A new method for the examination of mixtures of diastereoisomeric peptides

During the synthesis of dipeptides from optically active amino-acids the possibility of racemisation occurring at one or other of the amino-acid residues is always present. Such racemisation will produce a mixture of diastereoisomers. It may be detected by measurement of the optical rotation of the synthesised peptide, provided a stereochemically pure specimen is available for comparison. Mixtures of diastereoisomers that are available in appreciable amount may be separated by fractional crystallisation[1]. KENNER[2] has separated the diastereoisomers of a tetrapeptide containing two asymmetric carbon atoms by prolonged counter-current distribution in a Craig apparatus.

Recent work has demonstrated that diastereoisomers of amino-acids such as threonine[3], hydroxyproline[4], hydroxylysine[4] and lanthionine[5] may be chromatographically separated on columns of the ion-exchange resin Dowex-50. These results suggested that a similar separation of diastereoisomeric peptides might be possible. The present note describes such a separation of L-leucyl-L-tyrosine and D-leucyl-L-tyrosine. Both peptides were obtained from Roche Products Ltd., each gave only a single spot when examined by paper chromatography and spots corresponding to leucine and tyrosine after hydrolysis with 5 *N* HCl for 24 hours.

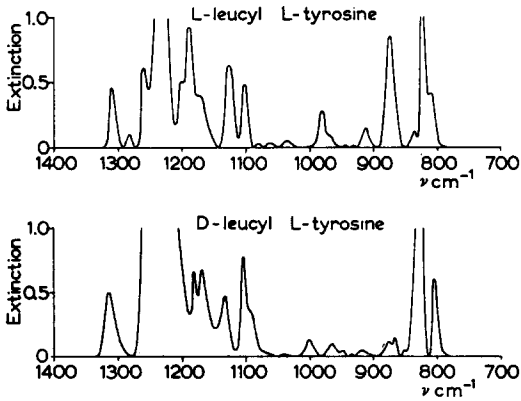

Fig. 1. The infra-red absorption spectra of diastereoisomeric peptides.

The infra-red spectra of the two diastereoisomeric peptides, which were measured in Nujol suspension in a Grubb-Parsons S3 infra-red spectrometer, showed marked differences (Fig. 1). OTEY AND GREENSTEIN[6] have recently observed differences between the spectra of isomeric cystine peptides.

The chromatographic method used was similar to that employed for the separation of lanthionine diastereoisomers[5], following the original method of MOORE AND STEIN[7] for the determination of amino-acids with only slight modifications. Dowex-50 with 4 % cross-linking agent was employed; separation on resin with a higher percentage of cross-linking agent was not as satisfactory. Separations of peptides are generally more effective on resins with lower percentages of cross-linking agents[8]. Resin columns of dimensions 100 × 0.9 cm were used, 0.2 *N* sodium hydroxide containing 5 ml of a 25 % solution of detergent BRIJ 35 per litre being passed through the column first, followed by the buffer solution.

The buffer used for the separation had the following composition:

500 ml of pH 5 citrate buffer (0.2 *M*)[9].
10 ml of *N* HCl.
490 ml water.

To it was added 5 ml of a 25 % solution of detergent BRIJ 35, 1 g of disodium versenate and 10 ml of benzyl alcohol. The pH measured at the glass electrode was 4.84. The benzyl alcohol included in the buffer should facilitate the separation of tyrosine-containing peptides[8,10].

The temperature of the column was maintained at 37.5° throughout the experiment. The peptide mixture dissolved in buffer (1 ml) was applied to the column which was then developed with the buffer, the eluate being collected in 1 ml fractions by means of a fraction cutter. The fractions were then treated with ninhydrin in concentrated sodium acetate buffer (1 ml)[11], so that pH adjustment of the fractions was not necessary, they were then diluted with 50 % (v/v) ethanol (5 ml), and the optical density of the solutions measured in 1 cm Wells at 570 mμ in a Unicam SP500 spectrophotometer.

