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The Separation of Small Amounts of Aromatic Amino Acids

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Tyrosine, phenylalanine and tryptophane can easily be separated from the other amino acids by adsorption on to carbon on account of their high affinity for this adsorbent, and can be displaced by a number of compounds with even greater tenacity for carbon such as benzyl alcohol. Under these conditions, however, difficulties are encountered in the separation of the individual amino acids especially if they are present in small quantities, owing to the narrow contiguous zones in which they appear in the effluent. Tiselius and Hagdahl [1] have described a method to which they have assigned the name "carrier displacement" whereby zones of inert substances of intermediate affinity are interposed between pairs of amino acids to enable the cutting of fractions to be carried out with greater ease. Hagdahl employed the interferometric technique of Tiselius and Claesson [2] for the identification of the fronts of the inert carrier substances. Owing, however, to the fact that the main steps in the refractive index/volume curve which act as markers for the positions of the amino acids, can, within considerable limits, be made of any desired height, solely depending on the concentrations and properties of the substances employed, the extreme sensitivity of this method is not required. The effluent was collected either in the linear, time operated collector of Drake [3], or in an apparatus of the drop Counting type of Moore and Stein [4]. Portions of 0.5, 1.0 or 2.0 ml were collected and 0.05 ml of each examined in a Zeiss dipping refractometer with contact prism for small quantities. The sensitivity of this instrument, 4×10^{-5} units of refractive index is quite adequate to distinguish the grosser steps of the curve, and (*cf.* Fig. 1) will also show up the amino acids as peaks if their concentration approaches the 100 μg level. No attempt was made to regulate the temperature, since such changes as might occur would not alter the position of the steps but only

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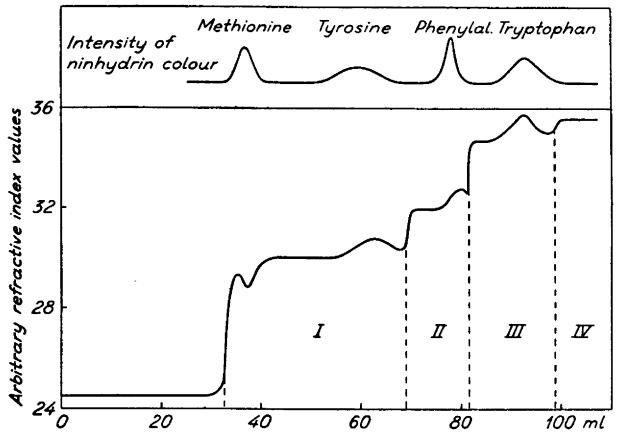

Fig. 1. The separation of aromatic amino acids in 100 μg quantities by carrier adsorption on 14.1 ml of carboraffin supra/super cel 1:3 mixture. I = n-butanol, II = 2-methyl-2-butanol, III = 3-methyl-1-butanol, IV = benzyl alcohol.

their height. The results obtained were sufficiently constant and predictable to permit the inert carriers to be completely ignored in later stages of the work. The separation of any pair of amino acids was proved by the treatment of each fraction with ninhydrin, regardless of the distribution of the carrier substances.

Preliminary work by Hagdahl [5] showed that a system containing certain amyl alcohols does not permit the separation of tyrosine and phenylalanine, although separating these two from tryptophane, and also from other less strongly adsorbed amino acids. The former pair always appeared together in the effluent after 2-methyl-2-butanol and before 3-methyl-1-butanol which emerge in that order. Of the eight isomeric amyl alcohols, only the two mentioned above and 1-pentanol were readily available at the commencement of the work.

Claesson [6] has shown that in an homologous series the degree of adsorption can be correlated with the boiling point, always rising with increases in that function. This has also proved to be the case where isomeric compounds with similar functional groups are concerned. Thus the boiling points of the three amyl alcohols referred to above are respectively 101.8, 130.5 and 138° C., whereas the retention volumes observed in a typical experiment in which 1 % solutions of each were forced through 14.1 ml of a 3:1 mixture of Super Cel and Carboraffin Supra to give a frontal analysis, are 76, 97 and 113 ml.

On the basis of these results it could be deduced that the three amyl alcohols, 2-methyl-1-butanol, (b. p. 128°) 2-pentanol (b. p. 119°) and 3-pentanol (b. p. 115.6°) would be more strongly adsorbed than 2-methyl-2-butanol and less than 3-methyl-1-butanol, and might be useful in the separation of tyrosine and phenylalanine. Subsequent experiments, however, using mixtures of 2 % solutions of 2-methyl-2-butanol with each of these three other isomers showed no separation of the two amino acids concerned.

