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SIMULTANEOUS QUANTITATION OF TWENTY-TWO AMINO ACIDS IN NANOLITRE SAMPLES OF BIOLOGICAL FLUID

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SUMMARY

The technique of thin-layer chromatography and ^{14}C-dansylation has been used for simultaneous measurements of 22 individual amino acids, taurine, serotonin, and γ-aminobutyric acid in nanolitre samples of glomerular and renal tubule fluid. Standard curves of each amino acid mixture containing all the others showed excellent linearity at amounts ranging from $5.7 \cdot 10^{-13}$ to $1.45 \cdot 10^{-11}$ mole (regression coefficients all greater than 0.95). With careful standardization of dansylation conditions, the technique permits highly reproducible measurement of less than 1 pmole of an amino acid.

INTRODUCTION

The analysis of free amino acids in nanolitre volumes of renal tubule fluid requires methods with a sensitivity in the picomole range. The most sensitive method for such analysis, well characterized by Neuhoff and co-workers[1–3], depends upon the reaction of amino acids with dansyl chloride (1-dimethylaminonaphthalene-5-sulphonyl chloride) and subsequent microchromatography on polyamide layers. Utilizing ^{14}C-labelled dansyl chloride, less than 1 pmole of an amino acid can be measured accurately. The methodology used for the simultaneous quantitation of more than 20 amino acids in 50 nl volumes of glomerular filtrate and renal tubule fluid is detailed here.

METHODS

Calibration curves and chromatographic techniques

Calibration curves for 22 individual α-amino acids, taurine, serotonin and γ-aminobutyric acid (GABA) were obtained in triplicate with four groups of 5–7

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amino acids each. Group 1 contained lysine, glycine, glutamic acid, aspartic acid and ornithine; Group 2 contained phenylalanine, cystine, threonine, tyrosine, valine, taurine and tryptophan; Group 3 consisted of alanine, proline, methionine, isoleucine, hydroxyproline and serotonin; histidine, asparagine, glutamine, serine, leucine, and GABA were contained in Group 4. The amino acids in a given group were dissolved together, each at 0.01 m*M* concentration in 0.05 *M* $NaHCO_3$ buffer adjusted to pH 10 with NaOH (Solution 1). In preparing calibration curves for members of a given group, amino acids of all the other groups were combined at equal concentration (0.01 m*M*) in the same bicarbonate buffer (Solution 2) for subsequent mixing with Solution 1.

^{14}C-Dansyl chloride solution, specific activity 104 mCi/m*M*, 0.5 mCi/ml acetone (CEA, Saclay, France, purchased from Research Products International, Mount Prospect, Ill., U.S.A.) was concentrated four-fold by evaporation prior to use.

Aliquots (250 μl, 200 μl, 100 μl, 50 μl, 20 μl or 10 μl) of Solution 1 containing the amino acids for calibration were added to 1 ml test tubes containing 250 μl of standard amino acid Solution 2, and the final volume of each mixture was made up to 500 μl with 0.05 *M* $NaHCO_3$, pH 10 buffer. Phenylglycine and norvaline were incorporated in precise amounts to serve subsequently as internal standards. After mixing, 2-μl volumes of each amino acid mixture and 2 μl of ^{14}C-dansyl chloride solution were mixed in a 10-μl constant-bore capillary tube (Drummond, Broomall, Pa., U.S.A.) which was sealed, the volume derived from the length of the fluid column, and the sample incubated at 37° for 30 min. Precisely 50-nl aliquots were applied slowly to 3 × 3 cm polyamide thin-layer chromatograms (Schleicher & Schüll, Dassel, G.F.R.) under microscopic visualization using *ca.* 40 μm O.D., 2 cm long micropipets. Rapid evaporation of the samples at the application point was achieved by heating to keep the chromatographic origin as small as possible. Because of the very small amounts of amino acids in the 50–100-nl volumes used in this study, non-radioactive dansylated amino acids were added to the same application point to facilitate recognition and complete harvesting of each spot after chromatography.

The microchromatograms were developed in the first dimension with 4% formic acid, and with benzene–glacial acetic acid (8:2) in the second[2]. Chromatography in the second dimension was repeated with ethyl acetate–methanol–glacial acetic acid (20:1:1), to completely separate dansylated NH_2 from alanine, cystine from impurities of dansyl-OH, glutamine from asparagine, and threonine from serine. Taurine was separated from dansyl-OH with a 1.5% diethylamine solution[4]. Histidine and leucine were separated using benzene–glacial acetic acid in a ratio of 9:1 instead of 8:2 in repeated chromatography in the second dimension. Arginine could not be separated from ε- and α-lysine. Since cysteine forms several dansyl compounds by oxidation[5], a calibration curve was not attempted.

