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A METHOD FOR THE SEPARATION AND IDENTIFICATION OF BASIC AMINO ACIDS AND HEXOSAMINES*

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SUMMARY

A method is described for separating and quantifying basic amino acids and hexosamines simultaneously from the column chromatography on Dowex 50W X8 of serum acid hydrolysates. Based on color development with 2,4,6-trinitrobenzene-1-sulfonic acid, differences in the absorption spectra of the trinitrophenylation products of the two types of compounds are characteristic. The ratio of O.D. 355: O.D. 475 provides a quick identification of the type of compound, amino acid or hexosamine.

Spectrophotometric methods based on the reaction of 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) with primary amino groups have been used for the determination of amino acids, peptides and amines at acid pH[1] and hexosamines at alkaline pH[2]. This paper describes the application of the method at alkaline pH to eluates from the column chromatography of acid hydrolysates of whole serum proteins, a technique which permits the identification and quantitation of the basic amino acids and hexosamines. The trinitrophenylated (TNP)-amino acids and TNP-hexosamines produced by trinitrophenylation at alkaline pH may be differentiated by their absorption spectra in alkaline solutions. The absorption spectra of the TNP-hexosamines change characteristically after acidification of the buffered reaction mixtures[2].

MATERIALS

Standard solutions

D-Glucosamine·HCl (Eastman Organic Chemicals) and D-galactosamine·HCl, L-leucine, L-lysine, L-phenylalanine, L-alanine, L-histidine·HCl, L-tyrosine, and L-tryptophan (Sigma Chemical Company) were used at a concentration of 1.0 m*M*/ml in 0.1 *M* phosphate buffer, pH 7.8.

Reagents for column chromatography

Dowex 50W X8 resin, 200–400 mesh (Sigma Chemical Company) was prepared

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according to the method of MOORE AND STEIN used for the chromatography of basic amino acids[3].

The following buffers were prepared following the technique of MOORE AND STEIN[3]: (a) 0.1 *M* citrate buffer, pH 3.42; (b) 0.1 *M* citrate buffer, pH 5.0; (c) 0.1 *M* phosphate buffer, pH 6.75[4]; (d) 0.2 *M* citrate buffer, pH 6.5.

Reagents for the spectrophotometric assay

Borate buffer. KOH pellets were added to 1.0 *M* H_3BO_3 to give a solution of pH 8.6.

TNBS reagent. 2,4,6-Trinitrobenzene-1-sulfonic acid, 1 mg/ml in distilled water. TNBS supplied by Eastman Organic Chemicals or Sigma Chemical Company was satisfactory.

METHODS

One milliliter aliquots of whole rat serum were precipitated with 10.0 ml 95 % ethanol, washed with 10.0 ml 95 % ethanol and hydrolyzed in 2 ml 3 *N* HCl for 4 h in a 100° paraffin bath. The uncentrifuged acid hydrolysates were evaporated to dryness over calcium carbonate and sodium hydroxide pellets *in vacuo* and redissolved in 1.0 ml 0.1 *M* citrate buffer pH 5.0.

An 0.5 ml aliquot of the redissolved hydrolysate or of a synthetic mixture of amino acids and hexosamines was applied to a 1 × 15 cm Dowex 50W X8 column in 0.1 *M* citrate buffer, pH 3.42. The sample was washed on with 0.1 *M* citrate buffer, pH 5.0, and the column was eluted initially with 25 to 45 ml of this buffer. The column was then eluted with 100 to 150 ml 0.1 *M* phosphate buffer, pH 6.75, followed by 100 to 150 ml 0.2 *M* citrate buffer, pH 6.5. One milliliter fractions were collected into tubes containing 0.11 ml 1.0 *N* HCl. The hexosamines were stabilized in excess hydrochloric acid. The column was eluted at room temperature, but the collection tubes were maintained at 4° in a circulating bath. Later studies showed that refrigeration of the collected fractions was not necessary in the presence of excess hydrochloric acid. Column flow rate was controlled at 2 ml/h by adjusting the height of the buffer reservoir.

For the colorimetric assay, 0.5 ml aliquots of the column fractions were mixed with 0.5 ml distilled water, 3.0 ml 1.0 *M* borate buffer, pH 8.6, and 2.0 ml TNBS reagent. Standards of 0.1 ml of 1.0 m*M* solutions of D-glucosamine·HCl and L-leucine plus 0.9 ml distilled water plus buffer and TNBS were run with each determination. The color was allowed to develop for 15 to 18 h at 15°. Absorption spectra and absorbances at 355 mμ and 475 mμ were read in a Beckman Model DU spectrophotometer. Reaction mixtures were acidified by the addition of 0.1 ml concentrated HCl and the absorption spectra were read again.

