occur readily with free amino acids and probably involved exchange at CH sites in the α position.

Treatment of proteins in alkaline solution has long been known to cause racemization at certain asymmetric carbon atoms (see, e.g., Neuberger, 1948; Groh and Nyilasi, 1952; Nyilasi, 1956–57). Racemization of free amino acids in alkaline solution also occurs, although at a slower rate. The methods used to study these processes have usually involved bacteriological assays (e.g., Groh and Nyilasi, 1950, 1952) or the measurement of changes in optical rotations. The former method is somewhat laborious and the latter is of low precision, particularly when only limited amounts of material are available and the specific rotations involved are small.

Changes in the optical rotations of proteins on treatment with acids have also been observed (Schein and Berg, 1946). However, racemization occurs less readily in acid than in alkaline solution, and measurable amounts of inversion occur only in concentrated solutions at elevated temperatures (e.g., Neuberger, 1948; Goodman *et al.*, 1962; Ikawa, 1964).

Optical inversion would be expected to be accompanied by an exchange between hydrogen ions in solution and the hydrogen atoms attached to the asymmetric α-carbon atoms in the susceptible amino acid residues. Indeed, it has been observed that when such amino acids or their derivatives are treated under racemizing conditions in the presence of deuterated water, deuterium atoms are incorporated irreversibly into the compounds (Neuberger, 1948). While every exchange event need not necessarily lead to an inversion, it has been found in the limited number of cases studied that in alkaline solutions racemization and deuterium-hydrogen exchange do in fact occur together. With hot acids however, a case has been reported in which exchange occurs at CH sites other than in the α position to the amino acid groups (Ratner *et al.*, 1940). Since radioactive assay methods are more simple and precise than methods for the assay of deuterium, it was considered that these exchange processes might be studied more conveniently by adding tritiated water to the aqueous medium.

The present study using tritium as a tracer was undertaken to provide a quantitative comparison of the extent to which hydrogen exchange occurs in amino acids, whether free or in protein combination. Two standard conditions for exchange were chosen. One of these, *p*H 12.5 at room temperature, may be encountered in carrying out protein-titration curves, during protein extractions and fractionations, or in selective hydrolysis of certain groups in peptides. The other, hydrochloric acid (6 M) at 105°, is normally used for protein hydrolysis prior to amino acid analysis using chromatographic procedures.

Materials and Methods

Proteins and Amino Acids.—The proteins used were: ribonuclease (recrystallized, bovine pancreatic, Armour lot BM 2470), insulin (recrystallized, bovine, Boots lot 2189, zinc-free), lysozyme (recrystallized, egg white, Armour lot ED 1990), and wool keratin (Lincoln, MW 144). Their concentrations in solution after tritium labeling were determined by Kjeldahl-N assay, assuming total-N values of 17.5, 15.85, 18.6, and 17.0% respectively.

The standard mixture of 17 L-amino acids (chromatographically pure, California Corp. for Biochemical Research) contained 200 μmole/l of each acid and the Kjeldahl-N content of the mixture was 13.55% (theory, N 13.72%).

Tritiated Water.—Tritiated water (specific activity approximately 5 c/ml, the Radiochemical Centre, Amersham, England) was diluted to 5 mc/ml with deionized water for use as a stock solution, and its specific activity was determined.

Optical Rotations.—Optical rotations (D line) of L-glutamic acid, L-aspartic acid, and L-tyrosine in HCl (6 M) were measured before and after heating. Meas-

* Present address: The Clayton Foundation Biochemical Institute, The University of Texas, Austin 12, Texas.

† To whom communications should be addressed.

urements were made in 10-cm tubes at 25° using a Stanley photoelectric polarimeter in conjunction with a Carl Leiss quartz prism monochromator and a sodium-light source.

Exchange in Hot Acid.—Proteins or free amino acids (25 mg) were added to the acid medium (AR concd HCl diluted 1:1 with tritiated water of 5 mc/ml to give a final concentration of 5.98 M; 1 ml) in a Pyrex tube. The tubes were sealed and heated inside a metal container at 105° for 24 hours or longer. The contents were then freeze-dried and "labile" tritium (i.e., attached at NH or OH sites) was removed by diluting the residue three times in water (1 ml), first at 25° and then twice at 40°, freeze-drying each time.[1] The residues were dissolved in water (250 μl) and separate aliquots (50 μl) were removed for radioactive assay, Kjeldahl-N estimation, or separation by two-dimensional chromatography.