To preserve a record of each chromatogram and identify the presence of very low concentrations of some amino acids in biological fluids, autoradiographs of each chromatogram were made using high-speed dental X-ray film (Ultra-speed, DF58, Eastman Kodak, Rochester, N.Y., U.S.A.). The film was placed on the chromatogram sandwiched between 3 × 4 cm glass plates, and exposed in the dark for 24 h before development. Thereafter, the spots of the dansylated amino acids were encircled with a pencil under UV light. The polyamide of each spot was quantitively scraped off under microscopic visualization and transferred to a scintillation fluid containing

2,5-diphenyloxazole (PPO), 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP), and toluene for counting in a liquid scintillation counter (Nuclear-Chicago, Des Plaines, Ill., U.S.A.). Sample areas of each chromatogram in which amino acid spots could not be detected on the autoradiographs were taken and counted for background radioactivity. This value was added to the background counts of the scintillation fluid in every vial. Having determined the net ^{14}C activity of each sample, amino acid concentrations were estimated from the standard curves. Because of possible variation in the degree of dansylation from sample to sample, values were corrected for the activity of the incorporated phenylglycine and norvaline internal standards.

Sample handling

Variation in the size of biological samples precluded the use of pre-calibrated micropipets for sample transfers. Instead, samples were measured and mixed with buffer and dansyl chloride solution in pre-calibrated 0.5 μl constant-bore capillary tubes. The tubes were mounted vertically in a pin vice in front of a dissecting microscope and connected at the bottom to an injection system with PE 40 tubing. The injection device consisted of a micrometer screw attached to a water-filled 1-ml tuberculin syringe. Turning the screw permitted precise movement of a column of water up or down in the lower third of the constant-bore capillary. Samples were thus readily introduced into the tube without accumulating at the top, and could be moved upward as collected into a collection pipet. Buffer and dansyl solutions were delivered and removed under microscopic visualization with 2 cm long, 40–60 μm O.D. capillaries attached vertically to a micromanipulator. A column of dansyl chloride solution, 120 nl volume, was introduced first and rapidly aspirated some 2 mm below the top of the capillary to minimize evaporation of the acetone. The length of the column was measured with a filar eye-piece and the volume derived. Samples to be assayed were introduced directly from their 8–10 μm O.D. collection pipets, and followed by a volume of 0.25 *M* $NaHCO_3$, pH 10 buffer (containing the internal standards, 0.01 m*M* phenylglycine and norvaline) which, added to the sample, would give a total volume of *ca.* 120 nl. These solutions, measured as above, were mixed by repeated aspiration and discharge back into the capillary tube. The combined fluids and the dansyl solution were then aspirated into a fresh capillary pipet and thoroughly mixed again. To avoid sample evaporation, the large end of the pipet was occluded with mineral oil injected through a 27-gauge needle, and the capillary end was sealed by proximity to a burning match. The reaction mixture was incubated at 37° for 30 min before transfer on to a polyamide sheet for chromatography.

RESULTS

To determine the accuracy and reproducibility of transferring 50-pl samples as performed here, the activity of three 10-μl samples of a ^{14}C-inulin solution was counted in a liquid scintillation counter. Seven nominal 50-nl aliquots of this solution, measured with a filar eye-piece in a 0.5-μl constant-bore capillary tube and washed out with water, gave a mean volume of 48.9 $\pm$ (SD) 2.0 nl.