RESULTS

Fig. 1 shows a chromatogram of a synthetic mixture of 0.5 mg each of L-leucine, L-phenylalanine, L-lysine, D-galactosamine·HCl and D-glucosamine·HCl. The difference in the relationship of absorbances at 355 mμ and 475 mμ for the amino acids and for the hexosamines clearly distinguished between these two types of compounds.

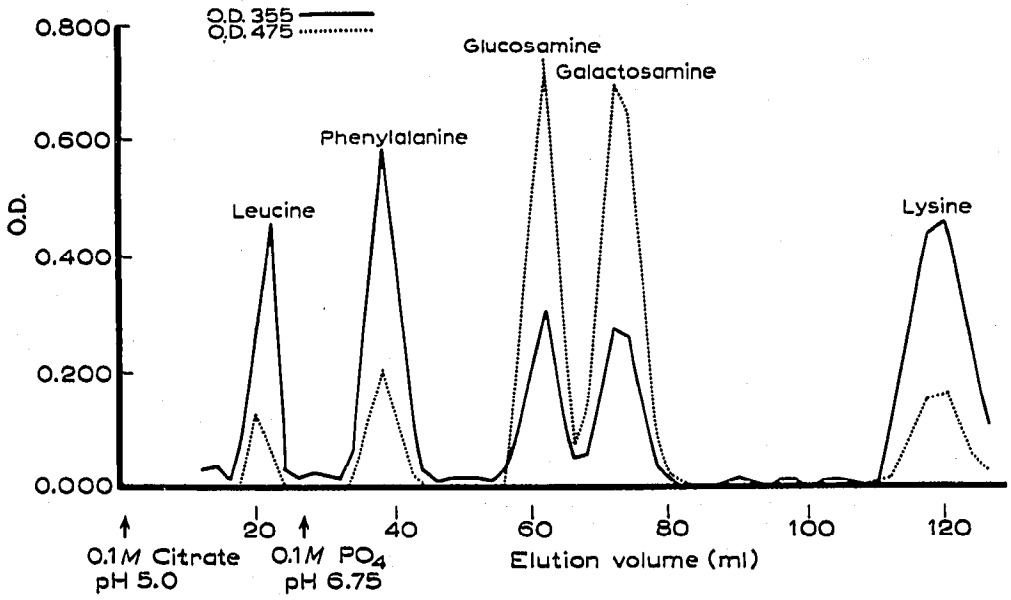

Fig. 1. Chromatogram of a Dowex 50W X8 column of a synthetic mixture of 0.5 mg each of L-leucine, L-phenylalanine, D-glucosamine·HCl, D-galactosamine·HCl and L-lysine. Optical density was read at 355 mμ (———) and 475 mμ (·····).

The ratio of O.D. 355:O.D. 475 for pure amino acids was approximately 2:1 to 4:1; for pure hexosamines, 1:2 to 1:2.5. Contamination of either type of substance with the other brought this ratio closer to 1. Absorbance of the hexosamine chromogen at 355 mμ and 475 mμ was proportional to concentration (Fig. 2a) and the ratio of O.D. 355:O.D. 475 remained constant above hexosamine concentrations of 0.03 m*M* (Fig. 2b).

The elution pattern of a whole serum acid hydrolysate (Fig. 3) contained an initial peak (A) of acidic and neutral amino acids and neutral sugars. Subsequent

