The method described for the separation of hydroxocobalamin and cyanocobalamin is quick and simple, can be easily carried out, and can be used for quantitative determination.

Department of Biopreparations and Vitamins,
Higher Institute of the Food Industry,
Plovdiv (Bulgaria)

YA. POPOVA
CH. POPOV
M. ILIEVA

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Ion exchange chromatography of some acidic and aromatic amino acids*

Available methods for separating phosphorylated and other acidic amino acids by ion-exchange column chromatography are time-consuming and do not provide good resolution. In view of the significance of glutamic acid and its metabolites in neural function and metabolism, and because the phosphoamino acids serve as the active sites of enzymes involved in phosphorus metabolism, it was desirable to develop an effective method for their separation.

Experimental

About 500 g of Dowex 1-X8 (AG 200–400 mesh BIO-RAD) was suspended in 2 l of water in a 3 l beaker and allowed to settle for five minutes. Approximately three-fourths of liquid containing the finer particles was decanted for subsequent use. After washing in the usual manner, the resin was converted to the acetate from the chloride form using 2 *M* sodium acetate.

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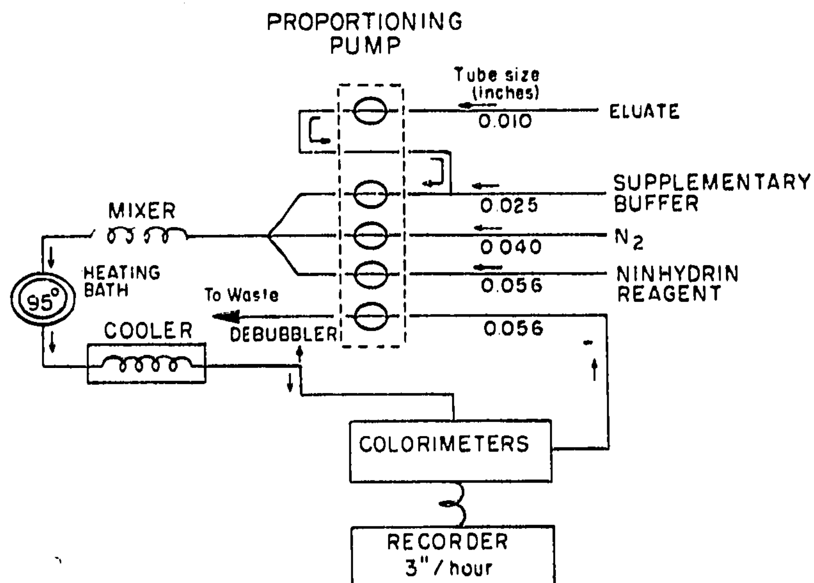

Fig. 1. Flow diagram.

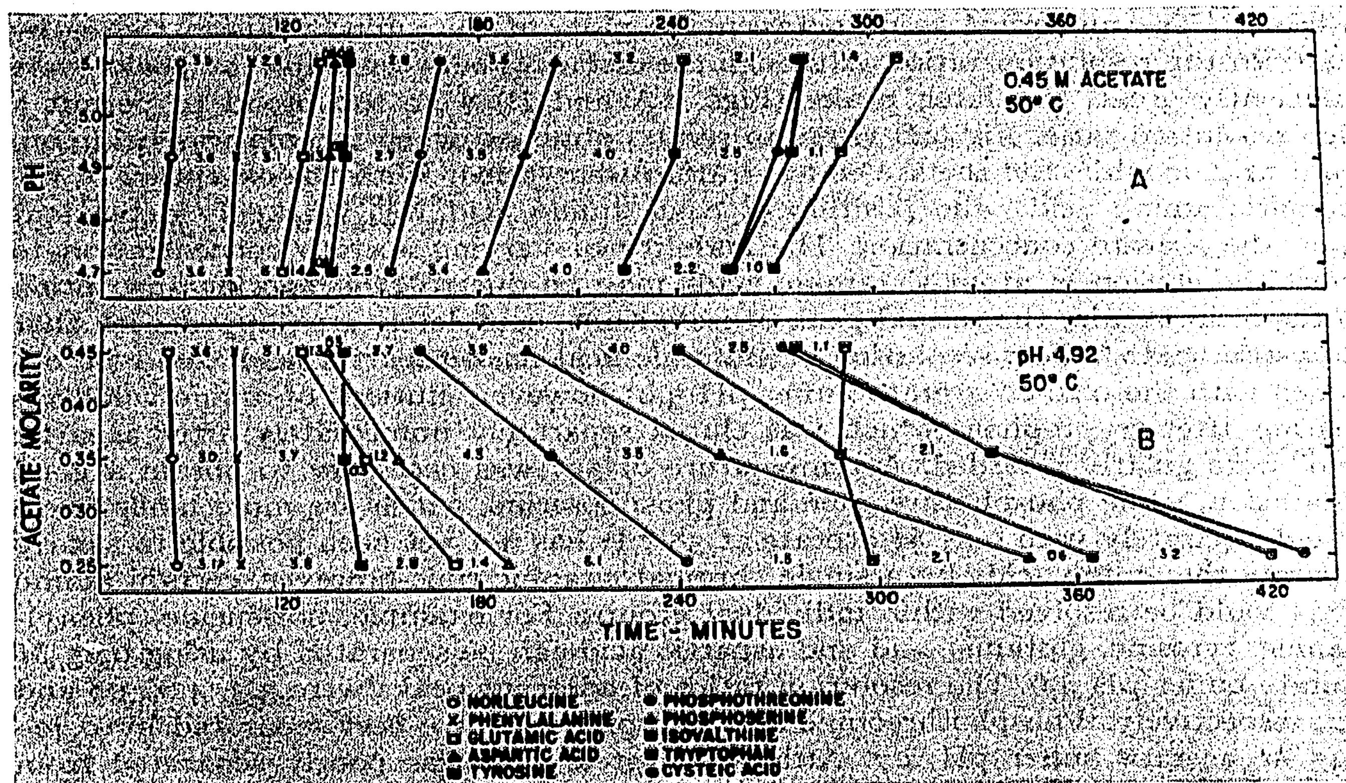

Fig. 2. Resolution of some acidic and aromatic amino acids as function of pH (A) and acetate concentration (B).