Exchange at pH 12.—Ribonuclease and wool (25 mg) were added to tritiated water (2.5 mc/ml, 2 ml) and adjusted to *p*H 12.5 using KOH (1 M, *ca.* 50 μl). After exchanging at 25° for 4–10 days, the proteins were freeze-dried and neutralized to *p*H 4 by adding acetic acid (1 M, 5 μl) in water (3 ml). The solutions were warmed and freeze-dried, the procedure being repeated three times in order to remove all "labile" tritium. The ribonuclease residue was finally diluted in water (500 μl), an aliquot (50 μl) was removed for tritium assay, and a further aliquot was hydrolyzed with HCl (6 M) at 105° for 24 hours in a sealed tube. The keratin (*ca.* 7 mg) had to be dissolved in KOH solution (0.2 M) at 90° before assay for "total tritium," the remainder being hydrolyzed in HCl as above. The hydrolysates in each case were then freeze-dried and washed as described in the previous paragraph, before being subjected to chromatography in order to determine the fraction of the total tritium label in each amino acid.

Chromatography.—The neutralized and washed radioactive mixtures of amino acids or hydrolysates were separated on Whatman 3MM paper by two-dimensional chromatography. Butanol–acetic acid–water (4:1:1) was used in the first dimension, and phenol–water (4:1) in the second dimension (Berry *et al.*, 1951). For a clearer separation of tyrosine and the three basic amino acids a system devised by Eager *et al.* (1964) was also used. This involved paper electrophoresis at *p*H 2.3 (1 M acetic acid) in one direction, followed by chromatography with 2-butanol–2-methoxyethanol–formic acid–water (8:2:1:3) at right angles. After drying, the papers were sprayed with dilute ninhydrin (0.04%) to locate the amino acid spots.

Radioactive Assay.—Assays were carried out with a Packard Tri-Carb liquid scintillation spectrometer (series 314EX) with samples, photomultiplier tubes, and preamplifiers, at −2°. The tritium contents of proteins, amino acids, or protein hydrolysates were estimated by adding a neutralized, aqueous aliquot (approximately 5 mg in 50 μl) to water (1.95 ml), adding dioxane-based scintillation mixture, and counting as described by Leach and Hill (1963). To estimate the number of exchanged hydrogen atoms, counts were compared with those of known volumes of the stock tritiated water *before* addition of hydrochloric acid, since the latter is a powerful quenching agent.

The radioactivities of the individual amino acids separated by two-dimensional paper chromatography were determined by cutting out the spots revealed by ninhydrin, and counting the disks of paper after immersion in a toluene-based scintillation mixture. These activities were compared with the radioactivity of a similar quantity of neutralized hydrolysate which had been spotted onto a disk of 3MM paper and treated with the two chromatographic solvents. The spot was sprayed with ninhydrin and counted in the toluene-based scintillation mixture. The activities of such paper disks, counted without chromatographic separation, were used to calculate the percentage recovery of radioactivity during chromatography.

As found by Nunez and Jacquemin (1961), the count was independent of the volume of scintillation mixture used. The residual activity on removing the paper disks from the counting vials was small, indicating little leaching out of amino acids; assays on separated amino acids were corrected for these small blanks. While the counting efficiency for a tritiated water standard in solution was 13.5%, the counting efficiency of tritiated water when absorbed on 3MM paper was only 1.5%. Quenching due to the paper and to traces of butanol and phenol from chromatography could account for the decrease in counting efficiency of hydrolysates on paper. The purple color produced by the ninhydrin reagent was found to have little or no effect on the count, but the characteristic strong yellow-brown color of lysozyme hydrolysates quenched the count appreciably.

[1] The effectiveness of this procedure for removing labile tritium was tested and found adequate using proteins which had been labeled under neutral or weakly acid conditions.