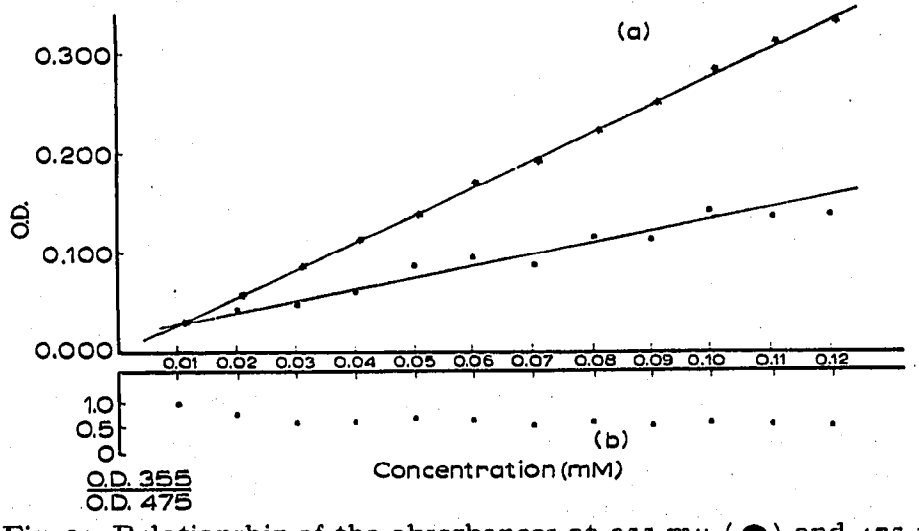

Fig. 2a. Relationship of the absorbances at 355 mμ (●) and 475 mμ (★) for a series of D-glucosamine·HCl concentrations. The series included the range of glucosamine concentrations expected in serum protein acid hydrolysates. Reaction mixtures consisted of D-glucosamine·HCl, 0.01 μmoles to 0.12 μmoles in 1 ml, + 3 ml 1.0 *M* borate buffer, pH 8.6, + 2 ml TNBS reagent.

Fig. 2b. The ratio of O.D. 355:O.D. 475 (■) was 0.5 for the concentrations above 0.03 m*M*.

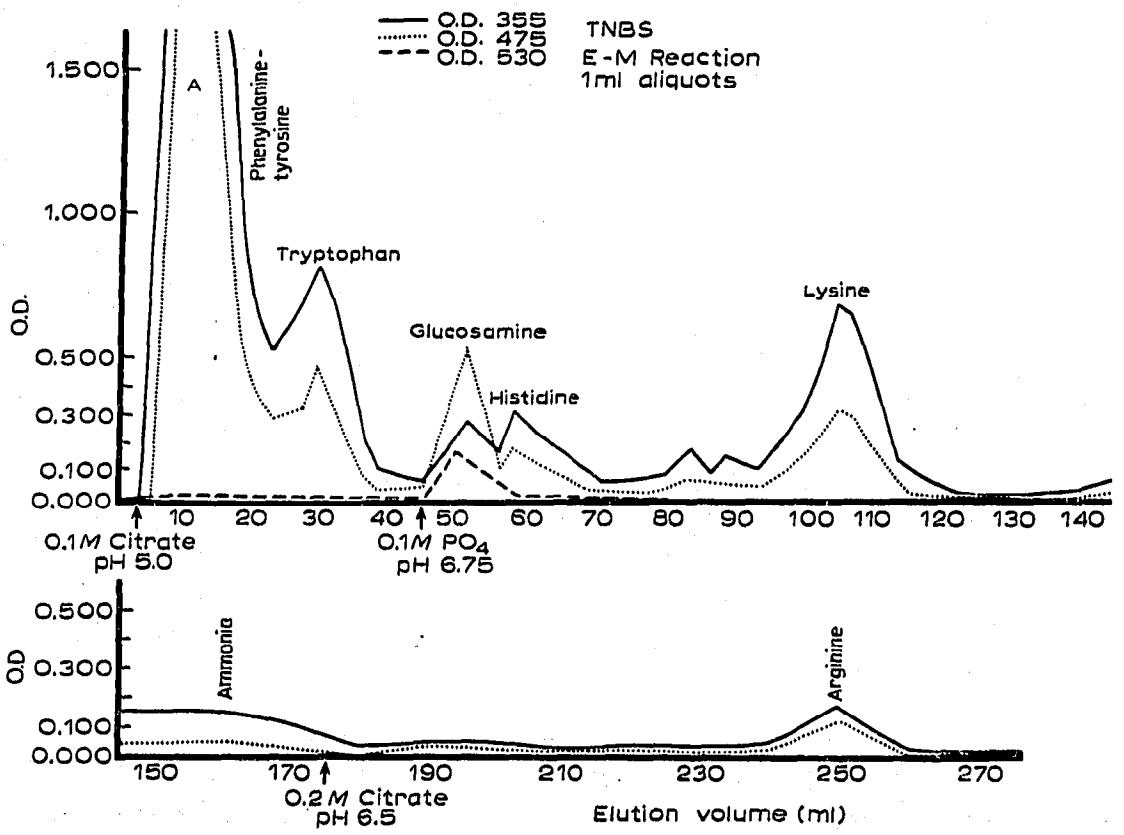

Fig. 3. Separation of the basic amino acids and glucosamine of 0.5 ml of a serum acid hydrolysate on a Dowex 50W X8 column. Optical density was read at 355 mμ (———) and 475 mμ (· · · · ·). Elution peak A represented the acidic and neutral amino acids and non-basic sugars. Results of the ELSON AND MORGAN method (------) confirmed the presence of a hexosamine.

