THE CHROMATOGRAPHY OF NINHYDRIN NEGATIVE COMPOUNDS ON AN AMINO ACID ANALYZER COLUMN

ARTHUR WAINER
Department of Biochemistry, Bowman Gray School of Medicine, Winston-Salem, N.C. (U.S.A.)
(Received June 3rd, 1966)

INTRODUCTION

Although the elution positions of numerous ninhydrin positive compounds have been established on various amino acid analyzers, very little is known concerning the elution of ninhydrin negative compounds on such columns. Some elegant methods have been developed for the elution of sugars and other compounds on anion exchange columns[1-3] and several studies on the absorption of non-electrolytes and weak electrolytes on cation exchange columns have been reported[4-6]. One generalization from the latter type studies is that extra —CH_2-groups increase absorption to resins[4,6-8]; however the specific effect of additional —CH_2-groups in relation to the chain length of a given compound has as yet not been made clear.

The purpose of the present investigation was to establish the elution positions of various ninhydrin negative compounds on an amino acid analyzer column using radioactively labeled compounds and an appropriate detection system. It was felt that with the increasing use of continuous flow radio-detection systems an established pattern of biological compounds would be of interest. The elution position of ninhydrin negative compounds may also be of interest in routine amino acid analysis since some ninhydrin negative compounds may interfere with or be necessary for normal color development. Sucrose would be an example of the former and HCN would be an example of the latter type compound[9].

Another purpose of this study was to investigate some of the non-ionic forces involved in the attraction of compounds to amino acid analyzer columns by using the patterns obtained above.

METHODS

The amino acid analyzer column (140 × 0.6 cm) of the Technicon Auto Analyzer system equipped with Type A Chromobeads (8% cross-linked, spherical particles having a specified diameter of 18 to 25μ) was used in this study[10]. The column was equilibrated and eluted with pH 2.87 citrate buffer (0.05 *M*; 0.20 *M* with respect to Na). The buffer most commonly used in this laboratory was purchased as a prepared Harleco reagent. It was discovered that this preparation contained twice the amount of Brij 35 detergent as prescribed by the Technicon manual which for one liter of buffer calls for 10 ml of a Brij 35 solution (prepared by dissolving 100 g of Brij 35 in 200 ml of water). The effect of Brij 35 concentration is described in the results. In

