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Tris(hydroxymethyl)aminomethane Buffer and Amino Acid Analysis

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Amino acid analysis of 14 commercially obtained lots of tris(hydroxymethyl)aminomethane buffer (TRIS) has revealed the presence of either one or two ninhydrin-positive components which chromatograph in the region of the basic amino acids.

The major TRIS component, found in all grades of the buffer tested here, is stable to acid hydrolysis and obeys Beer's Law in the concentration range of 1 to 100 mM.

Tris(hydroxymethyl)aminomethane (2-amino-2-hydroxymethyl-1,3-propanediol; $(HOCH_2)_3CNH_2$; commonly designated as TRIS) is a widely used buffer for *in vitro* biochemical and biological investigations. TRIS has the disadvantage however, that by virtue of its primary amino group, it interferes with the most commonly used protein assays:[1–2] the Folin-Lowry,[3] the Biuret,[4–6] and the Kjeldahl.[4] In practice this interference can be minimised by choice of suitable blanks and standards,[4–5] by removal of TRIS by dialysis [3] or, by precipitation of the protein, washing to remove TRIS, and analysis of the separated precipitate.[7]

In the course of recent experiments we observed that with proteins dissolved in TRIS buffer, amino acid analysis was complicated by the appearance of either one or two ninhydrin-positive peaks which eluted in the region of the basic amino acids. It was possible to eliminate these TRIS peaks by either dialysis of the protein solution against a non-TRIS containing buffer, or by precipitation of the protein and washing prior to acid hydrolysis. We chose however, to retain the buffer, and investigate more closely the chromatographic properties of TRIS.

MATERIALS

Tris (hydroxymethyl) aminomethane was obtained from the following sources:

1. Sigma Chemical Co., Missouri, U.S.A. "Sigma 7 – 9" lot 68B – 5220; lot 70B – 5630; lot 96B – 5280; lot 127B – 5110; lot 31C – 5230; lot 71C – 5220; lot 121C – 5140. "Trizma" lot 90C – 5400; lot 121C – 5430.

2. Merck, Darmstadt, Germany. Tris(hydroxymethyl)aminomethane Art. 8382 lot 2481999; Art. 8365 lot 2612546.

3. British Drug Houses, Poole, England, Tris(hydroxymethyl)methylamine Lab. Reagent 27119 lot 1205080; Analar 10315 lot 1261560; Aristar 45205 lot 1402350.

Standard amino acid mixture was obtained from Bio-Cal Instruments, Gräfelfing, Germany.

METHODS

Acid hydrolysis

Acid hydrolysis was carried out under a vacuum of 0.1 mmHg in 6 N redistilled hydrochloric acid at 110°C for 20, 24, 48, or 72 h.

Amino acid analysis

1. Bio-Cal single column system. A model BC-201 Bio-Cal amino acid analyser was used under the following conditions: Bio-Rad Aminex A-6 resin (15.5 – 19.5 μm); column 0.9 × 54 cm; flow-rate 80 ml/h buffer, 20 ml/h ninhydrin; buffers: A 0.2 N Na citrate, pH 3.25; B 0.2 N Na citrate, pH 4.25; C 0.2 N Na citrate, 1.0 N NaCl, pH 6.50; buffer changes A to B 59 min, B to C 99 min, C to NaOH 207 min; temperature 58°C.

2. Durrum system. A Durrum model D-500 amino acid analyser was used under the following conditions: Durrum resin (8 ± 2 μm, 8 % crosslinkage); column 0.175 × 50 cm; flow-rate 10 ml/h buffer, 5 ml/h ninhydrin; buffers: A 0.2 N Na citrate, pH 3.25; B 0.2 N Na citrate, pH 4.25; C 0.4 N Na citrate, 0.7 N NaCl, pH 7.9; buffer changes A to B 25 min 30 sec, B to C 44 min 30 sec, C to NaOH 76 min; temperature 0 to 35 min 36 sec 52°C, 35 min 36 sec to 76 min 75°C.

3. Bio-Cal short basic column system. A model BC-201 Bio-Cal amino acid analyser was used as follows: Bio-Rad Aminex A-5 resin (11.5 – 15.5 μm); column 0.9 × 15 cm; flow-rate 80 ml/h buffer, 20 ml/h ninhydrin; buffer D 0.35 N Na citrate, pH 5.28; temperature 58°C.

Ninhydrin reaction

1. Manual reaction. The manual ninhydrin method was similar to that of Moore and Stein [8] with some minor modifications. The ninhydrin solution was identical to that used in the Bio-Cal BC-201 analyser and contained per liter: Recrystallized ninhydrin 20 g, $SnCl_2$ anhydrous 0.336 g, methyl cellosolve 750 ml, 4 N K/Na acetate buffer (3 N K acetate, 1 N Na acetate, $3H_2O$, 12 mM K citrate, pH 5.13) 250 ml.