Calibration curves for six concentrations of 22 α-amino acids, taurine, serotonin and GABA in amounts ranging from $5.7 \cdot 10^{-13}$ to $1.45 \cdot 10^{-11}$ mole were ob-

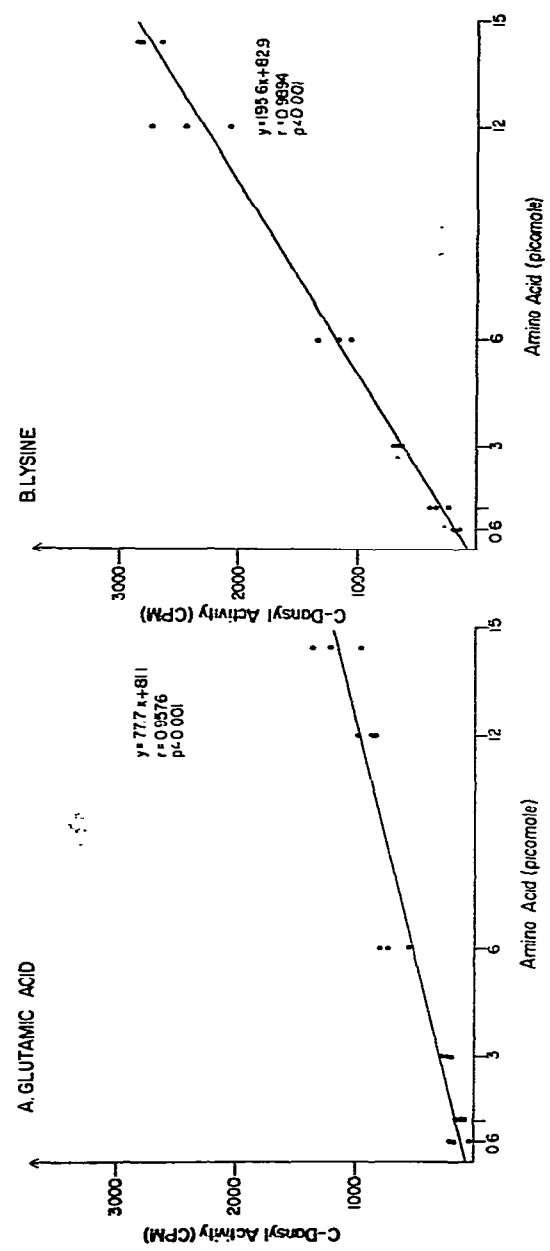

Fig. 1. Calibration curves for representative amino acids measured by ^{14}C-dansylation and thin-layer chromatography.

tained in triplicate. The relationship between ^{14}C-dansyl activity and the nominal amounts of four representative amino acids is shown graphically in Fig. 1; the regression coefficients for all other amino acids studied are given in Table I. All plots had linear regression coefficients greater than 0.95, $p < 0.001$.

TABLE I

REGRESSION EQUATIONS OF STANDARD AMINO ACID CALIBRATION CURVES

Values for the amino acids shown in Fig. 1 are not repeated. All regression coefficients are significant at the 0.1% confidence limit.

Amino acid	*Regression coefficient*	*Correlation coefficient*
Alanine	$y = 116.5x + 14.7$	0.9830
Asparagine	$y = 154.1x - 11.0$	0.9867
Aspartic acid	$y = 65.4x + 81.9$	0.9565
Cystine	$y = 234.8x + 16.9$	0.9858
GABA	$y = 154.3x + 131.6$	0.9931
Glutamine	$y = 156.7x + 73.8$	0.9868
Glycine	$y = 220.2x + 50.0$	0.9935
Histidine	$y = 199.6x - 51.4$	0.9961
HO-Proline	$y = 218.2x + 5.4$	0.9885
Isoleucine	$y = 163.7x + 47.3$	0.9928
Leucine	$y = 189.2x + 32.0$	0.9878
Methionine	$y = 163.2x - 79.5$	0.9892
Ornithine	$y = 242.9x + 8.2$	0.9919
Phenylalanine	$y = 186.6x + 11.0$	0.9917
Serine	$y = 146.1x + 28.8$	0.9815
Taurine*	$y = 233.4x + 175.6$	0.9892
Threonine	$y = 111.1x + 28.4$	0.9722
Tryptophan	$y = 153.3x + 166.5$	0.9905
Tyrosine (bis)	$y = 97.4x + 41.0$	0.9528
Tyrosine (*n*)	$y = 110.7x - 58.9$	0.9662
Serotonin (bis)*	$y = 39.5x + 56.0$	0.9694
Serotonin (*n*)*	$y = 50.7x - 14.9$	0.9608

* No amino acid.

The mean standard deviation of the linear regression lines of all the compounds for which calibration curves were derived was $\pm 4.6\%$ (range ± 2.6 to $\pm 8.0\%$). An autoradiograph of a chromatogram obtained in establishing the calibration curves demonstrates the position of the different amino acids, serotonin and GABA (Fig.2). Here, all amino acids are present in the amount of $1.45 \cdot 10^{-11}$ mole. As shown by this autoradiograph and the linear regression coefficients in Table I, the degree of dansylation of individual amino acids differed under the same incubation conditions. Glycine, leucine and phenylalanine consistently reacted more with dansyl chloride than, for example, aspartic acid and tyrosine. This is as expected[1].