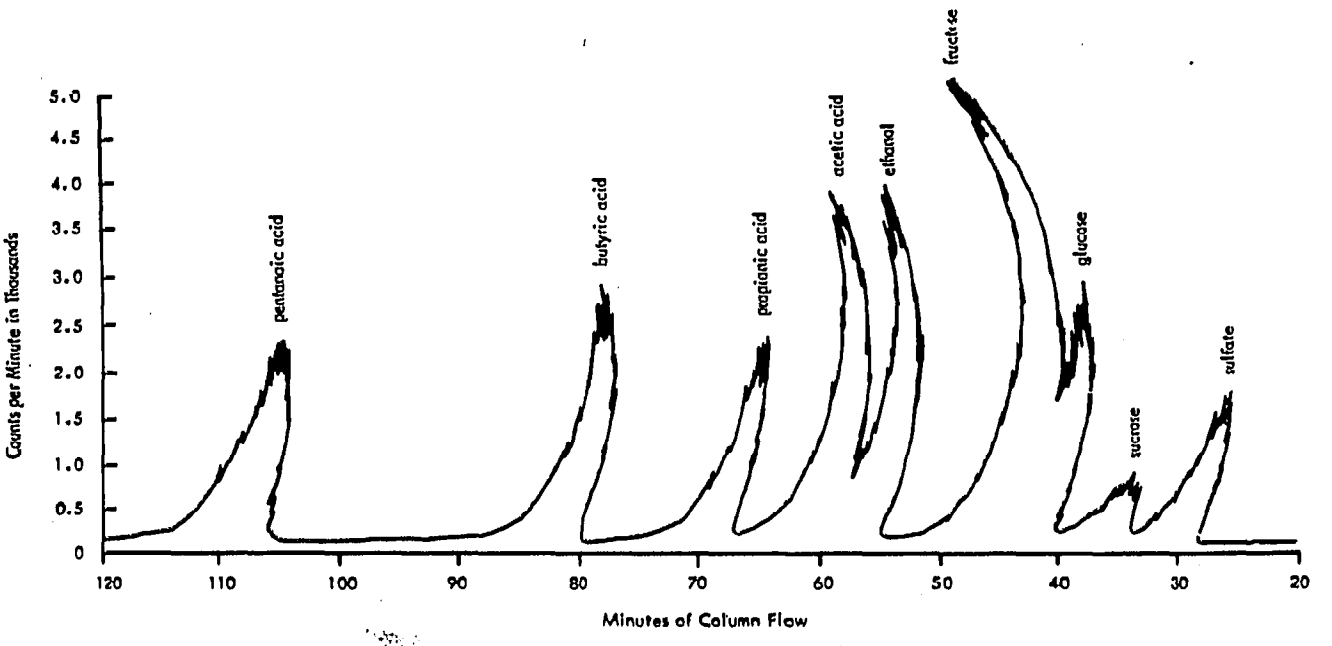

Fig. 1. An actual elution pattern obtained with a mixture of ^{14}C labeled compounds on the Technicon amino acid analyzer column. The flow rate was 0.45 ml/min and the column temperature was 60°. The equilibrating and eluting buffer was at pH 2.87, containing 20 ml of detergent per l.