peaks were phenylalanine–tyrosine, tryptophan, glucosamine, histidine, lysine, ammonia and arginine identified by comparison of their elution positions with chromatograms of synthetic mixtures of the amino acids and hexosamines. The identification of the glucosamine peak was confirmed by the ELSON AND MORGAN reaction[6]. Addition of glucosamine labeled with tritium or carbon-4 to the serum both before and after acid hydrolysis provided further proof that the hexosamine peak was glucosamine. Elution of the radioactivity from the Dowex 50W X8 column coincided with the elution of hexosamine. Decreasing the elution volume of the initial 0.1 *M* citrate buffer, pH 5.0, allowed a cleaner separation of the hexosamine (Fig. 4). Since the color absorption is proportional to concentration over a wide range, the amino acids and hexosamines may be quantified by reference to the molar extinction coefficients of the trinitrophenylation products of the pure standard solutions.

A comparison of the absorption spectra of the trinitrophenylation products of isolated amino acids and hexosamines differentiated the two types of compounds. Fig. 5 shows the absorption spectra of the reaction mixtures of standard solutions of D-galactosamine·HCl and L-leucine. Absorption maxima of the TNP-galactosamine were at 355 mμ and 475 mμ; absorption maxima of the TNP-leucine were at 355 mμ and 420 mμ. Acidification of the buffered reaction mixtures by the addition of 0.1 ml concentrated HCl had little effect on the absorption spectrum of the TNP-leucine. However, the absorption peak at 475 mμ of the TNP-galactosamine was completely

eliminated and the acidified mixture showed an absorption maximum at 380 mμ. The trinitrophenylation products of L-lysine, L-phenylalanine, L-alanine, L-histidine·HCl, L-tyrosine and L-tryptophan gave absorption curves similar to L-leucine; D-glucosamine·HCl gave an absorption curve similar to D-galactosamine·HCl. The molar extinction coefficients varied among these compounds (Table I).

Spectral analysis was applied to fractions from the Dowex 50W X8 columns to confirm the identification of the isolated substances. Aliquots from the amino acid peaks and from the glucosamine peak demonstrated absorption spectra compa-

TABLE I

MOLAR EXTINCTION COEFFICIENTS FOR THE TRINITROPHENYLATION PRODUCTS OF SOME AMINO ACIDS AND HEXOSAMINES

Reaction mixtures = 0.1 ml of 1.0 m*M* solution of each standard + 0.9 ml distilled water + 3.0 ml 1.0 *M* borate buffer, pH 8.6, + 2.0 ml TNBS reagent.

Compound	*O.D. 355* $\times 10^{-6}$	*O.D. 475* $\times 10^{-6}$	*O.D. 355 : O.D. 475*
D-glucosamine·HCl	1.30	3.12	0.417
D-galactosamine·HCl	1.37	3.57	0.384
L-leucine	1.94	0.49	3.96
L-phenylalanine	1.89	0.87	2.17
L-tyrosine	1.70	0.45	3.78
L-tryptophan	1.58	0.85	1.86
L-histidine·HCl	1.38	0.40	3.46
L-lysine	4.48	1.40	3.20
L-arginine	2.28	1.21	1.88
L-alanine	2.40	0.85	2.82