The correlation between b. p. and position of the adsorption isotherm as measured by the retention volume can also be extended to the isomeric aliphatic acids. A set of retention volumes for 2 % solutions of 2-methyl propionic, *n*-butyric, 3-methyl butyric, *n*-valeric and 4-methyl valeric acids are given in Table 1.

Table 1.

Substance	B. p. °C	Retention volume
2-methyl propionic acid	154.4	9.0
n-butyric acid	163.5	18.5
3-methyl butyric acid	176.7	20.1
n-valeric acid	187.0	27.5
4-methyl valeric acid	207.7	42.0

The figures refer to a displacement experiment with 1 % benzyl alcohol as displacer, using 8.5 ml of the same Super Cel/Carboraffin Supra mixture. The 4-methyl valeric acid was dissolved in 5 % ethanol, as was the benzyl alcohol in this case on account of the low solubility of the acid in water. The effect of this was merely to lower the retention volume, hence the correlation of boiling point with retention volume was still valid.

Tyrosine and phenylalanine were, however, not separated by any of these organic acids, invariably appearing in both frontal and displacement analysis between *n*-butyric and 3-methyl butyric acids. They might be separated by 2-methyl butyric acid, but not by the other isomeric 5-carbon acid pivalic acid (b. p. 163.8°). The former was, however, not readily available, and hence other methods have had to be employed.

Separation of organic acids has been achieved by partition chromatography using heavily buffered columns (Moyle, Baldwin and Scarisbrick [7]) and a similar system has been shown to facilitate the separation of the aromatic amino acids by displacement techniques. In a typical experiment, 104 μg of tyrosine, 120 μg of phenylalanine, 97 μg of methionine and 140 μg of tryptophane were dissolved in 0.1 *M* sodium carbonate/bicarbonate buffer (pH 9.7) and added to a column composed of filters having a total volume of 14.1 ml

and containing 3 parts of Super Cel to 1 of Carboraffin Supra. Through this column which had already been thoroughly saturated with the buffer solution, was passed a mixture of *n*-butanol, 2-methyl-2-butanol, 3-methyl-1-butanol and benzyl alcohol in 1 % concentration in the same buffer. Small peaks appeared in the refractive index/volume curve just before each of the main steps corresponding to the alcohols, (Fig. 1.). Paper chromatographic examination of the fractions representing these portions of the effluent showed that all the methionine was located at the foot of the first step, *i. e.* between the buffer and the *n*-butanol; the tyrosine at interface where the 2-methyl-2-butanol broke through; the phenylalanine was displaced by the 3-methyl-1-butanol, and the tryptophane by the benzyl alcohol. Between the fractions containing the acids the following volumes giving no ninhydrin colour were collected: 6.0, 3.5 and 4.5 ml. These are small but quite adequate to enable a separation to be effected since tailing of the amino acids was negligible.

Quantitative experiments using similar concentrations of the aromatic acids, and also lower amounts down to 20 μg showed that the recovery of the amino acids was of the order of 96 %. The method employed for the estimation of the amino acids was that of Moore and Stein [8] using ninhydrin. It was found that the method could not be employed directly to samples as they left the column since the presence of the small and unpredictable amounts of alcohols effected the colour production by the amino acid. The fractions were evaporated to dryness *in vacuo* and dissolved in water. The colour production was compared with a standard curve prepared from a pure sample of the amino acid concerned dissolved in sodium carbonate/bicarbonate buffer.

For quantitative experiments, it was necessary to purify the carbon and the Super Cel. Both were washed repeatedly with hydrochloric acid and ammonia, and finally extracted with butanol before use. This decreased the amount of soluble material which was extracted by the solvents during a run to a negligible amount.

As yet columns of this volume (10 to 20 ml) have not proved adequate for the separation of tyrosine and phenylalanine from a whole protein hydrolysate (casein hydrolysed with 6 *N* HCl). It will presumably be necessary to effect a group separation first [9] and then employ the above technique.

SUMMARY

1. Figures are given for the retention volumes of two series of compounds, showing that the correlation of boiling point with retention volume can be applied to isomeric substances having similar functional groups.

2. Such a series of isomers having closely similar retention volumes has been utilised in attempts to separate the aromatic amino acids by "carrier displacement chromatography" on carbon.

3. Buffered alkaline solutions of certain pentanols have been proved adequate for the separation of tyrosine and phenylalanine.

4. The recovery of these two amino acids from a column is 96 %.

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