# A NANOMOLE ADAPTION OF THE AUTOMATIC AMINO ACID ANALYSIS ACCORDING TO SPACKMAN, STEIN, AND MOORE (1958).

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The automatic method described in the following represents a scale reduction of the procedure according to Spackman, Stein, and Moore (1958). Teflon tubing of 0,5 mm inside diameter was employed as the columns. For a complete analysis of both acidic, neutral and basic amino acids (the latter being analyzed on a seperate column) a sample containing approximately 2 nanomoles* of each amino acid (a.a.) was required, i.e., about 2 mg of liver, or muscle (fresh wt.), or 0,03 ml of blood plasma, or 5 µg of protein.

Columns. The resin preparation Aminex-MS, blend Q 150** was suspended in buffer and allowed to settle in a polystyrene pressure bottle. Tubing 1 (fig.1) leading down from a pump (syringe type, piston fit pressuretight up to 10 atm.) and tubing 2 serving as the column were both tightly fitted into the bottle's two stuffing - box type connectors. Tubing 2 (column) was dipped with one end into the settled resin. The free end of the column was compressed by an adjustable clamp, so that

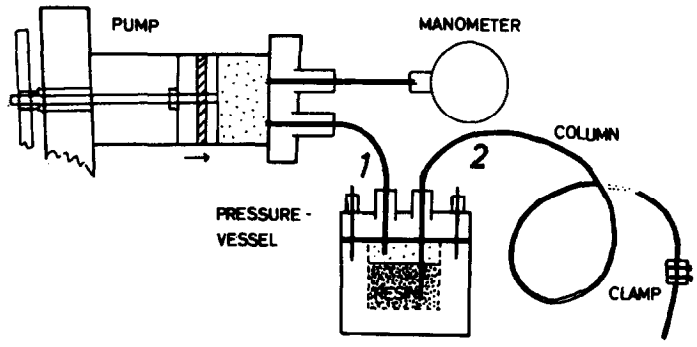



Figure 1 : Preparation of the column.

*1 nanomole = $10^{-9}$ moles

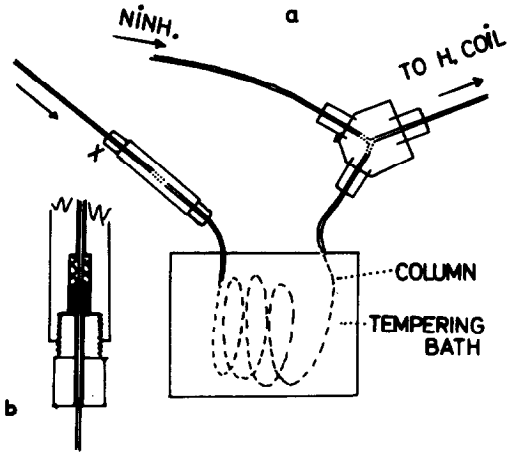
**California Corporation for Biochemical Research. (Standardized particle size distribution, specifically prepared for the method of Spackman, Stein, and Moore (1958)).

resin particles were retained but fluid allowed to pass. The filling and "packing" of the column was performed by applying pressure (1-3 atm.) to the polystyrene bottle by means of the pump. The packing process of the particles within the column took approximately 1 hour in case of a 150 cm column and could be observed by means of a lense. Subsequently, the column was removed from the pressure bottle and attached to the pump's outlet tube by means of a special fitting piece (fig.2b).

Flow rate; changing the eluent. To obtain the same flow speed within the column as employed by Spackman, Stein, and Moore (1958) it was observed that, since the column's cross sectional area (c.s.a.) was reduced from ~ 64 $mm^2$ to 0,19 $mm^2$, the eluent volume entering the column per unit time had to be decreased by a factor of ~ 300. Therefore, the output of the pumps was chosen to be 100 µl/hr.

With each change of the eluent, juncture X (fig.2a) was disconnected and, avoiding air bubbles, newly connected to the outlet of a pump filled with the subsequent medium. Though this procedure is not too laborious, it should be substituted by a more convenient device, which allows the switching from one pump to the other without disconnecting the system.

Figure 2 :
Scheme of column's arrangement (a) and fitting piece.




Operation times, pressure. Since it was endeavored to maintain the flow speed, it should have been possible to perform the column's regeneration and buffer equilibration within the same time periods as Spackman, Stein, and Moore (1958) describe. Nevertheless, we increased those periods slightly, since it proved that the elution times of the amino acids were somewhat higher than those of the large scale model. The elution of the acidic group took 17 hours (from applying the sample to the column to the

reaching of the base line after the alanine peak). This was obviously due to the fact that the column's inside diameter was slightly different from the ideal dimension, so that the flow speed could not be actually maintained. The operating pressure at the 150 cm column was 1,4 atm. at 50°C and 1,8 atm. at 37°C. The pressure at each pump outlet was registered by a 1-10 atm. manometer. In case of a blockage the fitting in front of the column began leaking at 6-8 atm.

