being used. The residue was in each case a glass. The ultraviolet spectra of these glasses were determined and those of dihydrohelvolic and helvolic acids were qualitatively the same as the spectrum of pyrohelvolic acid in the 260–300 mμ region. Pyrotetrahydrohelvolic acid showed no absorption in this region. The pyrolyses were then repeated with 10–30 mg. samples. The volatile distillate from helvolinic acid was isolated, and its infrared spectrum proved to be identical with that of acetic acid. The distillate from another sample of helvolinic acid was titrated and found to correspond to 96% of one mole of acetic acid per mole of helvolinic acid taken. A similar titration showed that tetrahydrohelvolic acid evolved 81% of one mole of acetic acid under these conditions.

Selenium Dehydrogenation of I.—A 24.0-g. sample of I was first reduced to the octahydro derivative with 1 g. of platinum oxide in 60 ml. of acetic acid. The reduction was complete in 3 hours and, after removal of the catalyst by filtration, the solvent was distilled in vacuum. The residual white froth was powdered and the powder was added portionwise to a suspension of 9 g. of lithium aluminum hydride in 800 ml. of ether. The mixture was heated under reflux for 3 hours, and the complex was then decomposed by the addition of 10 ml. of ethyl acetate followed by water. After the proper amount of water was added, the ether phase was decanted from the solid sludge and the solvent was evaporated. The residue was dried at 100° under vacuum, and then was mixed with 21 g. of selenium powder. The mixture was kept at 150–250° for one hour and then at 360–370° for 28 hours under a nitrogen atmosphere. The cooled residue was extracted with ether and, after filtration of the solution, the volume was reduced to 5 ml. The concentrated solution was then placed on a column containing 10 g. of neutral alumina of activity I,[32] and the material was eluted with pentane, then pentane–ether, 20-ml. fractions being collected. The first three fractions contained oils which were not investigated. Fractions 4 and 5 contained a total of about 150 mg. of a brown semi-solid. Later fractions contained only tarry materials and were not investigated. Fractions 4 and 5 were combined and rechromatographed on a similar column of alumina with 10% ether in pentane. In this case fractions 2–4 contained about 50 mg. of a gummy yellow solid. Ten ml. of ethanol was added to the solid and, after warming, the solution was decanted from the insoluble tar. The solution was diluted with a little water and allowed to cool. The yellow solid was collected by filtration, wt. 15 mg., m.p. 170–195°. This material was recrystallized and gave 7 mg. of a yellow solid, m.p. 195–210°. The ultraviolet spectra were determined for both of the samples and were identical (Fig. 2).

LOS ANGELES, CALIFORNIA

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF DENTAL RESEARCH AND THE NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE]

C^{14} Isotope Effect on the Ion-exchange Chromatography of Amino Acids

BY K. A. PIEZ AND HARRY EAGLE

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The ion-exchange chromatography on Dowex 50 of amino acids labeled with C^{14} was studied. Labeling of the carbon adjacent to an ionized atom, such as the 1- or 2-position in an α-amino acid, resulted in slower movement of the labeled molecules on the column with the result that the specific activity of successive fractions within the amino acid band increased. The presence of the isotope in positions separated from a charged center, such as serine-3-C^{14} and valine-4-C^{14}, had no effect on the ion-exchange behavior. The possibility is discussed that an inductive effect related to the heavier isotope is responsible for the observed differences.

In a preliminary communication,[1] we reported a systematic effect of C^{14}-labeling on the ion-exchange chromatography of randomly labeled[2] amino acids. The labeled amino acid molecules traveled somewhat more slowly on a column of Dowex 50 eluted with a citrate buffer (*p*H gradient) with the result that the specific activity of successive fractions within a peak was not constant but increased progressively. The fact that the magnitude of the isotope effect was related (inversely) more closely to the number of carbon atoms than to the molecular weight suggested that the effect of a C^{14} atom depended on its occupying a particular position in the molecule rather than solely on its higher mass. As here reported, this hypothesis has been confirmed by chromatographing specifically labeled amino acids.

