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Radioactive Markers in Analysis of Amino Acid Chromatograms*

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Summary

Radioactive markers in ion exchange chromatography of amino acid mixtures of biological samples is one of several methods used to identify a particular amino acid. It is shown that in fractions of column effluents C^{14}-labeled amino acids can be detected relatively easily and economically by liquid scintillation or gas flow systems. The interference of the small quantity of labeled amino acid required to give a significant counting rate with the color yield of the amino acids under investigation has been shown to be negligible.

Automatic ion exchange chromatography as described by Spackmann, Stein, and Moore (*1*) is now in general use for colorimetric determination of amino acids in mixtures. A persistent problem, however, in using this method of analysis is the identification of unknown peaks on a chromatogram. The usual procedure for identification is to add standards of the suspected amino acids to the sample and then to repeat the analysis to see if added standards appear in the same position as unknown peaks. In practice, however, one often adds a number of different standards in several runs without successfully identifying the amino acid represented by the un-

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known peak. In order to substantiate the identification of selected amino acids in chromatograms and to provide better points of reference along the elution curve so that identification of most common protein amino acids can be made in biological specimens (tissue hydrolysates, serum, and urine), we have added tracer amounts of ten C^{14}-labeled amino acids (of relatively high specific activity) to the sample to be analyzed. Radioactivity of the column effluent before and after the ninhydrin reaction was monitored by planchet as well as liquid-scintillation counting. We have determined the significance of recovered radioactivity and the effect on quenching of elution buffer and the ninhydrin color products. The advantages and disadvantages of sample collection before and after ninhydrin reaction and comparison of results obtained by planchet and liquid-scintillation counting have also been considered.

MATERIALS AND METHODS

A Technicon amino acid analyzer (Technicon Corp., Ardsley, N.Y. 10502) was used with norleucine as an internal standard. Column dimensions were 140×0.6 cm with a buffer flow rate of 0.5 ml/min. Column temperature was held constant at 60°C throughout the run, and pressure within the column was 200 psi. Chromobeads Type A (lot no. 128A) were used as the separating resin. A pH gradient of 2.875 to 5.000 was obtained using a Technicon Autograd with nine interconnected chambers. Ninhydrin flow rate was 1.06 ml/min. Measurements were made by colorimeters set at wavelengths of 570 mμ, 570 mμ, and 440 mμ with light paths of 15, 8, and 15 mm, respectively. Chart speed was either 6 in./hr or 1.8 in./hr, and the total time of a single run was 22.5 hr. A stream splitter was interposed before reaction with ninhydrin with 0.32 ml/min of the column eluate going to the manifold and 0.10 ml/min diverted to the fraction collector. The stream diverted to the fraction collector was segmented with 0.32 ml/min of air through a PT-2 fitting (Technicon Corp., Ardsley, N.Y. 10502). Segmentation with air was necessary to obtain sharp separation of the radioactive fractions. Samples of 1.5 ml from the diverted stream were collected directly either onto planchets or into glass scintillation vials. Samples on planchets were dried down immediately by a 250-W infrared heat lamp positioned approximately 15 cm above the planchet. Fractions were also collected after ninhydrin reaction from the effluent of the amino acid analyzer after cor-

rection for hold-up time of the reaction mixture between colorimeter and exit tube.

Fractions were collected at timed intervals of 15 min with a Model 6510C fraction collector (Microchemical Specialties Co., Berkeley, Calif.). Planchet counting was done in a gas flow detector (Model D47, Nuclear-Chicago Corp., Des Plaines, Ill. 60018) with automatic sample changer (Model C 110B, Nuclear-Chicago Corp.). Scintillation counting was carried out at 10°C in a liquid scintillation spectrometer (Model 1517A Beckman Instruments, Fullerton, Calif. 92634) equipped with automatic external standardization. The Triton-liquid scintillation cocktail (1.5 ml of collected fraction plus 19.0 ml of cocktail) described by Patterson and Greene (*2*) was used. Glass scintillation vials were obtained from Demuth Glass Co., Parkersburg, W. Va. 26101. Duplicate samples were taken for each radioactive assay and a minimum of three 10-minute counts were obtained on every sample. Average values were reported as counts per minute. The statistical counting error (two sigma) was less than 2% at the 95% confidence level.