All samples were adjusted to pH 6 and 2 ml aliquots containing 2 μmol of amino acid or 1000 μmol of TRIS were added to 1 ml of ninhydrin solution. The samples were mixed for 30 sec on a Whirlmixer and after sealing with aluminium foil, heated in a boiling water bath for 10 min. After cooling for 10 min the samples were diluted 15 times in 1:1 ethanol/water and spectra measured within 30 min in a Cary model 17 spectrophotometer. Blank samples contained water in the place of amino acids and were otherwise treated as above.

When the reaction was carried out under nitrogen both the samples and ninhydrin solution were extensively washed in dry nitrogen and the heating carried out in sealed ampoules under nitrogen.

2. Bio-Cal BC-201. The ninhydrin solution was that described above for the manual method. The sample/ninhydrin ratio used in the BC-201 was 2:1 and the absorbance was measured at 570 and 440 nm after 8 min heating at 100°C in a closed system.

3. Durrum D-500. The ninhydrin solution used was Nin-Sol obtained commercially from Pierce Chemical Company, (Rockford, Illinois) and contained per liter: 20 g ninhydrin, 0.625 g hydrindantin, $2H_2O$, 250 ml 4 M Li acetate buffer, pH 5.2, and 750 ml dimethyl sulphoxide. Reaction conditions in the Durrum were 2:1 sample/ninhydrin ratio, heating for 1.5 min at 125°C, absorbance measured at 590 nm.

RESULTS

TRIS buffer from all of the sources here tested showed a major peak which chromatographically behaved as a basic amino acid (Fig. 1). On the one column system of the Bio-Cal BC-201 the major TRIS peak appeared between phenyl-

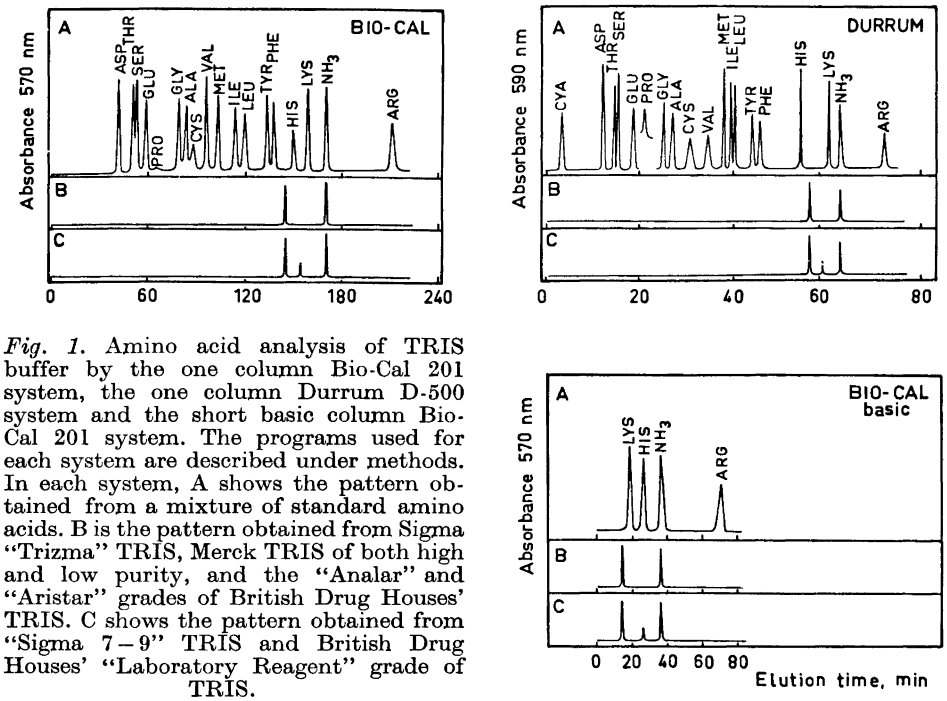

Fig. 1. Amino acid analysis of TRIS buffer by the one column Bio-Cal 201 system, the one column Durrum D-500 system and the short basic column Bio-Cal 201 system. The programs used for each system are described under methods. In each system, A shows the pattern obtained from a mixture of standard amino acids. B is the pattern obtained from Sigma "Trizma" TRIS, Merck TRIS of both high and low purity, and the "Analar" and "Aristar" grades of British Drug Houses' TRIS. C shows the pattern obtained from "Sigma 7 – 9" TRIS and British Drug Houses' "Laboratory Reagent" grade of TRIS.

alanine and histidine. In the one column high-speed Durrum D-500 system the major TRIS peak appeared between histidine and lysine. On the short basic column of the Bio-Cal BC-201 a system similar to the second column of the Beckman 120 C system, the major TRIS peak ran off slightly before lysine.