Fig. 3 illustrates the autoradiographs obtained with *ca.* 100 nl volume samples of Necturus glomerular and renal proximal tubule fluid, the larger volume being chosen for clarity of illustration in print. Some 21 amino acids are identified in glomerular fluid by their position (legend to Fig. 3). The amounts and concentrations of each amino acid in these samples are given in Table II. The internal standards,

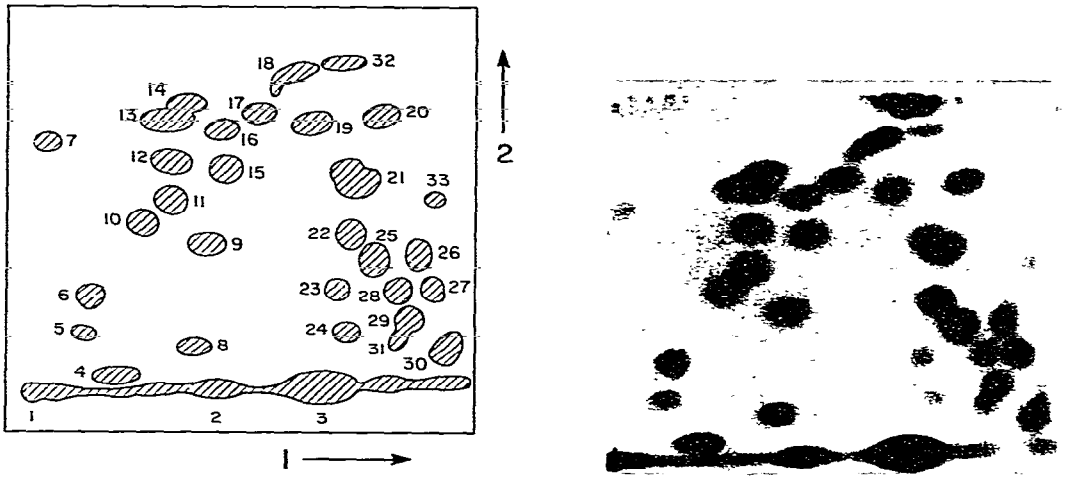

Fig. 2. Autoradiograph of a standard mixture of amino acids in amounts of $1.45 \cdot 10^{-11}$ mole each. Exposure time was 14 h. Spot identification: 1, starting point; 2, taurine; 3, dansyl-OH; 4, cystine; 5, *n*-serotonin; 6, tryptophan; 7, bis-tyrosine; 8, *n*-tyrosine; 9, ornithine; 10, bis-lysine; 11, phenylglycine; 12, phenylalanine; 13, leucine and histidine; 14, isoleucine; 15, methionine; 16, norvaline; 17, valine; 18, proline; 19, GABA; 20, δ-aminolevulinic acid; 21, alanine and dansyl-NH_2; 22, glycine; 23, glutamic acid; 24, aspartic acid; 25, hydroxyproline; 26, glutamine; 27, asparagine; 28, threonine; 29, serine; 30, arginine, ε-lysine and α-lysine; 31–33, unknown dansylation products.