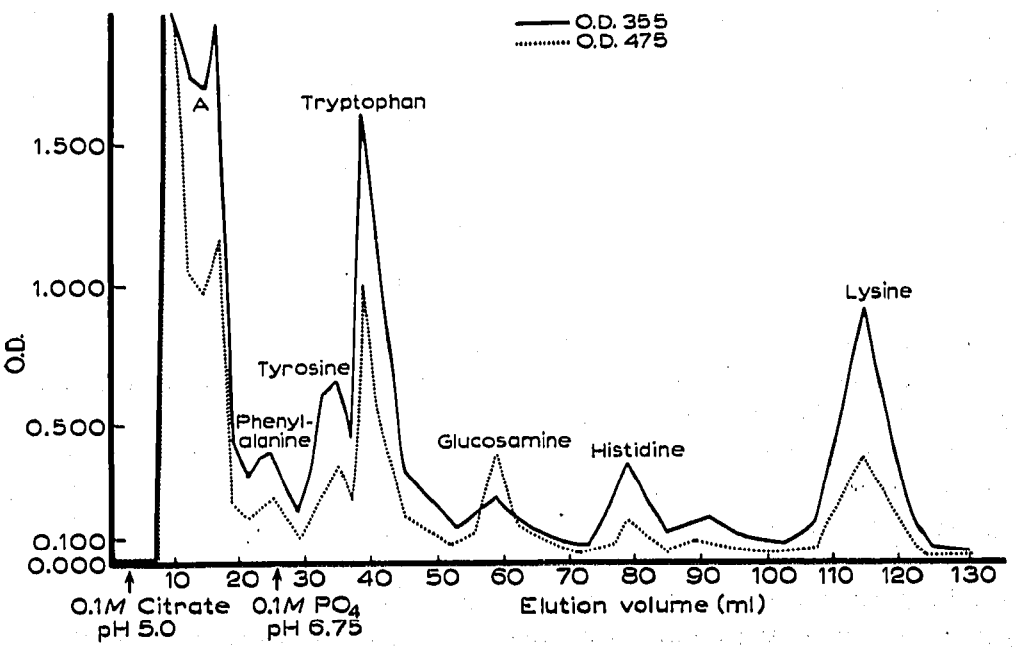

Fig. 4. Chromatogram of a serum acid hydrolysate demonstrating the effect of decreasing the elution volume of the initial 0.1 *M* citrate buffer, pH 5.0, from 47 ml in Fig. 3 to 27 ml. The glucosamine was eluted as a symmetrical peak. O.D. 355 mμ (———), O.D. 475 mμ (·····). Elution peak A represented the acidic and neutral amino acids and non-basic sugars.

