CHROM. 5503

Improved quantification of imino acids poorly resolved by automated amino acid methods

A common feature of most automated amino acid procedures is the recording of chromatographic data at wavelengths of 570 and 440 mμ, the latter being used primarily for quantifying the two imino acids, proline and hydroxyproline. The slight reactivities of imino acids with ninhydrin, coupled with their occurrence in relatively low concentrations in physiological fluids, render their accurate measurement somewhat difficult. Another factor that further reduces the precision of imino acid analyses is the frequent occurrence of inadequately resolved peaks; *e.g.*, the incomplete separation of hydroxyproline from aspartic acid and of proline from either glutamic acid or citrulline, or both.

This report describes a procedure which provides a more precise analysis of imino acids, even when their chromatographic peaks are fused to such an extent that their presence is not otherwise discernible. The modified procedure features the splitting of the chromatographic effluent into two streams and the simultaneous use of two ninhydrin reagents, one of which is specifically designed to react preferentially with imino acids.

Experimental

Instrument. An automatic amino acid analyzer (Technicon Corporation, Tarrytown, N. Y.) that utilized the single column, gradient elution procedure of PIEZ AND MORRIS[1] was used in this study. Except as noted below, the operation of the analyzer, including the solutions and materials used therewith, was in accord with the operation manual[2] furnished by the manufacturer of the analyzer. The chromatographic system consisted of a 140 × 0.636 cm column, packed to a height of 130 cm with Technicon Chromobeads (Type B) and maintained at 60° by the circulation of water through the jacket.

Procedures. Alterations in the recommended operational procedure[2] may be deduced from the flow scheme shown in Fig. 1; three modifications are particularly noteworthy. First, the effluent stream was split into two approximately equal parts, one of which was mixed with the conventional ninhydrin reagent recommended by the manufacturer[2] and denoted in Fig. 1 as Nin-I. The second modification consisted of mixing a novel ninhydrin reagent, Nin-II, with the other half of the effluent stream. The preparation of Nin-II was identical to that of Nin-I except that hydrindantin was omitted. The third modification was in the equipping of the three colorimeters; as is shown in Fig. 1, two of the colorimeters were equipped in the usual manner[2], to permit absorbance measurements at 440 and 570 mμ, both with an optical path of 15 mm. The reactant stream passing through these colorimeters resulted from the admixture of Nin-I with effluent. The third instrument (colorimeter No. 1, Fig. 1), which ordinarily was equipped with an 8 mm flow cell for attenuated reading at 570 mμ, was equipped instead with a set of 440 mμ filters and a 15 mm flow cell to measure the absorbance resulting from the reaction of the effluent with Nin-II, the hydrindantin-free reagent. This latter modification was made solely to permit direct experimental comparison of the two ninhydrin reagents for use