Results and Discussion

Exchange at CH *Sites in Hot Acid.*—The total number of g-atoms of hydrogen exchanged at CH sites per 10,000 g of each protein is shown in Table I. After hydrolysis for 24 hours at 105° the number varies between 30 and 42, with reproducibility of ±2 g-atoms of hydrogen between triplicate experiments. It is seen in the case of wool that the exchange increases slowly with the time of hydrolysis. In the case of lysozyme the yellow color of the hydrolysate might exert a quenching effect on the scintillator, so that the value of 30 might be a low estimate.

Table I

Number of Gram-Atoms of H Exchanged at CH Sites per 10,000 g of Various Proteins during Hydrolysis with HCl (6 M) at 105° for 24 Hours, in the Presence of Tritiated Water

Protein	H Atoms Exchanged[a]
Insulin	42
Lysozyme	30
Ribonuclease	33
Wool keratin	33
Wool keratin	41[b]
Wool keratin	48[c]
Amino acid mixture	10

[a] Mean of three experiments (±2 H atoms). [b] Hydrolysis for 32 hours. [c] Hydrolysis for 48 hours.

The proportion of radioactivity in each hydrolysate (measured on paper) which was recovered from the seventeen radioactive spots on the paper chromatograms is shown in Table II. In addition, up to 10% of the total radioactivity remained at the origin of the chromatograms. The apparently high recovery for lysozyme (above 100%) is no doubt due to quenching of the count in the unchromatographed hydrolysate spot by the yellow humin deposited on the paper. This material is of course absent in the amino acid spots.

The amount of radioactivity in each amino acid spot

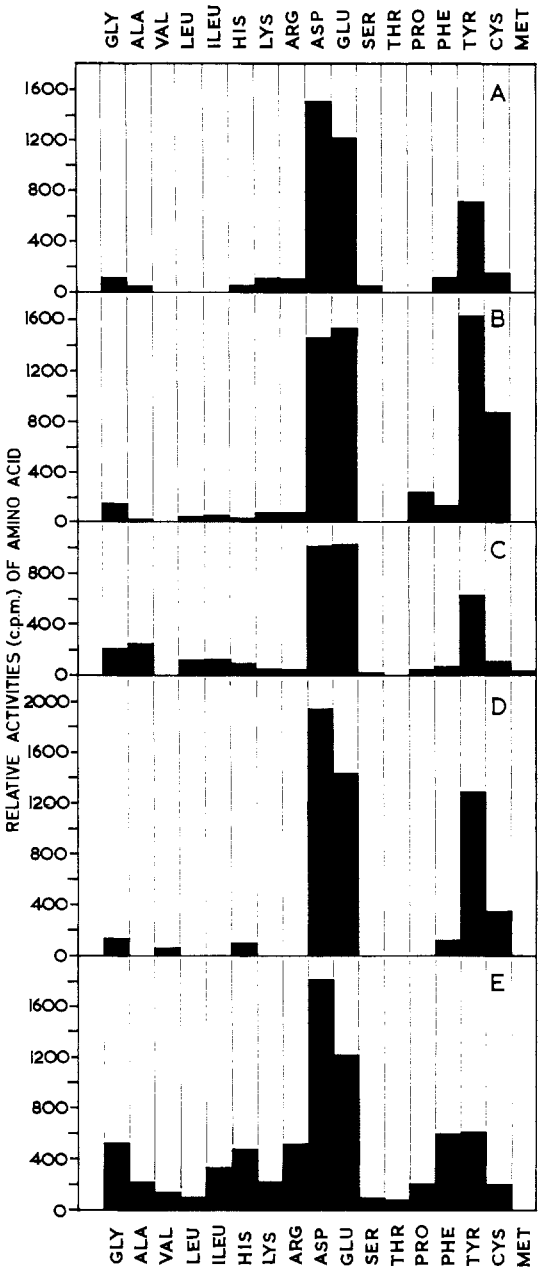

FIG. 1.—Radioactivity incorporated in various amino acid residues during hydrolysis with HCl (6 M) at 105° for 24 hours in the presence of tritiated water: (A) insulin, (B) lysozyme, (C) ribonuclease, (D) wool keratin, (E) wool keratin (84-hour hydrolysis). Ordinate values (cpm) are calculated for 10 mg of protein and corrected for the relative abundance of each amino acid in the different proteins by dividing by the number of residues per 10,000 g.