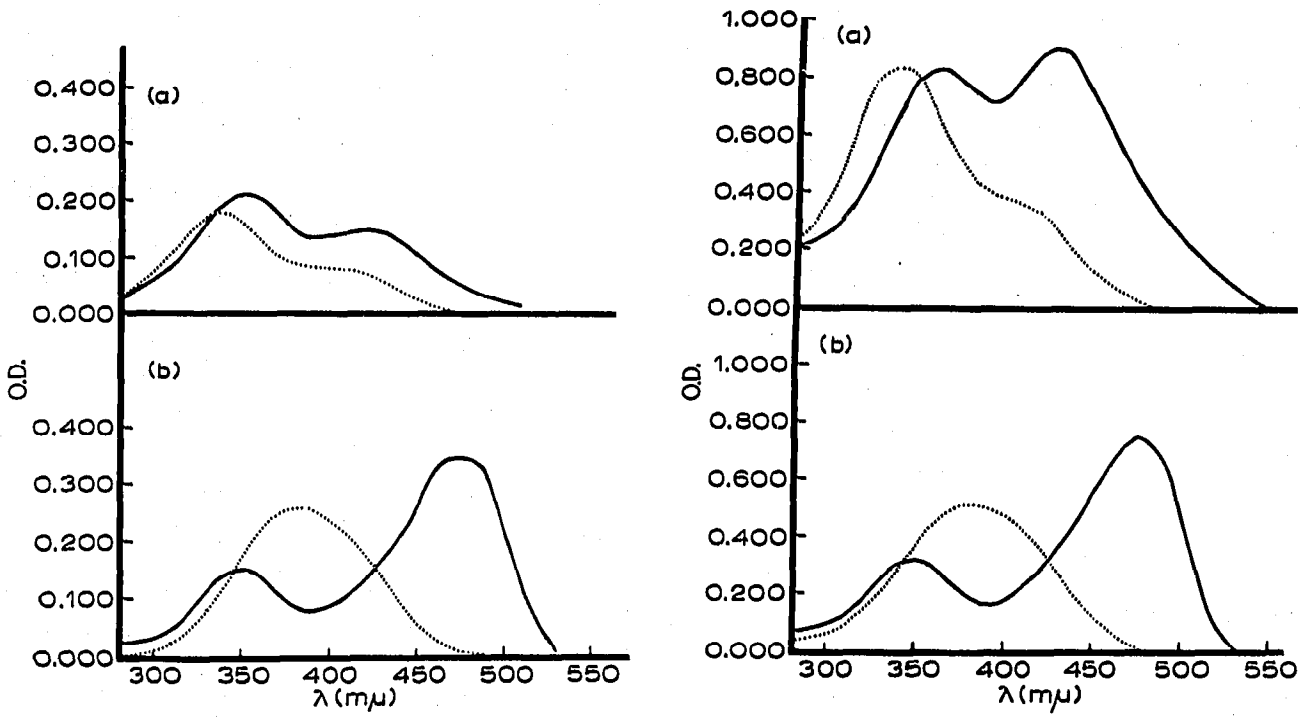

Fig. 5. Absorption spectra of TNP-leucine (a) and TNP-galactosamine (b). The spectra for the buffered alkaline reaction mixtures (———) showed absorption peaks for both compounds at 355 mμ, for the amino acid at 420 mμ and for the hexosamine at 475 mμ. The absorption spectra of the reaction mixtures after the addition of 0.1 ml concentrated HCl (· · · · ·).

Fig. 6. Absorption spectra of fractions from a Dowex 50W X8 column on a serum acid hydrolysate. (a) is a fraction from the phenylalanine-tyrosine peak; (b) is a fraction from the glucosamine peak. (———) and (· · · · ·) denote the spectra of the buffered alkaline reaction mixtures and acidified reaction mixtures respectively.

rable to those obtained with known amino acid and hexosamine solutions (Fig. 6). The addition of concentrated HCl produced a similar elimination of the 475 mμ peak in the column fraction identified as glucosamine.

DISCUSSION

Various procedures have been described for the spectrophotometric assay of amino acids and of hexosamines by separate means. The ninhydrin method[5] does not provide a differentiation between the two types of compounds. The less sensitive and less reliable ELSON AND MORGAN reaction for hexosamines must be used in conjunction with the ninhydrin method to enable a distinction to be made. As shown in Fig. 3, the ELSON AND MORGAN reaction, utilizing twice the test volume of the TNBS method, gave approximately one-third the optical density.

For a complex mixture of amino acids and hexosamines such as a serum protein acid hydrolysate, a method was needed to separate and identify easily the basic amino acid and hexosamine components. Column chromatography on Dowex 50W X8 by the GARDELL method[7] using 0.3 *N* HCl as the solvent did not separate the amino acid and hexosamine components of the serum acid hydrolysates. The TNBS procedure performed on eluates from a GARDELL column showed absorption spectra charac-

teristic of amino acids throughout the entire effluent volume. These spectra were not due to the effect of the 0.3 *N* HCl; hexosamines prepared in this solvent gave absorption curves similar to hexosamines in the 0.1 *M* phosphate buffer, pH 6.75. The GARDELL column method was also slower than the method described in this paper. Both columns were eluted at a flow rate of 2 ml/h; the hexosamines were eluted in 60 to 75 h with the GARDELL method and in 27 to 35 h with the modified MOORE AND STEIN method. Separation of the constituents of a serum acid hydrolysate by paper or thin layer chromatography was likewise unsatisfactory. The hydrolysate was so complex that a solvent system appropriate to separate all components simultaneously was not possible. However, modifying the MOORE AND STEIN method for the chromatography of basic amino acids on Dowex 50W X8 by careful regulation of the elution volume of the initial 0.1 *M* citrate buffer, pH 5.0, gave hexosamine peaks free from contamination by amino acids. Tryptophan not destroyed by acid hydrolysis did not overlap the hexosamine as reported by EASTOE[4] with this adjustment of the initial buffer.

The application of the GALAMBOS AND SHAPIRA TNBS procedure[2] at alkaline pH to the effluent fractions of the Dowex 50W X8 columns provided a simple simultaneous identification and quantitation of the amino acids and hexosamines. The ratios of O.D. 355:O.D. 475 of the absorbances of their trinitrophenylation products were characteristically 2:1 to 4:1 for the amino acids and 1:2 to 1:2.5 for the hexosamines. Additional confirmation of the type of compound, amino acid or hexosamine, was obtained by absorption spectra on the TNBS reaction mixtures. Absorption maxima occurred at 355 mμ for both amino acids and hexosamines, at 420 mμ for amino acids and at 475 mμ for hexosamines.

The advantages of the modified MOORE AND STEIN Dowex 50W X8 column used in conjunction with the TNBS method are obvious. Complex mixtures of amino acids and hexosamines may be separated reproducibly, absorbance is linear with concentration over a wide range, and amino acids and hexosamines may be distinguished and quantified simultaneously.

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