Experimental

Ion-exchange Chromatography.—The ion-exchange methods were the same as those devised by Moore and Stein[3] except as indicated. For the separation of the acidic and neutral amino acids, *p*H gradient elution[4] of a 100 × 0.9 cm. column of Dowex 50-x12, minus 400 mesh[5] at 50° was used. The *p*H gradient was obtained by an equal level arrangement[4] employing a 300-ml. straight-sided bottle with an inside diameter of 56 ± 0.5 mm. for the mixing chamber and a 500-ml. Florence flask with a 12 cm. neck, 26 ± 0.5 mm. inside diameter, for the reservoir. The reservoir was filled with 0.25 *N* NaOH (containing 1% BRIJ 35 solution)[3] and the mixing chamber with 250 ml. of citrate buffer, *p*H 3.10 (prepared from 245 g. of sodium citrate (dihydrate), 600 g. of citric acid (monohydrate), 50 ml. of thiodiglycol, water to make 10 l. and 1% BRIJ 35 solution). Pressure was applied to both flasks to maintain a flow rate of 6 ml./hr. (about 2.5 lb. at 50 cm. below the top of the column). This provided a very gradual, slightly concave *p*H gradient, starting at *p*H 3.10 and reading *p*H 3.3 at about 200 ml. After 150 ml. of effluent had been collected, the mixing chamber was closed to provide a constant volume mixer,[4] which produced a sharply increasing gradient reaching *p*H 6 at about 330 ml. of effluent.

Collection of fractions was stopped at this point, after the emergence of phenylalanine. Elution was continued for at least 50 ml. after the effluent became alkaline. The column was washed with about 50 ml. of the *p*H 3.10 buffer before starting the next analysis.

The following specifically labeled amino acids were chromatographed in the amounts indicated (the figures showing

(1) K. A. Piez and H. Eagle, *Science*, **122**, 968 (1955).

(2) The expression "randomly labeled" is suggested as more accurate than the more common "uniformly labeled," since the latter term is true only in a statistical sense and does not necessarily apply to the individual molecule. In fact, in the usual sample of C^{14}-labeled material the level of activity is such that most of the labeled molecules contain only one C^{14} atom, randomly placed.

(3) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

(4) K. A. Piez, *Anal. Chem.*, **28**, 1451 (1956).

(5) Rescreened (wet) through 200 mesh. It has been our experience that approximately 90% of the resin as received can be washed through a 200 mesh sieve with a jet of water. One lot contained only 50% that passed 200 mesh. It was not suitable for chromatography, giving very poor resolution. The cause appeared to be too narrow a size range, that is, nsufficient very fine resin to give a solidly packed column.