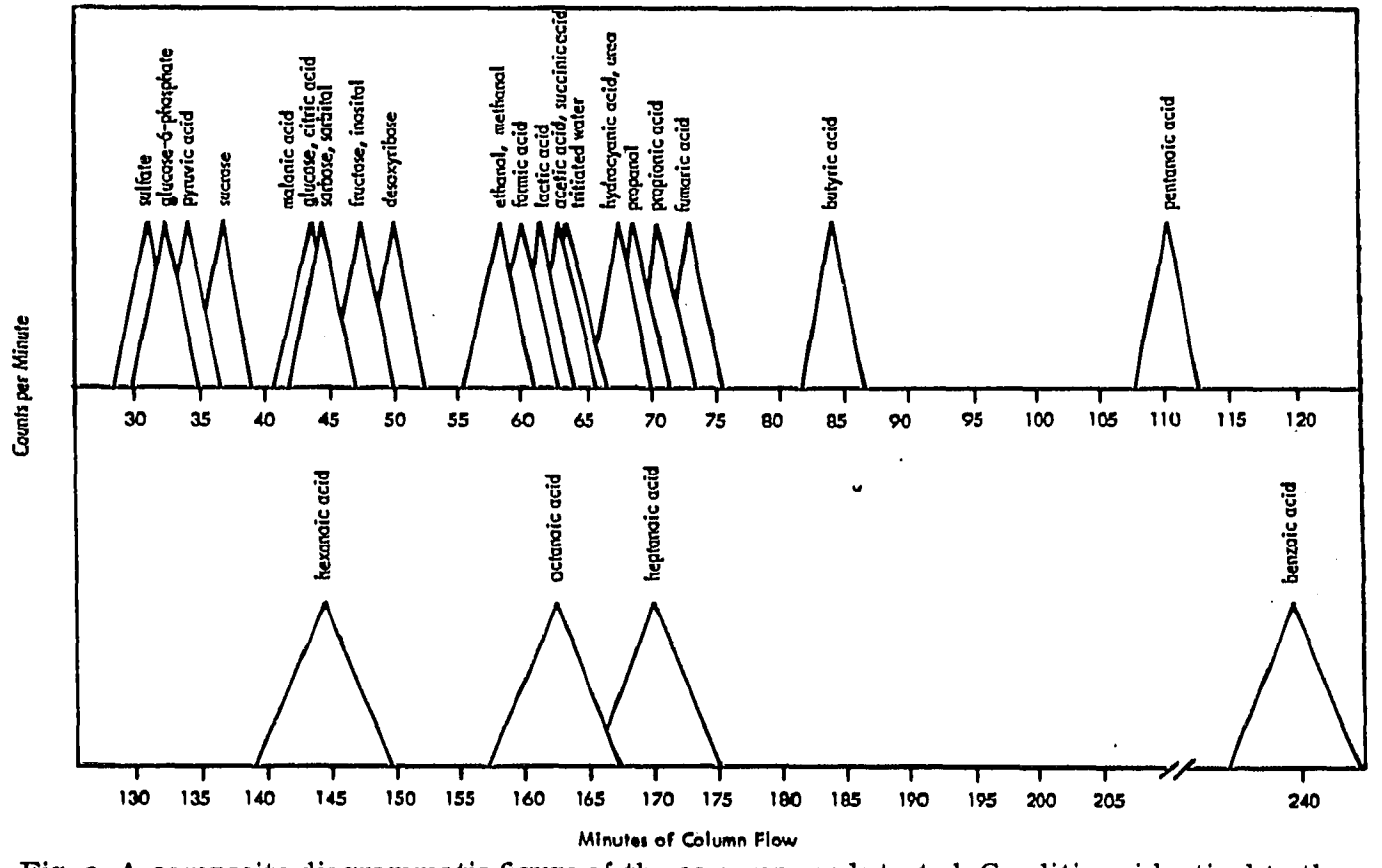

Fig. 2. A composite diagrammatic figure of the 30 compounds tested. Conditions identical to those in Fig. 1.

some experiments the buffer was adjusted to pH 1.87 and was used for both equilibration and elution. The effluent from the column was monitored by a liquid scintillation detector equipped with a flow cell (Nuclear Chicago). The compounds studied were usually ^{14}C labeled, purchased or donated by various suppliers. Amounts from 0.1 to 10 mμmoles containing 1 to 50 mμC were used with no difference in elution within the concentration range.

RESULTS

A typical elution pattern obtained from a mixture of several compounds is shown in Fig. 1. The pattern demonstrates the degree of separation that may be expected for the thirty compounds shown on the diagrammatic composite in the next figure.

Fig. 2 is a representation of the elution pattern obtained with all of the compounds tested. Included is urea, which is slightly ninhydrin positive but is shown to give orientation. It may be seen that in the homologous series of acids the effect of additional —CH_2-groups depends on the size of the acid to which they are added. Thus, there is little difference between the elution of acetic and propionic acids, however as the chain length increases the effect becomes more pronounced, being maximal between pentanoic and hexanoic acids. The effect then decreases and becomes negative between heptanoic and octanoic acids. Results with longer chain acids are shown in a following figure. Alcohols are eluted at a slightly more rapid rate than the corresponding acids. Sugars are most rapidly eluted, with most being in a tight group. Sucrose, glucose, and fructose can be separated as shown in Fig. 1.

Of specific interest is the common elution time of various unrelated compounds in the region of 60–70 min. All of these compounds seem to have only limited capacity to be attracted to the resin.

Several of the compounds tested are partially ionized at pH 2.87 and some of these were rerun at pH 1.87 as shown in Fig. 3. Citric, malonic and fumaric acids are retarded at pH 1.87 compared to 2.87, which is consistent with their dissociation constants (0.84, 1.61, and $1.00 \cdot 10^{-3}$ respectively). Other compounds with lower dissociation constants are unaffected by the pH change in this region. The effect of the double bond of fumaric acid on elution from the resin is evident at the lower pH, fumaric acid being eluted 35 min later than the corresponding saturated compound, succinic acid. Also, the relative positions of malonic and succinic acids at pH 1.87 where ionization is negligible demonstrates the slight effect of the —CH_2-groups on the non-ionic forces involved in relatively small molecules.

The elution of some longer chain fatty acids and the effect of detergent on their elution is shown in Fig. 4. The longer chain fatty acids are eluted more rapidly as the detergent concentration increases. The elution patterns of the shorter chain fatty acids and alcohols are not shown but are similar to that obtained in Fig. 1 at all detergent concentrations. The effects of detergent only become important starting with pentanoic acid.