TABLE II

AMINO ACIDS PRESENT IN GLOMERULAR AND TUBULE FLUIDS

Amino acid	*Glomerular sample (vol = 102 nl)*			*End proximal tubule sample (vol = 100 nl)*		
	Net cpm	*Amount ($\times 10^{-12}$ M)*	*Concentration ($\times 10^{-6}$ M/l)*	*Net cpm*	*Amount ($\times 10^{-12}$ M)*	*Concentration ($\times 10^{-6}$ M/l)*
Alanine	612	4.9	47.9	257	2.35	22.2
Asparagine	97	0.75	7.4	27	0.6*	6.0
Aspartic acid	145	0.84	8.0	47	0.6*	6.0
Cystine	337	1.31	12.8	497	2.12	20.0
GABA	28	0.6*	6.0	17	0.6*	6.0
Glutamine	501	3.14	30.8	20	0.6*	6.0
Glutamic acid	225	2.3	17.1	77	0.6*	6.0
Glycine	782	3.1	30.5	160	0.93	8.8
HO-Proline	128	0.62	6.1	97	0.6*	6.1
Isoleucine	1042	5.76	56.4	77	0.6*	6.0
Leucine	1657	8.23	80.9	89	0.6*	6.0
Lysine	1372	6.26	61.3	74	0.6*	6.0
Methionine	186	1.63	16.0	28	0.6*	6.0
Ornithine	99	0.6*	6.0	20	0.6*	6.0
Phenylalanine	884	4.36	42.8	85	0.6*	6.0
Proline	369	9.88	8.6	55	0.6*	6.0
Serine	491	3.02	29.6	141	0.85	8.0
Threonine	194	1.40	13.7	121	0.88	8.3
Tryptophan	307	0.82	8.0	0	0	0
Tyrosine (bis)	1353	12.6	123.1	94	0.6*	6.0
Valine	1529	8.1	79.0	110	0.6*	6.0
Internal Standards						
Phenylglycine	1043	7.5	25	1167	9.0	25
Norvaline	1083	7.5	25	1113	9.0	25

* Lower limits of quantitation. Values to be considered approximations.