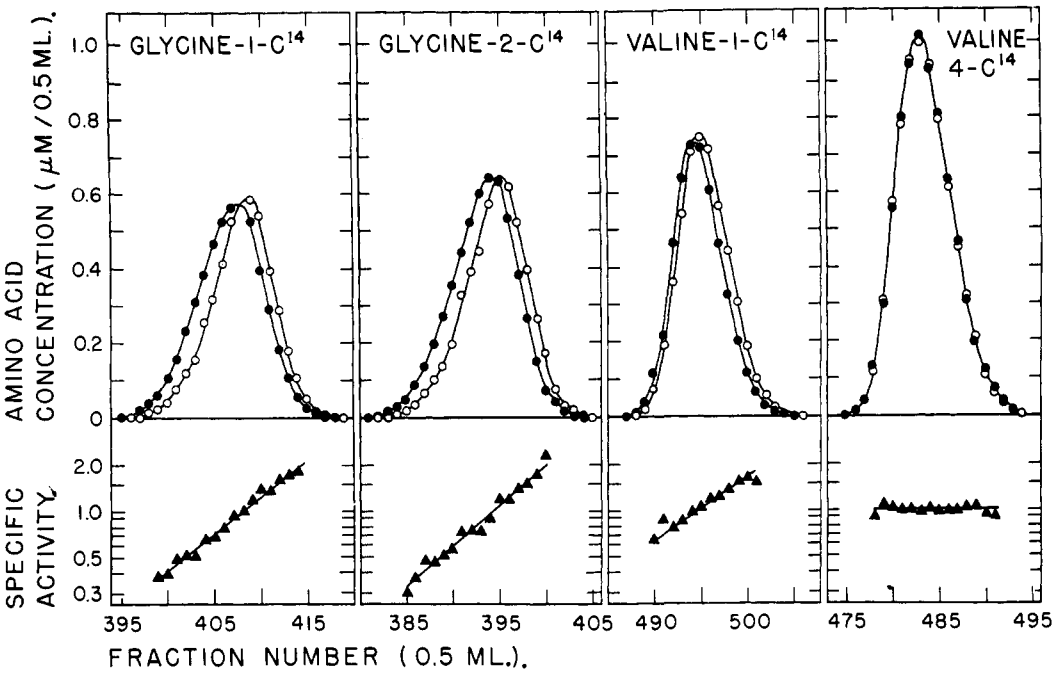

Fig. 1.—Portions of effluent curves from the ion-exchange chromatography of C^{14}-labeled amino acids on Dowex 50-x12: ●, amino acid concentration as determined by ninhydrin; O, amino acid concentration as measured by C^{14} count assuming no change in specific activity; ▲, specific activity found.

μmoles and μc., respectively): glycine-1-C^{14}, 4.8, 2.0; DL-alanine-1-C^{14}, 5.0, 2.8; DL-valine-1-C^{14}, 4.7, 2.4; DL-serine-3-C^{14}, 4.2, 2.0; DL-glutamic-2-C^{14}, 5.2, 1.4; glycine-2-C^{14}, 5.3, 2.8; DL-valine-4-C^{14}, 8.2, 1.7; DL-phenylalanine-2-C^{14}, 13.8, 2.4; DL-glutamic acid-1-C^{14}, 5.9, 2.0; DL-aspartic acid-4-C^{14}, 4.5, 2.0. One-half ml. fractions were collected. Each was diluted with 2 ml. of water and 1-ml. portions were taken for determination of total amino acid concentration with ninhydrin,[6] the remainder being kept for measurement of C^{14} activity.

The randomly labeled amino acids which were chromatographed have been described.[1] Randomly labeled glycylglycine was prepared from randomly labeled glycine through the diketopiperazine[7]; 6.52 μmoles, 0.13 μc., of the peptide was chromatographed.

Determination of C^{14}-Activity.—The fractions from the ion-exchange column were counted in a gas flow counter equipped with an automatic sample changer and printer-timer. Dilutions were made where necessary to bring the activity to a convenient range. One-half ml. portions were dried in 2.5 cm. copper planchets. When the samples were not diluted, a circular piece of lens paper approximately 2.4 cm. in diameter was placed in the planchet before the sample was dried. This allowed more even distribution of the buffer salts as the sample dried and correspondingly greater reproducibility of counts. Self-absorption due to solids in the eluting buffer varied with the composition and the correction factor ranged between 2 and 3. Within a single peak, however, the absorption was essentially constant. Its magnitude was determined by adding a known amount of the labeled amino acid to similar 0.5-cc. aliquots of occasional samples. The difference in counts between the sample and the sample plus the known amino acid gave the counts per μmole amino acid in the particular fraction.

Results

For each amino acid two effluent concentration curves were determined, one based on ninhydrin color and the other on C^{14}-activity assuming no change in specific activity. Four such pairs of curves for some representative amino acids appear in Fig. 1. It is apparent that a partial resolution of labeled from unlabeled molecules occurred in some cases. The curve determined by ninhydrin represents actual concentration, while the C^{14}-activity curve shows only an apparent concentration in those cases where partial resolution occurred. The ratio of the apparent concentration to the actual concentration provides a measure of relative specific activity in successive fractions.