C^{14} counting efficiency for either gas flow detector or the liquid scintillation spectrometer was between 76% and 78%. The integration of peaks on the chromatogram was performed by a wide dynamic range integrator (Model No. CRS-11AB/HS/42, Infotronics Corp., Houston, Texas). POPOP (dimethyl) and PPO were obtained from Packard Instrument Co., Downers Grove, Ill. 60515. L-methionine (methyl-C^{14}) 25 mC/mM, L-valine C^{14} (U) 125 mC/mM, L-leucine C^{14} (U) 150 mC/mM, L-histidine (ring-2-C^{14}) 25 mC/mM, L-3-phenylalanine-C^{14} (U) 255 mC/mM, L-alanine-C^{14} (U) 75 mC/mM, glycine C^{14} (U) 50 mC/mM, L-serine-C^{14} (U) 75 mC/mM, L-threonine-C^{14} (U) 100 mC/mM, and L-aspartic acid-C^{14} (U) 100 mC/mM were obtained from the Radiochemical Centre, Amersham, England. L-leucine-1-C^{14} 30 mC/mM was obtained from New England Nuclear Corporation, Boston, Mass. Standard solutions containing 2.5 micromoles of each amino acid per milliliter were obtained from Technicon Corp., Ardsley, N.Y.

RESULTS

Table 1 shows that 10^{-2} μC of each C^{14}-labeled amino acid added to the sample mixture is sufficient to give a level of activity which is significantly different from background when collected before the

TABLE 1

Recovered Radioactivity in Counts per Min Above Background

10^{-3} μC of each C^{14}-labeled amino acid was added to the sample mixture applied to an amino acid analyzer. Radioactivity in collected fractions corresponding to each of the amino acids was determined by liquid scintillation and planchet counting before as well as after reaction with ninhydrin.

	Liquid scintillation counting		Planchet counting	
C^{14}-Labeled amino acid	Before ninhydrin reaction	After ninhydrin reaction[a]	Before ninhydrin reaction	After ninhydrin reaction[a]
Aspartic acid	23412	2509	1221	265
Threonine	8895	1402	1582	111
Serine	43719	4911	1294	491
Glycine	20697	1954	1013	30
Alanine	26367	2629	2050	35
Valine	19452	2138	1714	137
Leucine-1-C^{14}	20767	3	1682	0
Methionine	13150	2955	1147	103
Phenylalanine	21104	2540	2098	263
Histidine	19867	2611	1366	388

[a] No correction has been made for the dilution factor of 3.52 due to ninhydrin reagent.

ninhydrin reaction. After ninhydrin reaction, glycine, alanine, and leucine-1-C^{14} lose practically all radioactivity due to loss of the carboxyl groups. The data in Table 1 also show that fraction collection before color reaction gives much higher counting rates because the concentration of amino acid in these fractions is much higher.

Table 2 shows the quenching observed in collected fractions before and after color reaction. The data demonstrate that there is no significant difference in quenching whether determined before or after the ninhydrin–amino acid reaction. This means that the measured quenching is mainly due to the eluted buffer. The colored ninhydrin reaction product does not significantly contribute to the quenching.

Although the addition of the labeled amino acids will also contribute to the color yields of those amino acids already present, calculation shows that this interference is less than 0.8% of the color yield obtained by that concentration (0.5 μM) of an amino acid whose peak rises to about three-fourths of the full width of the recorder chart. This interference of the marker with the measured color

TABLE 2

Percentage Quenching in Fractions Collected Before and After Ninhydrin Color Reaction with Amino Acids

Amino acid	Quenching, %	
	Before color reaction	After color reaction
Aspartic acid	39.0	38.8
Threonine	39.4	39.6
Serine	38.4	38.5
Glycine	36.9	37.2
Alanine	36.7	36.4
Valine	38.3	38.0
Methionine	39.0	41.5
Leucine-1-C^{14}	38.4	38.3
Phenylalanine	40.0	39.7
Histidine	41.2	40.7
Tyrosine	40.8	41.3

yield would be detectable only by a sensitive electronic integrator since the error of peak integration by hand in our experience is at least 5%.

DISCUSSION

The color of the ninhydrin–amino acid condensation products does not significantly increase quenching. This has already been shown in a different approach by Olson, White, and Noma (*3*). The findings are also in agreement with the observations of De Bersaques (*4*), Herberg (*5*), and Ross and Yerick (*6*), who have demonstrated a direct correlation between color quenching and absorbance at 4000–4300 Å. The colored ninhydrin–amino acid condensation products in our experiments have their maximum absorbance at 5700 Å, which may account for the fact that no significant difference was noted in quenching before and after color development. The relatively high quenching observed in our experiments is due principally to highly concentrated buffers which are used.

We have shown that radioactively labeled amino acids may be used as markers in analysis of amino acid chromatograms. Although a flow cell monitor which continuously records radioactivity in a liquid scintillation spectrometer has been described by Piez (*7*), a simple gas flow detector and scaler is sufficient for this purpose. The highest recovery of added marker, however, is obtained by means of liquid

scintillation counting. Sample collection before color reaction with ninhydrin by means of a stream splitting device has the advantage of providing fractions with greater activity and permits measurement of amino acids which carry their label in C-1-positions. The concentration of the amino acids under investigation may be calculated accurately from the chromatogram since the flow rates of the split stream are easily controlled.

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