Pretreatment of buffers; ninhydrin solution. Ninhydrin solution was filtered, buffers were boiled and filtered before aspiration into the pump cylinder. The volume capacity of the syringe pump's cylinder was ~ 40 ml. It provided a good reservoir for ninhydrin solution, which proved to be stable there for more than 6 weeks. The ninhydrin solution used was that according to Moore and Stein (1954), not the stannous chloride form.

Narrowing the effluent stream; heating coil. As one should expect, considerable tailing and blurring of the peaks occurs, if a bore of 0,5 mm was maintained throughout the entire system. A sufficient narrowing of the effluent stream was obtained by leading a stainless steel wire (diameter 0,4 mm) through the teflon tubing (bore 0,5 mm). The residual c.s.a. is then ~ 0,06 $mm^2$. A better reduction of the c.s.a. was obtained, if capillary stainless steel pipe 0,2 mm in bore was connected to the column's outlet, the c.s.a. being 0,03 $mm^2$. Though this reduction of the c.s.a. of the effluent stream was much smaller than on the column of Spackman, Stein, and Moore, the effect was sufficient. The ideal means, of course, would be teflon tubing smaller in bore than 0,5 mm, if it were available. A volume of 3 µl, equalling approximately a 1 ml fraction in a conventional large column, occupies a length of 10 cm in the steel capillary, and a length of 5 cm in the teflon tubing with inside wire.

The confluence of the eluate and the ninhydrin stream was performed by means of a fitting piece with Y-shaped bore. The mixing ratio was chosen to be 1 : 1 . Obstruction of the heating coil did not occur, if a halt in the flow of the mixture within the coil was avoided. If the apparatus should be stopped, the ninhydrin pump should be turned off at least half an hour before. By elevation of the system's outlet (behind the absorption cell) a pressure of about 1,5 m water was applied to the heating coil for avoiding bubble formation. As easily calculated, the length of the heating coil should be ~ 160 cm in case of 0,03 $mm^2$ c.s.a., and ~ 60 cm in case of 0,06 $mm^2$ c.s.a., respectively, if the mixture is to remain 15 minutes at 100°C.

Absorption Cell, Photometry, Recorder. To obtain good sensitivity of the photometric procedure without critical widening of the stream diameter we employed an ultramicro-cuvette designed by Ullrich and Hampel (1958). The fluid passed this platinum cell through a ⌐¬-shaped channel of 0,5 mm inner bore. The light beam transverses the horizontal part of the channel in the same direction as the fluid flows, the light path being 6 mm. The platinum cell, enclosed in a brass housing of suitable dimensions, was assembled in the normal tray holder of a photometer. The photometer must provide sufficient light intensity and focusing, and a sufficient zero point constancy over long periods. The Zeiss spectrophotometer PMQ II could be employed with a wide allowance of amplification remaining. Also a much simpler instrument, the filter - photometer "Eppendorf" (Beisenherz et al., 1953) proved to be suitable, if the elevated amplification was employed (step-up switch in drawn-out position, amplification step 12). We used the photometer "Eppendorf" in connection with a diode circuit* (transforming transmission to optical density) and a Philips Multi Channel Recorder Type PR 3210 A/00. Preliminarily, only one channel was utilized, measuring one cell at one wave length (578 mμ). Devices for continous measuring with more than one micro cell (with different light paths and two wave lengths, 578 mμ and 436 mμ) are in development**.

Quantity of substance applied to the column; technique of application. To obtain optimal peaks from a conventional column of 64 $mm^2$ c.s.a., approximately 0,5 micromoles of each amino acid must be applied. Since we reduced the c.s.a. by a factor of about 300, one could expect to obtain good peaks applying ~ 1,7 nanomoles. This proved to be approximately true. Fig. 3 shows a section of a chromatogram (the group aspartic acid, threonine, serine, glutamic acid, citrulline) obtained by applying 2,5 nanomoles of each amino acid to a 150-cm-column. By means of a lambda-pipette* 10 μl of a standard mixture*** were transferred to a small Misco centrifuge tube. From there this volume was drawn into the tubing immediate in front of juncture X (fig.2) by suction with aid of the pump piston. Subsequent to this, 5 μl of buffer pH 2,2 were transferred 3 times to the same tube and drawn in similarly. After this the tubing was reconnected at juncture X. Entering of air bubbles had to be avoided during the whole procedure. This is easily attained, if the

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*Netheler u. Hinz GmbH, Hamburg-Wellingsbüttel, Wellingsbüttler Weg 75

**Firms Carl Zeiss, Oberkochen/Württ., and Netheler u. Hinz GmbH, Hamburg.

***Spinco No. 120-220 Amino Acid Calibration Mixture Type 1 (containing 2,5 μMol/a.a./ml), diluted 1 : 10 with buffer pH 2,2.

initial disconnection at juncture X is performed carefully under simultaneous slight pushing of the piston.