If it is assumed that each pair of curves can be represented by normal distribution curves with the same standard deviations but not necessarily the same means, an equation relating specific activity to fraction number can be derived from the ratio of two distribution curves. Thus

$$\ln S = \frac{\mu_1 - \mu_2}{\sigma_2} X + \frac{\mu_2^2 - \mu_1^2}{2\sigma^2}$$

where S is the specific activity, μ_1 is the mean of the C^{14}-activity curve, μ_2 is the mean of the ninhydrin color curve, σ is the standard deviation, and X is the fraction number. It is apparent from this equation that a plot of the logarithm of the specific activity *versus* fraction number should be a straight line. That this is the case is shown in Fig. 1.

The slope of this line is a measure of the degree of resolution. It varies directly with the difference between the peaks and inversely with the width of the peak (expressed as the standard deviation squared). It is certainly a valid measure for comparing the isotope effects of the same amino acid labeled in different positions. The slope can also be used to compare different amino acids but not necessarily in a quantitative sense. Although amino acids are similar compounds, there are differences in exchange behavior, unrelated to labeling, which can affect the slope. For example, glutamic acid does not show as sharp an elution peak as glycine. Therefore, the slopes observed for

(6) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

(7) H. F. Schott, J. B. Larkin, L. B. Rockland and M. S. Dunn, *J. Org. Chem.*, **12**, 490 (1947).

TABLE I

SLOPES[a] OF LOG (SPECIFIC ACTIVITY) CURVES

Amino acid	Random	Position of C^{14}-label 1	2	3	4
Asp	5.4 ± 0.5[b]				3.0 ± 0.2
Thr	4.0 ± .2				
Ser	7.2 ± .7[b]			0.4 ± 0.5	
Glu	1.8 ± .4[c]	5.2 ± 0.3	5.0 ± 0.2		
Pro	2.6 ± .3		6.2 ± .4[d]		
Gly	12.5 ± .3[b]	9.6 ± .3	10.5 ± .4		
Ala	7.0 ± .2[b]	9.6 ± .4			
Val	2.8 ± .3	6.8 ± .6			−0.4 ± 0.3
Phe			2.4 ± .2		
Glygly	4.4 ± .4				

[a] 100 log (apparent concn./actual concn.) ± standard error. [b] Pooled value from three experiments. [c] Pooled value from two experiments. [d] Derived from glutamine-2-C^{14} biosynthetically.

these two amino acids can only be used for a qualitative comparison. There may also be differences in the sharpness of the peaks resulting from differences in the magnitude of the *p*H gradient used to elute the amino acids. However, in this case, any sharpening will be balanced by a relative pushing together of the two peaks.[8] The slopes will also be independent of the level of activity because of the logarithmic relationship.

The slopes of the log specific activity curves were calculated by the method of least squares. Since the ninhydrin determinations were subject to a relatively large error at the leading and trailing edges of the peaks, concentrations which gave an absorbance less than 0.1 were not used in the calculation. The slopes are presented in Table I, together with their standard errors. Also included are the data for randomly labeled amino acids. In some cases the slopes are pooled values from one chromatogram of known amino acids and two chromatograms of protein hydrolysates from Hela cells grown in tissue culture[9] in the presence of randomly labeled glucose. As anticipated, the slopes in the two types of experiment did not differ significantly despite several hundredfold differences in the specific activities.