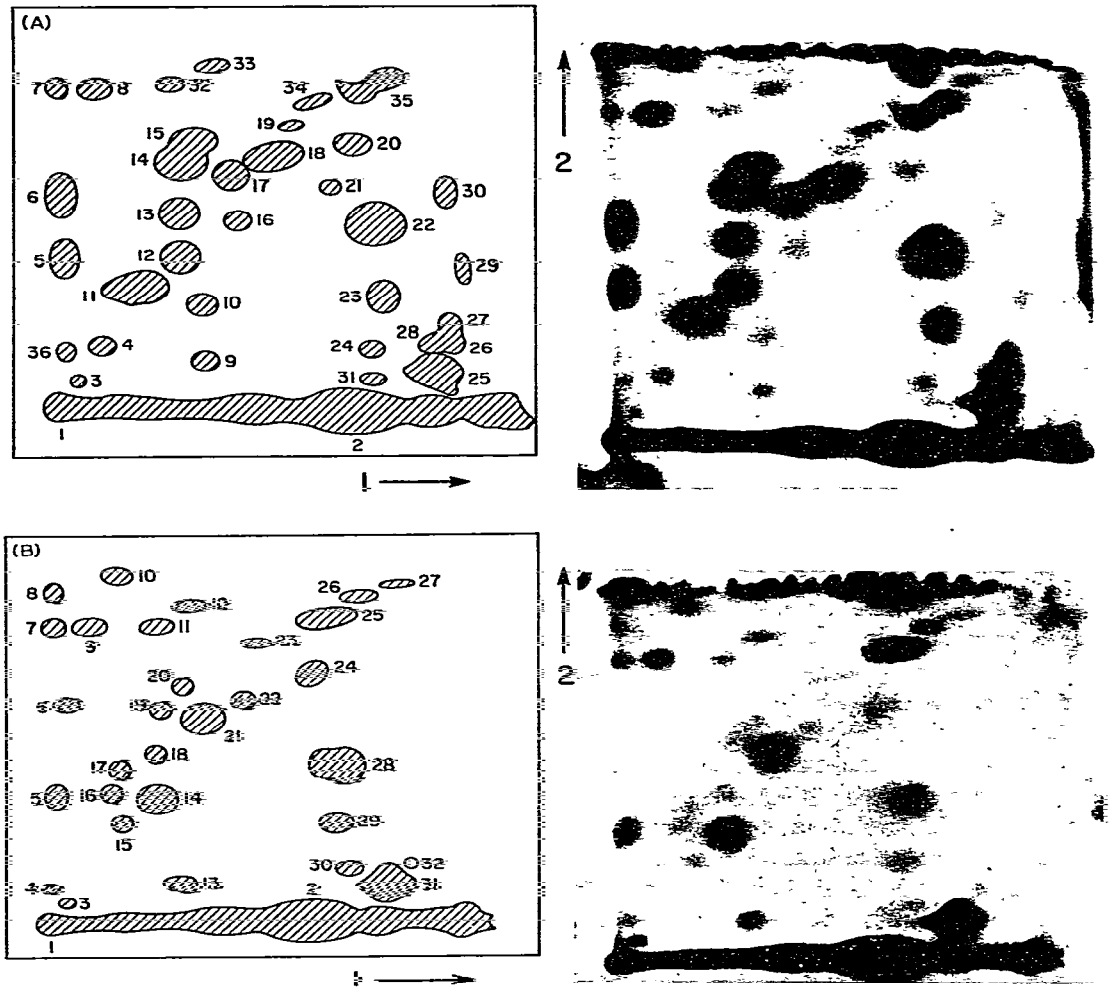

Fig. 3. (A) Fourteen hour autoradiograph of a 100-pl sample of Necturus glomerular fluid. Spot identification: 1, starting point; 2, dansyl-OH; 3, unknown; 4, tryptophan; 5, unknown; 6, bis-tyrosine; 7, 5-hydroxyindole; 8, unknown; 9, *n*-tyrosine; 10, ornithine; 11, lysine; 12, phenylglycine; 13, phenylalanine; 14, leucine and histidine; 15, isoleucine; 16, methionine; 17, norvaline; 18, valine; 19, proline; 20, δ-aminolevulinic acid; 21, GABA; 22, alanine and dansyl-NH_2; 23, glycine; 24, glutamic acid; 25, cysteine; 26, asparagine; 27, glutamine; 28, threonine; 29, unknown; 30, ethanolamine; 31–36, unknown. (B) Fourteen hour autoradiograph of a 100 pl sample of Necturus proximal tubule fluid. The amino acids are present at lower concentration than in Fig. 3A due to tubular absorption and are identified as: 1, starting point; 2, dansyl-OH; 3–5, unknown; 6. bis-tyrosine; 7, 5-hydroxyindole; 8–12, unknown; 13, *n*-tyrosine; 14, phenylglycine; 15, lysine; 16 and 17, unknown; 18, phenylalanine; 19, leucine; 20, isoleucine; 21, norvaline; 22, valine; 23, proline; 24, δ-aminolevulinic acid; 25–27, unknown; 28, alanine, dansyl-NH_2; 29, glycine; 30, glutamic acid; 31, cysteine; 32, glutamine.

phenylglycine and norvaline, are evident; unidentified spots present in both samples may represent peptides. The obvious difference in amino acid concentration in the two fluids relates to tubular absorption.

DISCUSSION

The technique of dansylation and chromatography of amino acids in small quantities has been well established and described in great detail by Neuhoff and co-workers[1–3]. The advent of ^{14}C-labelled dansyl chloride with a very high specific activity now permits the quantitation of even smaller amounts of individual amino acids. The method, although somewhat tedious, is accurate and highly reproducible when dealing with mixtures of numerous amino acids present in amounts above $5 \cdot 10^{-13}$ mole. Careful standardization is essential, however, since the reaction of amino acids with dansyl chloride varies not only with their pK values but also is affected by reaction of dansyl with the carboxyl groups of admixed amino acids. The pH at which the reaction occurs, proteins and peptides present in the sample, the dansyl chloride: water ratio and reaction time all influence the degree of dansylation[1]. Thus, in assaying picomolar amounts of admixed amino acids, it is necessary to establish calibration curves for each under optimal, standardized reaction conditions in the presence of all other amino acids in the fluid to be tested.

The standards gave excellent linearity with amounts of amino acids ranging from $5.7 \cdot 10^{-13}$ to $1.45 \cdot 10^{-11}$ mole. The intercepts of most lines did not pass through zero, however. Although background correction was made for radioactivity in areas without detectable amino acids, it seems likely that somewhat higher counts were present in the areas immediately adjacent to many amino acid spots.

The reaction conditions used in this study were very similar to those described by Schulze and Neuhoff[4]. A higher concentration of dansyl chloride was used, however, and, preparing the chromatogram immediately after incubation, it was found unnecessary to add diethylamine to destroy excess dansyl chloride[4]. In dealing with tubule fluid samples, it was necessary to reduce the volumes of all constituents of the reaction and develop methods of performing the reaction in variably sized, 50–100-nl volumes. The technique proved to give highly satisfactory results with a sensitivity of $5 \cdot 10^{-13}$ moles and overall reproducibility in replicate determinations of $\pm 4.6\%$. Twenty-one amino acids in 50-nl volumes of glomerular filtrate could be identified and 19 quantitated. Tubular absorption reduced the concentration of a number of amino acids to the point that they could not be quantified in end-proximal tubule samples, although their presence generally could be detected by autoradiography.

ACKNOWLEDGEMENT

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