is given in Figure 1. The counts are calculated[2] for 10 mg of each protein and corrected for the relative abundance of each amino acid by dividing by the number of residues per 10,000 g of protein. The pattern is very similar for all four proteins (Fig. 1A–D) showing preferential exchange at aspartyl, glutamyl, and tyrosyl residues. Cystyl and glycyl residues show some evidence of exchange and the other residues are only slightly radioactive even after 48 hours (Fig. 1E). The results for tyrosine were the most variable and the decrease in its radioactivity on continued hydrolysis suggests that decomposition may occur.

[2] The activities as measured were up to 20,000 cpm per chromatogram spot, using 3–4 mg of hydrolysate.

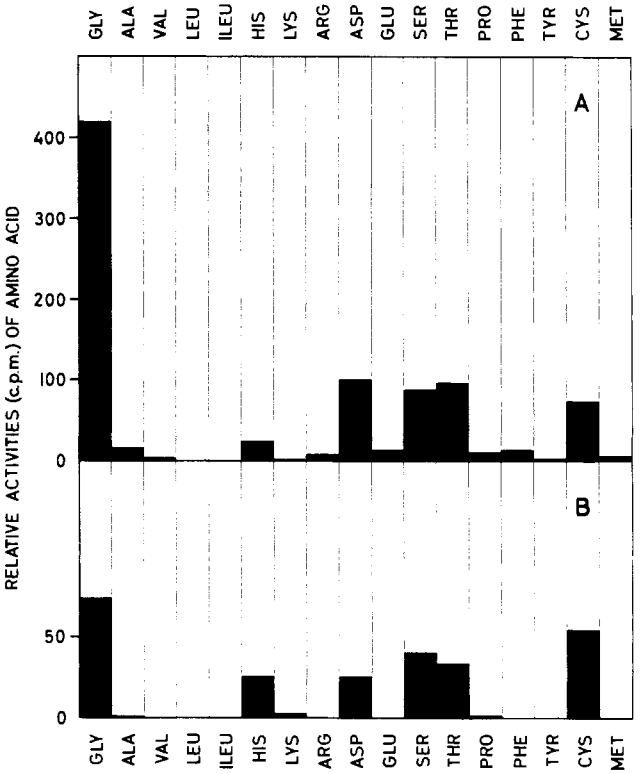

FIG. 2.—Radioactivity incorporated in various amino acid residues during incubation at *p*H 12.5 and 25° for 4 days in the presence of tritiated water and subsequent hydrolysis with HCl (6 M) at 105° for 24 hours. (A) ribonuclease, (B) wool keratin. Ordinate values (cpm) are calculated as in Fig. 1.

TABLE II

PERCENTAGE RECOVERY OF RADIOACTIVITY DURING PAPER CHROMATOGRAPHY

Protein	Recovery (%)[a]
Insulin	73
Lysozyme	>100
Ribonuclease	77
Wool keratin	91
Wool keratin	74[b]
Wool keratin	74[c]

[a] Mean of three experiments (±5%). [b] Hydrolysis for 32 hours. [c] Hydrolysis for 48 hours.

The number of g-atoms of hydrogen replaced in each amino acid has been calculated and the results are shown in Table III. Between one and two hydrogen atoms are replaced in each residue of aspartic acid, glutamic acid, or tyrosine, and there appear to be no major differences in this respect among the four proteins. The table also shows (last column) that of the synthetic mixture of seventeen free amino acids, those most susceptible to exchange are similar in this respect to the proteins, although their extent of exchange is somewhat less. When aspartic acid, glutamic acid, and tyrosine were each treated separately with hydrochloric acid (6 M) at 105° for 24 hours, the number of hydrogen atoms exchanged per molecule was again one or two and under these conditions, the changes in the specific optical rotation were comparatively small (see Table IV).