DISCUSSION

In the present study the ^{14}C-labeled compounds used were of two types: (1) compounds that are of biological interest and may be reactants or products of meta-

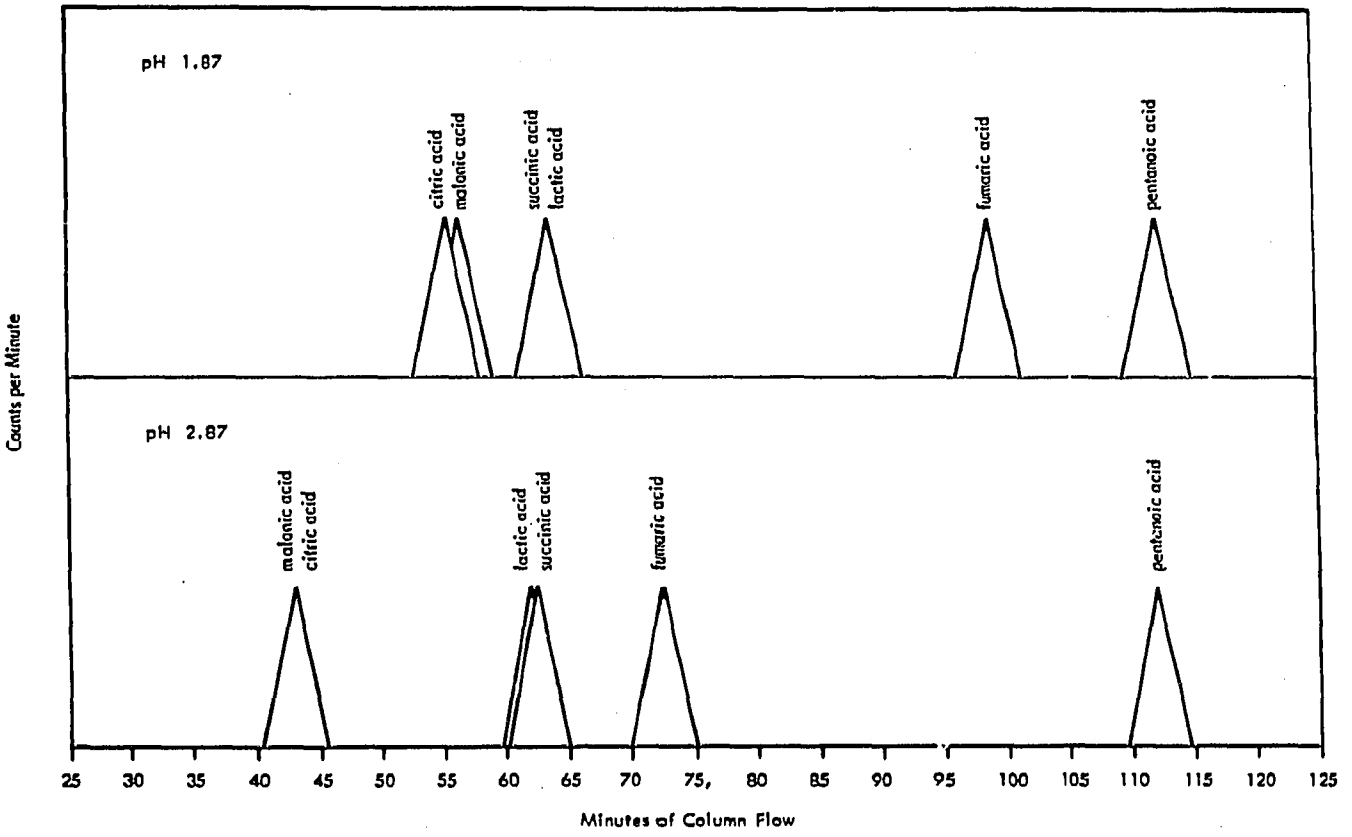

Fig. 3. The effects of pH on the elution of several compounds from the column. The conditions were similar to those in the previous figures with the exception that compounds were run at the two different pH values indicated.