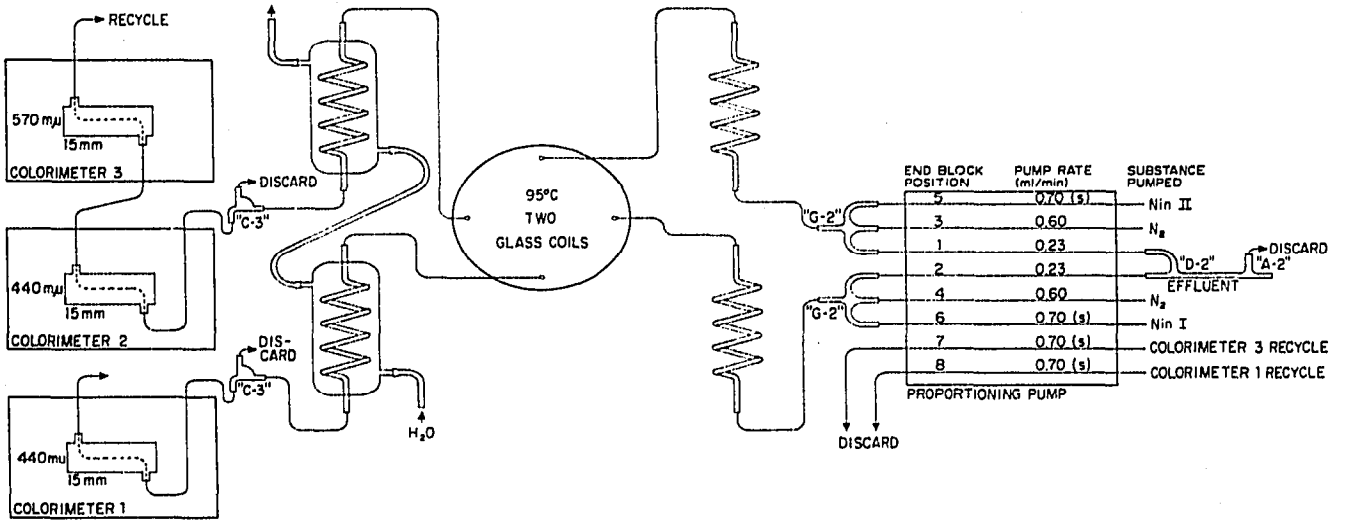

Fig. 1. Flow scheme of the experimental system used to study the analysis of imino and amino acids. The letters and digits enclosed in quotation marks (*e.g.*, "A-2") refer to Technicon glass connections, and the notation (S) under the heading PUMP RATE indicates the use of Solvaflex manifold tubing. Nin-I denotes the unmodified ninhydrin reagent; Nin-II is the hydrindantin-free reagent.

440 mμ and is not used in the routine operation of the system described in this paper.

In addition to the two ninhydrin reagents indicated above, two other ninhydrin reagents were prepared for limited experimental use. These were prepared in the same manner as described[2] for Nin-I, except that they contained one-third and two-thirds, respectively, of the quantity of hydrindantin recommended by the manufacturer of the instrument[2].

Two types of experiments were performed with the modified analyzer to demonstrate the application of the modified reagent. In one, the entire amino acid analyzer was utilized for separating and quantifying a mixture of amino and imino acids. The second type of experiment did not involve the chromatographic capability of the instrument, but utilized the analytical system as follows. Individually prepared solutions of glycine, proline, or hydroxyproline were introduced into the analytical system via the manifold tubes through which the two chromatographic effluent streams were normally pumped. This procedure was used for two purposes; first, to determine the reactivities of an amino and an imino acid with each of the four ninhydrin reagents, and second, to construct calibration graphs for the reactivity of proline and hydroxyproline with two of those reagents.

Results and discussion

The rationale for using the split-stream, two-reagent procedure described herein is apparent from the data illustrated in Fig. 2. It is evident from these data that the stepwise reduction in the quantity of hydrindantin in the ninhydrin reagent resulted in only a slight loss in 440 mμ absorbance of the hydroxyproline chromogen. In contrast, however, it is apparent that the ninhydrin reactivity of glycine was markedly influenced by the quantity of hydrindantin in the reagent, the absorbance of the glycine product at 440 mμ being scarcely detectable when hydrindantin was omitted from the reagent. The slight residual color produced by glycine in the absence of added hydrin-

dantin may be attributed to the small quantity of hydrindantin produced as an intermediate in the reaction of glycine with ninhydrin.

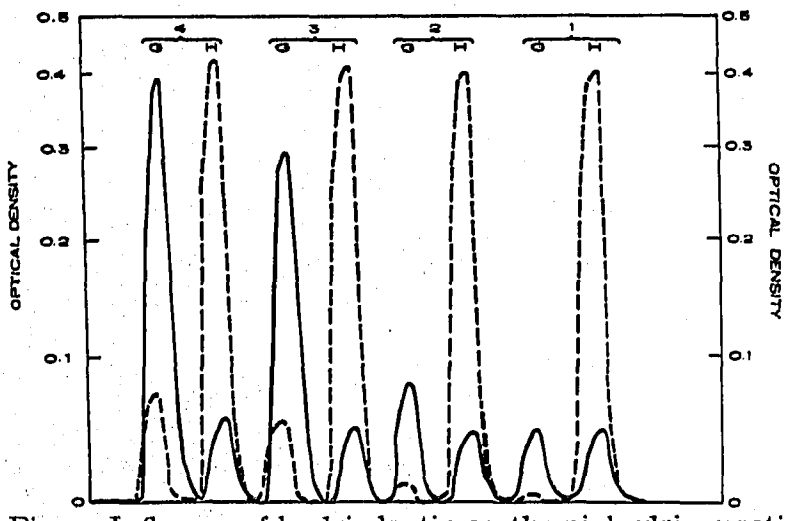

Fig. 2. Influence of hydrindantin on the ninhydrin reactivities of 0.1 mM glycine (G) and 0.48 mM hydroxyproline (H) solutions. Ninhydrin reagents containing 0, 0.5, 1.0, and 1.5 g/l hydrindantin, are designated by the numbers 1, 2, 3, and 4, respectively. Color production was monitored at 440 mμ (- - -) and 570 mμ (——).