The proportions of DL-glutamic and aspartic acids isolated from acid hydrolysates of proteins or polypeptides are usually no more than 5–10% (Neuberger, 1948; Chibnall *et al.*, 1943; Opsahl and Arnow, 1942;

TABLE III

NUMBER OF GRAM-ATOMS OF HYDROGEN EXCHANGED AT CH SITES PER RESIDUE WEIGHT OF AMINO ACIDS IN VARIOUS PROTEINS DURING HYDROLYSIS WITH HCl (6 M) AT 105° FOR 24 HOURS

	Insulin	Lysozyme	Ribonuclease	Wool Keratin	Wool[a] Keratin	Wool[b] Keratin	Amino Acid Mixture
Glycine	0.1	0.1	0.2	0.1	0.1	0.7	0.1
Alanine						0.3	
Valine						0.2	
Leucine			0.1			0.1	
Isoleucine			0.1			0.5	
Histidine		0.1	0.1			0.6	
Lysine	0.1					0.3	
Arginine	0.1					0.2	
Aspartic acid	2.2	1.1	1.2	2.0	2.7	2.4	0.7
Glutamic acid	1.7	1.4	1.3	1.5	1.9	1.6	0.4
Serine							
Threonine							
Proline		0.2				0.3	
Phenylalanine	0.1	0.1			0.2	0.8	
Tyrosine	1.0	1.4	0.8	1.4	1.0	0.8	0.6
Cystine	0.2	0.6	0.2	0.4	0.3	0.3	0.1
Methionine							

[a] Hydrolysis for 32 hours. [b] Hydrolysis for 48 hours. Values below 0.05 are omitted.

TABLE IV

NUMBER OF GRAM-ATOMS OF H EXCHANGED AT CH SITES, AND CHANGES IN SPECIFIC ROTATION OF FREE AMINO ACIDS AFTER HEATING WITH HCl (6 M) AT 105° FOR 24 HOURS

Amino Acid	H Atoms Exchanged	Specific Rotation[a] Before Heating (°)	After Heating (°)
L-Glutamic acid	1.0	+30.3	+27.3
L-Aspartic acid	1.9	+24.6	+21.3
L-Tyrosine	1.6	− 7.8	− 5.8

[a] Specific rotation using the D line.

Goodman *et al.*, 1962; Ikawa, 1964), agreeing with our observations on the free amino acids. The large number of hydrogen atoms exchanged and the small magnitude of the changes in optical rotation preclude the α-carbon CH groups as the main sites of exchange in hot acid. Ratner *et al.* (1940) have already shown that the hydrogen atoms attached to the α- and β-carbon atoms in free glutamic acid are stable to boiling hydrochloric acid but that the two hydrogen atoms attached to the γ-carbon atom may be exchanged under these conditions. The present observations suggest that the activating influence is the COOH side chain which labilizes only those hydrogen atoms which are attached to the *adjacent* carbon atom. In tyrosine the activating influence of the phenolic –OH group is presumably transmitted via the benzene ring to the side-chain $-CH_2-$ group. If the main activating influence originates in the acidic or phenolic side chain, this would explain why the three types of residue show a similar susceptibility to exchange whether combined in different proteins (Table III) or in the free state (Table IV). The catalysis of hydrogen exchange at CH sites by strong acids has been well documented for compounds other than amino acids (see, e.g., Olsson, 1959; Gold and Satchell, 1963).

Exchange at CH *sites at* pH *12.5.*—The total number of g-atoms of hydrogen exchanged at CH sites after 4 days at pH 12.5 varied with the protein. Of the two proteins studied, ribonuclease[3] consistently incorporated three times as many g-atoms of tritium (viz., 30–35 per 10,000 g protein), as did wool keratin. However, the amino acid residues responsible for the uptake were the same in both cases. Figure 2 shows that glycyl, seryl, threonyl, aspartyl, and cystyl residues are the most susceptible to exchange with no significant amounts of radioactivity detectable in other residues. Although it may be noted that the measured amounts of radioactivity were considerably smaller than after labeling in acid, this decrease was caused by poor chromatographic recoveries, and replicate experiments showed qualitatively a very similar pattern to that of Figure 2.