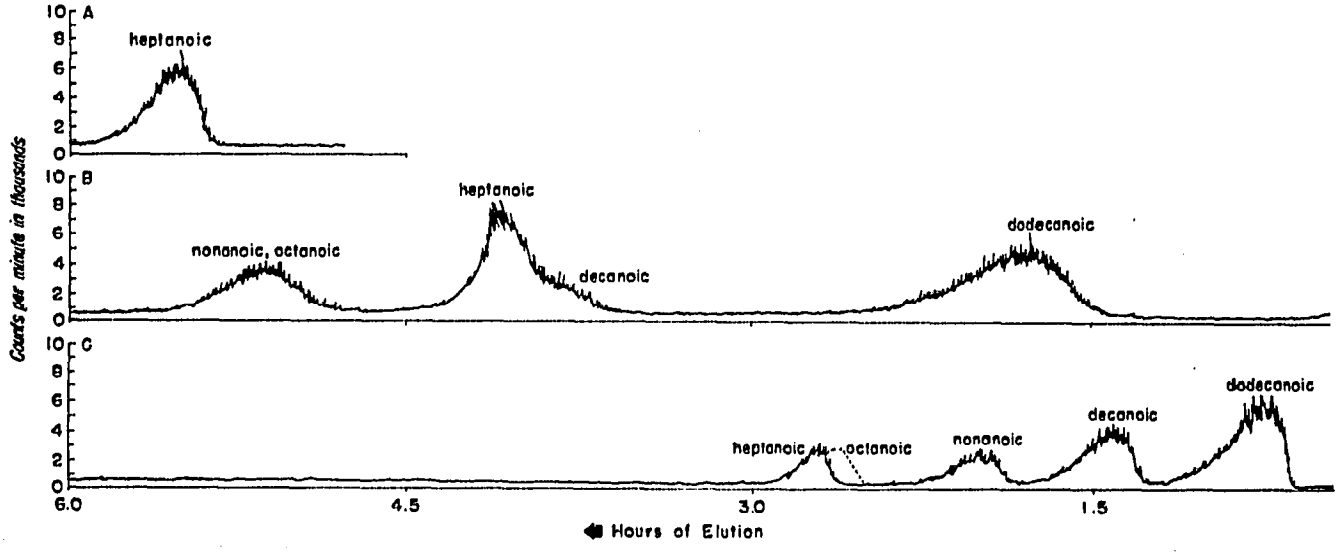

Fig. 4. The elution of longer chain fatty acids and the effect of detergent on the elution patterns. Part A is the elution of fatty acids with a buffer solution containing no detergent. Only heptanoic acid emerges by 6 h. Octanoic acid (not shown) comes at 12.5 h and nonanoic, decanoic and dodecanoic acids do not emerge even after 17.5 h. Part B is the elution pattern obtained using the buffer containing 10 ml of detergent per l (see methods) and Part C is the pattern obtained using the buffer containing 20 ml of detergent per l. The last buffer was that used in the experiments shown in the previous figures.

bolic reactions, for example, acetic, lactic, pyruvic acids and some of the intermediates of the citric acid cycle and (2) compounds whose elution patterns might aid the understanding of the forces operating in chromatography on the column. Examples of the latter are the series of fatty acids and the alcohols.

Some compounds of metabolic interest are well separated with the pH 2.87 buffer, such as pyruvic and acetic acids. The production of these metabolites from ^{14}C-labeled alanine and cysteine has been studied in this laboratory. Also, the mitochondrial production of acetate from pyruvate has been studied. The separation of acetic and lactic acids could probably be effected by increasing the pH of the eluting buffer, causing lactic acid to dissociate while acetic acid would remain largely undissociated. Intermediates of the citric acid cycle such as citric, succinic and fumaric acids are well separated at pH 1.87. The principal advantage of such column chromatography as compared to paper or thin layer chromatography is the large volume and amount of material that can be placed on the column.