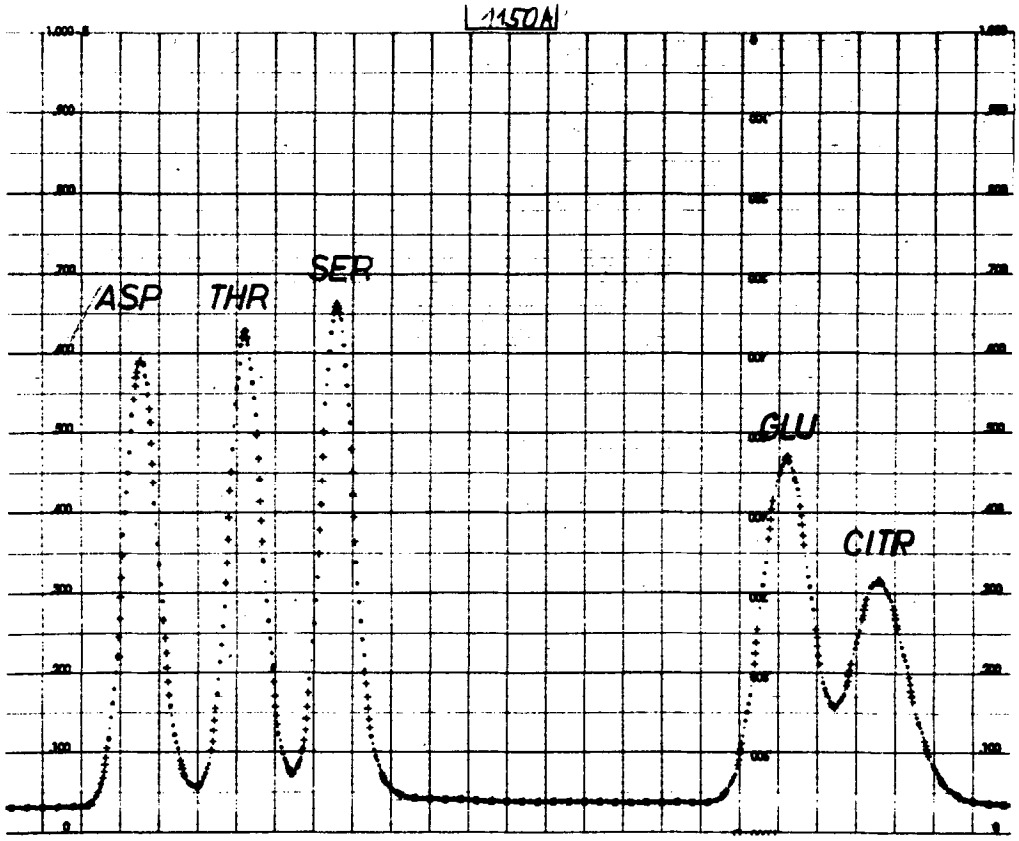


Figure 3 : Section of a typical chromatogram obtained by applying 2,5 nanomoles of each amino acid to a 150 cm column. Abscissa: time; distance between two vertical lines =30 minutes Ordinate: Absorbance.

Evaluation; precision of the method. Absorbance curves were obtained. The areas of the peaks (above the zero line) were measured by means of planimetry*. In table 1 the means of the planimetric values (number of planimeter units) obtained from a series of six chromatograms of the acidic amino acids are listed, together with the relative standard deviation.

Presumably, the precision can be improved, if the metering of small amounts of fluid and their application to the column were facilitated by a suitable device.

*Aristo-planimeter 1137 L, Aristo-Werke, Hamburg-Altona.

Table 1

Reproducibility of results with known mixtures of amino acids. Data obtained from six chromatograms. Load 2,5 nanomoles of each amino acid (1,2 nanomoles in case of phosphoserine and taurine, 56 nanomoles in case of urea).

| | Mean (planimeter units) | Relative Standard Deviation, % |
|---|---|---|
| Phosphoserine | 156 | 7,0 |
| Taurine | 154 | 11,4 |
| Urea | 360 | 5,9 |
| Aspartic Acid | 332 | 4,9 |
| Threonine | 330 | 5,0 |
| Serine | 340 | 5,6 |
| Glutamic Acid | 350 | 1,6 |
| Glycine | 328 | 1,2 |
| Alanine | 342 | 3,6 |

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