Solutions of free amino acids, either singly or in a mixture, showed little evidence of tritium exchange at CH sites at pH 12.5 (see Table V). Cystine was the most susceptible amino acid under these conditions, exchanging 0.2 g-atoms of hydrogen per mole.

TABLE V

NUMBER OF GRAM-ATOMS OF H EXCHANGED AT CH SITES PER MOLE OF FREE AMINO ACID AT pH 12.5 AND 25° FOR 10 DAYS, IN THE PRESENCE OF TRITIATED WATER

Amino Acid	H Atoms Exchanged
Glycine	0.02
Aspartic acid	0.03
Serine	0.02
Threonine	0.01
Cystine	0.20
Amino acid mixture	0.02

The CH sites in proteins at which exchange occurs in alkaline solutions are probably in the α positions of the amino acid residues. Hydrogen exchange at such sites would be expected to lead to optical inversion. Indeed, three of the five amino acids responsible for tritium labeling at CH sites in proteins, viz., serine, threonine, and cystine, are known to racemize readily in alkalis (Neuberger, 1948; Groh and Nyilasi, 1952; Bohak and Katchalski, 1963). The racemization of aspartyl residues in alkali has not been investigated thoroughly, while glycine is of course optically inactive.

The stability of hydrogen atoms at α-CH sites should be more dependent upon amino acid environment than should the side-chain hydrogen atoms which

[3] This is similar to an earlier estimate for ribonuclease under similar conditions (Leach and Hill, 1963).

are more remote from peptide bonds. This would explain why, comparing ribonuclease and wool, the same amino acids show different susceptibilities, and why free amino acids are much more resistant to exchange than combined amino acids. This variability is in marked contrast with the type of CH exchange occurring in acid solution (Tables III and IV) where the β- or γ-carbon atoms are involved. Variations have been noted in the susceptibilities of serine, threonine, and other amino acids to racemization in alkaline solutions and this provides further justification for equating hydrogen exchange with racemization. It is interesting to note that glycine, with two hydrogen atoms in the α position, shows a greater amount of exchange at *p*H 12.5 than any other amino acid.

The quantitative assessment of data such as those of Figure 2, obtained after exchange at *p*H 12.5, is complicated by the necessity for acid hydrolysis before the amino acids can be separated. It might be anticipated that some of the tritium atoms incorporated at CH sites during tritiation at *p*H 12.5 would be lost later owing to acid hydrolysis. However, it was found that the loss of radioactivity in the hydrolysis step was only 15%. We have seen that the CH sites involved during alkaline treatment are predominantly in the α position, whereas the β or γ positions are mainly involved during treatment with hot acid. The small magnitude of the loss in activity during acid hydrolysis is further evidence that two different types of CH site are involved, especially when it is considered that three of the amino acid residues (aspartyl, glycyl, and cystyl) are susceptible to labeling in *both* treatments.

Acid hydrolysis precludes the possibility of examining the exchange behavior of tryptophan during acid or alkaline treatments, since this amino acid is largely destroyed under the hydrolytic conditions. One might expect extensive hydrogen exchange in the indole ring under acid or alkaline conditions (see, e.g., Challis and Long, 1963) and losses in radioactivity during hydrolysis may be caused in part by the destruction of C-tritiated tryptophanyl residues.

Conclusions

It has been shown that, in addition to the amino acid residues (seryl, threonyl, and cystyl) which are reported to racemize readily, two others (glycyl and aspartyl) show hydrogen exchange at CH sites in alkaline solution. The weight of evidence suggests that this exchange occurs at the α-carbon atoms. In hot concentrated hydrochloric acid, conditions under which little racemization occurs, extensive hydrogen exchange also takes place. However, in this case the residues concerned (aspartyl, glutamyl, and tyrosyl) are probably labeled at the β- or γ-carbon atoms.

It is therefore possible to estimate the extent to which racemization may occur[4] during alkaline titrations, extractions, or fractionations of proteins, or in the coupling stages of peptide synthesis, by incorporating tritiated water into the solvent medium. To distinguish between α-labeling and β- or γ-labeling in the product, it is necessary merely to test the lability of the tritium label toward alkaline and acid conditions, respectively.

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[4] An exact correlation would require that every exchange event led to an optical inversion.