A few general observations concerning the attractive forces of the column may be given. The effect of adding $-CH_2-$groups is to increase the Van der Waals forces as previously noted. Such forces are probably too slight to be very significant in the small molecules such as methanol, ethanol, acetic acid etc. but when combined in the longer chain molecules, the forces of the individual groups seem to potentiate each other. The attractive forces of the aliphatic side chains of amino acids may also act to potentiate the ionic forces and may therefore exert more influence than predicted on the basis of their individual attractions. The effect of detergent concentration on the elution of the longer chain fatty acids is understandable and may be of use in the elution of compounds with long nonpolar chains such as di- or tripeptides[11].

Previously, SARGENT AND RIEMAN[5] have described the elution of organic acids on both anionic and cationic resins using salting out chromatography. Although they were successful in separating the acids on an anion exchange resin (due to ion exchange) their results were less successful on cation exchange columns. The present success is due to the high resolution column used as well as the very high sensitivity of the radiodetection method. However, longer chain fatty acids could be potentially separated on other columns, the limiting factor being their solubility in the acidic buffer.

Using the data obtained above a concluding observation may be made concerning the distribution of the water in the column. Since the water in the column is both around and within the resin, molecules may occupy both water spaces in similar or vastly different proportions[12]. It is likely that the sulfate anion is almost entirely excluded from the interior of the resin and its elution time represents the time necessary to pass through the external water of the column. At the elution rate of 0.45 ml/min and subtracting two minutes delay time necessary to be detected by the liquid scintillation counter, the external volume of water calculates to be about 12.5 ml. The total column volume is 37 ml and thus the resin plus internal water would be 37—12.5 or 24.5 ml. The elution time (about 60 min) of the several neutral and often unrelated small molecules which probably penetrate the interior of the resin with ease may be an estimate of the internal plus external water volume, equal to 26 ml. Thus, the internal volume would be 26—12.5 or 13.5 ml and the resin volume would be 37—26 ml or 11 ml.

A more complete understanding of such factors as water distribution in a resin

column as well as the attractive forces of various groups may enable the accurate prediction of the elution of any given molecule from such a column.

ACKNOWLEDGEMENTS

The author would like to acknowledge the advice and consultation of Dr. J. S. KING. Also, this work was supported in part by a grant (07233) from the NIH.

SUMMARY

The elution of 30 radioactive ninhydrin negative compounds from an amino acid analyzer column is described. Some compounds of metabolic interest are shown to be well separated on the column. The effect of $—CH_2$-groups on the elution of compounds is shown to depend on the size of the given molecule. Based on the elution of the compounds tested some calculations are made concerning the volume occupied by the resin, the water in and surrounding the resin.

REFERENCES

1 O. SAMUELSON AND B. SWENSON, *Acta Chem. Scand.*, 16 (1962) 2056.
2 O. SAMUELSON AND B. SWENSON, *Anal. Chim. Acta*, 28 (1963) 426.
3 O. SAMUELSON AND L. O. WALLENIUS, *J. Chromatog.*, 12 (1963) 236.
4 D. REICHENBERG AND W. F. WALL, *J. Chem. Soc.*, (1956) 3364.
5 R. SARGENT AND W. RIEMAN, *J. Org. Chem.*, 21 (1956) 594; *Anal. Chim. Acta*, 17 (1957) 408; 18 (1958) 197.
6 S. S. BHATNAGAR, A. N. KAPUR AND M. L. PURI, *J. Indian Chem. Soc.*, 13 (1936) 679.
7 S. S. BHATNAGAR, A. N. KAPUR AND M. S. BHATNAGAR, *J. Indian Chem. Soc.*, 17 (1940) 361.
8 C. W. DAVIES AND G. G. THOMAS, *J. Chem. Soc.*, (1951) 2624.
9 H. ROSEN, C. W. BERARD AND S. M. LEVENSON, *Anal. Biochem.*, 4 (1963) 213.
10 *Technical Manual, Amino Acid Autoanalyzer*, Technicon Instruments Corp., Chauncey, N.Y.
11 J. S. KING AND A. WAINER, *J. Chromatog.*, 24 (1966) 215.
12 D. REICHENBERG, in C. CALMON AND T. R. E. KRESSMAN (Editors), *Ion Exchangers in Organic and Biochemistry*, Interscience, New York, 1957.