The finding that Nin-II resulted in a slight decrease in 440 mμ absorbance of the hydroxyproline product suggests that the modified reagent may have resulted in some loss in sensitivity. Irrespective of this possibility, it is evident from the calibration data presented in Fig. 3 that Nin-II was superior to Nin-I with respect to quantitative accuracy. Although a linear response was found for proline and hydroxyproline with both reagents, close scrutiny of the calibration data indicates that the plots of absorbance against concentration for both of these imino acids did not pass through the origin when Nin-I was used.

The chromatogram depicted in Fig. 4 was selected from among many others

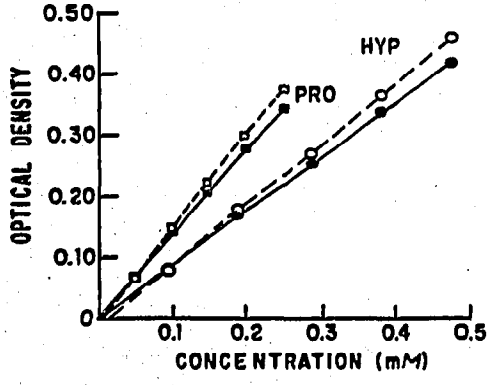

Fig. 3. Calibration curves of ninhydrin products of imino acids at 440 mμ. Proline product produced from Nin-I (□ - - - □) and Nin-II (■ —— ■); hydroxyproline chromogen produced from Nin-I (○ - - - ○) and Nin-II (●—— ●). Each solution was pumped into the manifold for a sufficient time to permit the recording of a 5-min plateau at maximum absorbance.

Fig. 4. Influence of the ninhydrin reagent on the quantitation of imino acids that are poorly resolved from neighboring amino acids. The recordings shown were made at 440 mμ using Nin-I(---) and Nin-II (——) reagents. Hyp, hydroxyproline; Cit, citrulline; other amino acids are designated by the commonly used abbreviation.

because it exemplified the poorly resolved imino acid peaks that one encounters, and also illustrates the marked improvement in the quantification of those peaks by the modified system described herein. Because absorbance values at 440 mμ were of primary interest in the present experiments, the 570 mμ absorbance recording resulting from use of the unmodified reagent, Nin-I, is not shown in Fig. 4. It is worthy of mention, however, that the 570 mμ recording in this chromatogram gave no indication of the presence of hydroxyproline or proline.

In contrast, the 440 mμ tracing obtained with Nin-I revealed that the hydroxyproline peak was fused with that of aspartic acid and the proline peak was merged with the citrulline peak. Resolution was so poor that area calculations based on the ascending limb of the hydroxyproline peak and the descending side of the proline peak yielded erroneously high values for both imino acids. Thus, when Nin-I is used, proline and hydroxyproline exert an inconsequential influence on the analysis of amino acids, whereas amino acids drastically interfere with the quantitation of imino acids. This interference was virtually eliminated by the use of Nin-II as the reagent, with the result that quantitative analysis of proline and hydroxyproline was notably improved.

The basis for our improved system for quantitation of imino acids lies in the fact that hydrindantin is not required for the reaction between imino acids and ninhydrin to yield the yellow-colored product[3], whereas the reaction between amino acids and ninhydrin to produce the characteristic purple color does involve hydrindantin. Thus, omission of hydrindantin and reducing agents in the preparation of Nin-II yielded a reagent that reacted normally with imino acids but gave only a slight color reaction with amino acids.

The system described herein has been adapted to the routine analysis of both imino and amino acids for which it has proved to be thoroughly satisfactory. Because Nin-II is the reagent of choice for the analysis of proline and hydroxyproline, no essential purpose is served by a 440 mμ recording of the effluent that is mixed with Nin-I. For routine use, therefore, colorimeter No. 2 was re-equipped with its original set of 570 mμ filters and an 8 mm flow cell to permit its use for the quantitation of amino acids.

This work was aided by Grant A-003 of the ROBERT A. WELCH Foundation, Houston, Texas, to J. M. PRESCOTT.

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1 K. A. PIEZ AND L. MORRIS, *Anal. Biochem.*, 1 (1960) 187.
2 Technicon Corporation, *Amino Acid Analyzer Instruction Manual*, AAA-1, Ardsley, New York, 1967.
3 J. P. GREENSTEIN AND M. WINITZ, *Chemistry of the Amino Acids*, John Wiley, New York, 1961, pp. 1301–1303.

Received June 14th, 1971