In samples of TRIS of lower purity ("Sigma 7 – 9" and BDH laboratory reagent) a minor peak was seen which occurred between histidine and lysine on the one column Bio-Cal system, between the major TRIS peak and lysine on the Durrum D-500, and together with histidine on the basic Bio-Cal column (Fig. 1). The magnitude of this minor peak varied from 10 % to 32 % of the area under the major peak. It was noted, however, that the absolute area under the major peak was constant in all cases where the minor peak was observed. The major TRIS peak is characterized by its high 440 nm absorbance in relation to the absorbance at 570 nm (Fig. 2). The spectrum of the major TRIS ninhydrin product, as determined after a manual ninhydrin reaction is qualitatively and quantitatively different from that of leucine, valine, or proline. The magnitude of the TRIS/ninhydrin absorbance at 570 nm is greatly affected

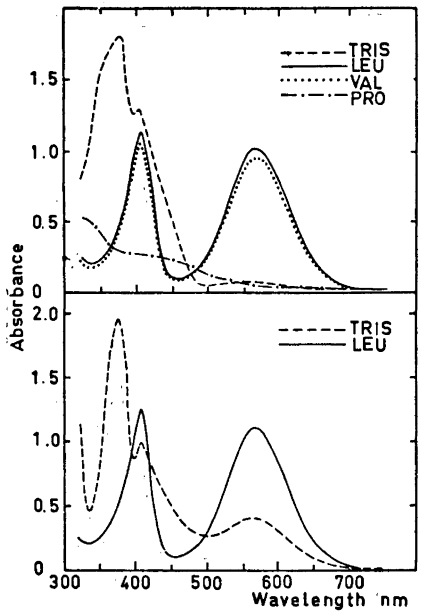

Fig. 2. Visible spectra of the manual ninhydrin reaction products of TRIS (500 mM), leucine (1 mM), valine (1 mM) and proline (1 mM). The manual ninhydrin reaction was carried out as described under methods. Upper half – heating carried out in air; Lower half – heating carried out under oxygen-free nitrogen.

by the presence of oxygen in the system. The bottom half of Fig. 2 shows the increase in absorbance at 570 nm as a result of carrying out the manual ninhydrin reaction in sealed ampoules under nitrogen. The colour yield of the major TRIS peak in the red region of the spectrum is therefore variable depending upon the exact reaction conditions used while remaining constant for any one set of conditions (Table 1).

The area under the major TRIS peak is linearly dependent on the concentration of TRIS in the range of 1 to 100 μmol/ml. Varying amounts of pro-

Table 1. Colour yield of TRIS.

Method	Colour yield Leucine equiv.	
Manual ninhydrin	0.0001	±3 %
Manual ninhydrin under N_2	0.0007	±3 %
Bio-Cal 201	0.0013	±2 %
Durrum D-500	0.0039	±3 %

The colour yield of Sigma "Trizma" lot 90C–5400 was measured after manual ninhydrin determination as described in Fig. 2. The colour yield on the Bio-Cal BC-201 of the same lot of "Trizma" TRIS was determined after amino acid analysis of a mixture containing 25 nmol of leucine and 50 μmol of TRIS. On the Durrum D-500 the colour yield was determined with a mixture containing 2.5 nmol of leucine and 200 nmol of the same lot of TRIS as above.

tein do not influence this relationship. TRIS is not significantly affected by acid hydrolysis for times up to 72 h, the differences observed over 72 h being within the limits of error of the amino acid analyser.

DISCUSSION

TRIS buffer and ninhydrin react in a manner which bears some similarities to the reaction between α-amino acids and ninhydrin. The TRIS/ninhydrin reaction mechanism appears, however, different, as evidenced by the low colour yield and the visible spectrum of the reaction product(s). The colour yield of the reaction at 570 nm is constant for one set of reaction conditions but variable between different reaction conditions. The absorbance of the TRIS/ninhydrin product(s) at 570 nm obeys Beer's Law in the concentration range of 1 to 100 μmol/ml (1 – 100 mM) under any one set of conditions.

TRIS yields either one or two ninhydrin-positive peaks on amino acid analysis. The major peak is present in all the grades of TRIS examined in the course of this work, whilst a minor peak was observed in TRIS grades of lower purity. Both the major and the minor TRIS peaks chromatographed together with the basic amino acids on the three column systems tested here. In both the higher and lower purity TRIS grades the elution position and magnitude of the major TRIS peak was not significantly affected by hydrolysis in 6 N hydrochloric acid for periods up to 72 h.

It can thus be noted that if TRIS is suspected as a contaminant during amino acid analysis the major TRIS peak can be immediately identified by its position and in the case of analysers equipped with 440 nm optics, by the high relative absorbance of the TRIS peak at this wavelength. If it is not possible or desired to remove TRIS from samples before acid hydrolysis, it is therefore suggested that grades of TRIS which are free from the minor component are used to simplify the interpretation of resulting chromatograms.

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