Discussion

From the data in Table I, the following conclusions can be drawn: (1) The presence of a C^{14} atom adjacent to a charged atom results in an isotope effect. (2) The 1- and 2-positions are essentially equivalent. This was found with both glutamic acid and glycine and may be presumed to apply generally to α-amino acids. (3) A C^{14}-atom in a carboxylate group at a distance from the ammonium group, such as the 4 position of aspartic acid, has a smaller isotope effect than if it were in the 1-position. This follows from the data obtained for aspartic and glutamic acids. A slope for aspartic acid-1-C^{14} can be calculated from the observed values for the randomly labeled and the 4-labeled compounds and the reasonable assumptions that the 1- and 2-position are equivalent and the 3-position is without effect. The value obtained is 9.3 ± 1.1, approximately three times the observed value for the 4-labeled amino acid. Similarly, glutamic acid-5-C^{14} should show little or no isotope effect since the observed value for the randomly labeled amino acid can be accounted for, within experimental error, by the observed slopes for the 1- and 2-labeled compounds. (4) The presence of C^{14} in a position not bearing an ionic group is without an effect on the ion exchange behavior of the amino acid. Two of the amino acids, serine-3-C^{14} and valine-4-C^{14}, give direct evidence on this point. Further evidence is supplied by the fact that the slopes observed for randomly labeled glutamic acid, proline, glycine, alanine and valine can be fully accounted for by the isotope effect resulting from the corresponding 1- and 2-labeled amino acids. In the case of glycine it is necessary to correct the slope obtained for the randomly labeled amino acid for the presence of doubly labeled molecules, amounting to approximately 20% of the total number of labeled molecules.[1] (5) The α-amino structure is not uniquely involved since aspartic acid-4-C^{14} and randomly labeled glycylglycine show an isotope effect. In the case of the peptide the observed slope is approximately the value that would be calculated if it were assumed that two of the four carbons were effective positions and that glycine provides a reasonably good comparison. Presumably the effective positions are adjacent to the free carboxylate and ammonium groups.

The mechanism by which this isotope effect occurs is not readily apparent. It is obviously not a direct result of the increase in molecular weight since the position of the label has a large effect. Also, it is difficult to formulate a mechanism from the change in zero point energy, although this consideration has been suggested as an explanation of an isotope effect observed in the ion exchange chromatography of Li, K and N.[10] In addition, many isotope effects in organic reactions can be satisfactorily explained by the increase in bond strength that is predicted from the change in zero point energy. An example of this is the decarboxylation of C^{14}-labeled malonic acid.[11] However, in the ion-exchange chromatography of amino acids, bonds to the isotope are not made or broken in the exchange process. Nor can it be easily imagined that a change in ability to assume some sort of a transition structure during sorption or desorp-

(8) B. Drake, *Arkiv. Kemi*, **8**, 1 (1955).

(9) H. Eagle, *J. Biol. Chem.*, **214**, 839 (1955); *Science*, **122**, 501 1955).

(10) T. I. Taylor and H. C. Urey, *J. Chem. Phys.*, **5**, 597 (1937).

(11) P. E. Yankwich, A. L. Promislow and R. F. Nystrom, THIS JOURNAL, **76**, 5893 (1954).

tion is involved since the isotope exerts an effect whether it is close to the point of attachment of the amino acid to the resin or at a distance. The only requirement is that it be adjacent to a charged atom.

It therefore appears certain that the isotope exerts its effect on the ionic state of the molecule. The possibility of an inductive effect owing to a shift of electrons away from the heavier isotope as compared to the lighter atom might be considered. This would require that C^{14} be less electronegative than C^{12}. The result would be that in the 2-position of an α-amino acid, the heavier isotope would tend to stabilize the adjacent ammonium group and thereby increase the statistical chances of attachment to the sulfonate groups of the resin. In the 1-position the isotope would tend to increase the chances of the carboxylate group being protonated and thereby decrease the average repulsive force between this group and the resin. In the case of a carboxylate group located further from the ammonium group, such as the 4-position in aspartic acid, the effect would be less since at the *p*H of the eluting buffer this group would be largely protonated and its importance as a repulsive group would be less. In addition, its greater separation from the point of attachment might be expected to decrease its contribution to the repulsive forces.

This inductive effect can be considered more simply, but less mechanistically, as a decrease in acidity of either a carboxyl or ammonium group which is adjacent to the C^{14} atom. The more basic ionic species travel more slowly on a cation-exchange resin.

All of the data fit this suggested explanation. However, there is apparently no independent evidence in support of the necessary requirement that C^{14} be less electronegative than C^{12}. At the same time it must be remembered that a difference too small to be observed by most methods might be sufficient to explain the observed isotope effect. The effect is small and ion-exchange methods provide an extremely sensitive procedure for observing small differences.