A 0.9 × 60 cm column was prepared with the resin and equilibrated at 50° for 2 h with the same acetate buffer used as an eluent in each run. The column was attached to the Technicon Auto Analyzer, and the amino acid analysis was conducted according to the standard micro-method recommended by the manufacturer with the following modifications: A Milton Roy mini pump was adjusted to supply 20 ml of buffer per hour, which was the maximal rate permitted to obtain a clear separation with the column used. The eluate from the column was channeled through an orange-green Tygon manifold tube where it was supplemented with acetate buffer. (Supplementation was necessary because of the inadequate flow rate.) The combined mixture was passed through an orange-white Tygon tube, mixed with the ninhydrin reagent, segmented with nitrogen gas, and incubated in the heating bath for 15 min at 95°. The speed of the chart recorder was 3 in./h. Technicon's certified 4 N sodium acetate buffer pH 5.51 was used to prepare the eluent, after appropriate dilution and adjustment of pH with glacial acetic acid. Standard amino acids were obtained from Sigma Chemical Company. Isovalthine (L-isovalthine + L-alloisovalthine) was a gift from Dr. S. OHMORI.

Results

The chromatographic separation of acidic and aromatic amino acids was studied as a function of pH and concentration of acetate buffer (Figs. 2A and B). With an increase in pH in the range 4.7–5.1 at an acetate concentration of 0.45 M, there was a slight retardation in the elution but no significant improvement in the resolution of the amino acids examined. Its hould be noted that isovalthine possesses two peaks, the second of which is overlapped completely with that of cysteic acid (see "Discussion"). The numerical value between the plots of the amino acids in the figure refers to the resolution ratio[1]. Although the distances between adjacent peaks were sufficiently great, the later peaks (Figs. 2A and B) were too broad to permit a high resolution ratio. Fig. 2B refers to results obtained when the pH was held constant at 4.92 while the acetate concentration was varied. With the exception of the aromatic amino acids, the position of each amino acid was markedly dependent upon the acetate concentration. The inability of varying acetate concentration to affect the elution of aromatic amino acids may be due to their specific adsorption to the resin. Again, the second peak of isovalthine completely overlapped with that of cysteic acid. A clear resolution of glutamic acid from aspartic acid could be obtained with the 0.20 M acetate buffer pH 5.0; however, elutions of amino acids appearing after tryptophan, which was eluted separately immediately after aspartic acid, were greatly retarded. A temperature of 50° was necessary to obtain a good separation, while phosphothreonine and phosphoserine, which are more labile, were eluted separately with 0.25 M acetate at 38°. It has not yet been possible to clearly separate the amino acids by a single chromatographic run; however, certain amino acids could be resolved within individual groups. For example, norleucine, phenylalanine, tyrosine, glutamic acid and aspartic acid can be separated by using 0.25 M acetate buffer pH 4.6. The resolution ratios of adjacent peaks were 3.3, 4.0, 3.8 and 2.2, respectively. Phosphothreonine and phosphoserine are best separated by 0.40–0.45 M acetate buffer pH 4.7. Although cysteic acid was poorly separated from tryptophan and the second peak of isovalthine, its analysis is made possible by virtue of the fact that the tryptophan can be destroyed by acid hydrolysis, while isovalthine

is only present in trace amounts, if at all. Amino acids such as phenylalanine, tyrosine, glutamic acid, aspartic acid and cysteic acid were quantitatively recovered, while the recovery of phosphothreonine, phosphoserine, tryptophan and isovalthine was 75.5, 86.0, 78.5 and 57.0 per cent respectively. The reproducibility of the position of each peak was within ± 1.0 per cent and the reproducibility of the areas of the peaks ± 4.0 per cent. No decrease in the exchange capacity of the resin was noted after use for two months.

Discussion

Although KENNEDY *et al.*[2] used a Dowex 1 acetate column to isolate phosphoserine from a partial protein hydrolysate by employing a gradient of acetate buffer, the time required for the elution of phosphoserine was 26 h, as compared to 4 h in the method described. If a finer spherical resin were to become available, the flow rate could then be increased to permit a still faster and better separation.

The decomposition of dicarboxylic amino acids on the Dowex 1 acetate column which was described by HIRS *et al.*[3] was not observed. The loss of significant quantities of phosphoserine, phosphothreonine, tryptophan, and, especially, isovalthine would seem to be related to the high temperature used as well as to some characteristic of the resin. Isovalthine was completely resolved into its components, L-isovalthine and L-alloisovalthine[4] by the present method. The first peak was presumed to be L-isovalthine and the second L-isoallovalthine from chromatography on a strongly acidic cation exchanger[4].

Center for Brain Research, University of Rochester, Rochester, N.Y. (U.S.A.)

KENGO KURAHASI
LEO G. ABOOD
G. GOMBOS*

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* Present address: Department of Biochemistry, University of Strasbourg, Strasbourg, France.