The period of heating of the eluate fractions with ninhydrin was 15 min, as with amino-acids. Experiment showed that reaction of the dipeptide L-leucyl-L-tyrosine with ninhydrin, as judged by the optical density of the blue colour, was not complete under the above conditions even after heating for 1 hour. As the period of heating was prolonged beyond 15 min, however, the optical density of the "blank" with no peptide present also increased, and tubes containing only small amounts of peptide could not readily be distinguished owing to the elevation of the "baseline". The time of reaction with ninhydrin was therefore limited to 15 min.

Peptides differ widely in the values of their "colour yields"[9] relative to leucine (*cf.* DOWMONT AND FRUTON[10]). This may be because the reaction of ninhydrin with peptides follows a different

course to its reaction with amino-acids. Standard curves showing the relation between optical density of colour and concentration were plotted for both L-leucyl-L-tyrosine and D-leucyl-L-tyrosine by heating known amounts of peptide dissolved in 1 ml of buffer with ninhydrin for 15 min. They were linear over the concentration range 0–50 μg of peptide per ml. The "colour yield"[9] was 1.31 for L-leucyl-L-tyrosine and 1.22 for D-leucyl-L-tyrosine; the values of "colour yields" for particular compounds however are known to vary slightly with variations in experimental conditions. This difference in the "colour yields" of the two peptides (7%) is small, but is probably greater than the experimental error involved in the measurement.

The curve relating optical density to volume of effluent obtained when a mixture of 1.55 mg of D-leucyl-L-tyrosine and 2.03 mg of L-leucyl-L-tyrosine dissolved in 1 ml of buffer was applied to the Dowex-50 column is shown in Fig. 2. On similar chromatograms each peak was found to be derived from a single peptide, the first from D-leucyl-L-tyrosine, the second from L-leucyl-L-tyrosine. From the area under each peak in Fig. 2 the amount of peptide emerging from the column was calculated using the standard curves previously described. Each peak corresponds in amount to the quantity of peptide applied to the column, the "recovery" of the peptide (95% in each case) being quantitative within the limits of error of the experiment. It may be pointed out that as the "spread" of each peak is somewhat broad, the experimental "recovery" might be expected to be less than for a compound giving a sharp peak. The "scatter" of the experimental points about the curve is a little greater than is normally observed with amino-acids; this may well be due to the fact that after 15 min heating with ninhydrin (the reaction time chosen) the optical density of the solution is still increasing at an appreciable rate with time.

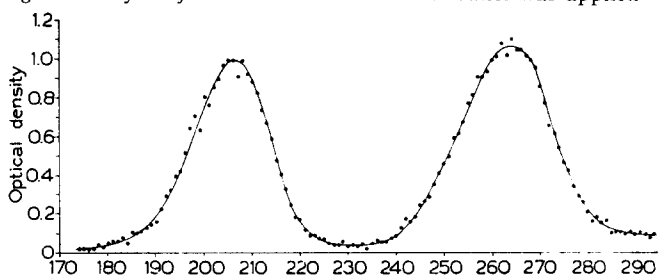

Fig. 2. The separation of D-leucyl-L-tyrosine and L-leucyl-L-tyrosine on a Dowex-50 column.

No extensive investigation of the variation in the degree of separation of the two diastereoisomers with the pH of the buffer has yet been carried out, but it appears that as the pH of the eluting buffer is raised the sharpness of the peaks increases while the separation between them decreases and they emerge from the column sooner.

In suitable cases the present experiments should afford a means of examining synthetic peptides prepared from optically active amino-acids for the occurrence of racemisation, provided suitable buffers for the separation of the diastereoisomers can be found. It might also be found possible to separate diastereoisomeric peptides on a preparative scale by using fairly large columns.

Racemisation of an amino-acid residue may occur during the partial hydrolysis of a protein to peptides for determining the sequence of the amino-acid residues. Large columns of Dowex-50 are commonly used to separate these peptide mixtures, and the possibility arises that two peaks on the chromatogram curve (corresponding to two diastereoisomeric peptides) might be obtained from one dipeptide sequence as a result of such racemisation.

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