BETHESDA 14, MARYLAND

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, STATE UNIVERSITY OF IOWA]

Hydrogen Ion Equilibria of Ribonuclease[1]

BY CHARLES TANFORD AND JACK D. HAUENSTEIN[2]

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The dissociation of hydrogen ions from crystalline ribonuclease has been studied at 25° at three ionic strengths. The number of dissociating groups of any one kind, *i.e.*, carboxyl, imidazole, amino, guanidyl, etc., agrees precisely with the known amino acid content of the molecule. The *pK* values suggest that the dissociating groups are located at the surface of the molecule, except that three phenolic groups may be buried in the interior, as previously reported. About half the carboxyl groups appear formally to have an abnormally low *pK*. The possible structural significance of this is discussed in light of the structure of ribonuclease proposed by Hirs, Moore and Stein and by comparison with other proteins.

This paper reports a study of hydrogen ion titration curves of ribonuclease and their interpretation in terms of the intrinsic dissociation tendencies of the various types of dissociating groups and in terms of the electrostatic effect of the protein charges. Previous studies of a similar nature have been made for ovalbumin,[3] β-lactoglobulin,[4] human serum albumin,[5] bovine serum albumin,[6] insulin[7] and lysozyme.[8] The experimental technique and underlying theory have been previously summarized.[9]

Experimental

The ribonuclease used in this study was lot 381-059, purchased from Armour and Co. Chromatographic analysis has shown that this particular lot consists of one principal and two minor components. The minor component present in larger amount appears to differ from the principal component only in having a single additional free carboxyl group.[10]

Isoionic, salt-free stock solutions of the protein were prepared by passing a solution of the protein down an ion exchange column.[11] Solutions for measurement were prepared by weight from such stock solutions by addition of standard HCl, KOH and KCl. Conductivity water was used throughout. The final protein concentration was usually about 0.5%. In some solutions a 2% concentration was used, with no apparent difference.

Most of the *p*H measurements were made using the precision potentiometer on a Beckman Model GS *p*H meter. The scale, rated at 0.200 mv. per division, was calibrated and shown to correspond to 0.1992 mv. per division. The full scale of the potentiometer enables one to measure a potential difference corresponding to about 3.3 *p*H units. By means of this scale we evaluated accurately the *p*H of two commercial buffers (*p*H 7 and *p*H 10), comparing them with Bureau of Standards potassium hydrogen phthalate (*p*H 4.005 at 25°), which was used as primary standard. This provided standard solutions lying within the desired range of 3.3 *p*H units of any *p*H between 1 and 13.

Some of the measurements at ionic strength 0.03 and 0.15, near the isoionic point, were made by continuous titration (under nitrogen) in a beaker, with volume addition of acid or base. Most of these are not shown in Fig. 1, for lack of space. There was no indication that data obtained in this way differed from those evaluated in the usual manner. (One would expect a lack of precision by this method only as one moves far from the isoionic point, and at low ionic

(1) Presented at the 129th National Meeting of the American Chemical Society, Dallas, Texas, April, 1956.

(2) Abstracted in part from the Ph.D. thesis of Jack D. Hauenstein, State University of Iowa, August, 1955.

(3) R. K. Cannan, A. C. Kibrick and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 243 (1941).

(4) R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

(5) C. Tanford, THIS JOURNAL, **72**, 441 (1950).

(6) C. Tanford, S. A. Swanson and W. S. Shore, *ibid.*, **77**, 6414 (1955).

(7) C. Tanford and J. Epstein, *ibid.*, **76**, 2163, 2170 (1954).

(8) C. Tanford and M. L. Wagner, *ibid.*, **76**, 3331 (1954).

(9) C. Tanford in T. Shedlovsky, ed., "Electrochemistry in Biology and Medicine," John Wiley and Sons, Inc., New York, N. Y., 1955.

(10) C. Tanford and J. D. Hauenstein, *Biochim. Biophys. Acta*, **19**, 535 (1956).

(11) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.