THE SEPARATION OF TRYPTIC PEPTIDES BY GEL FILTRATION

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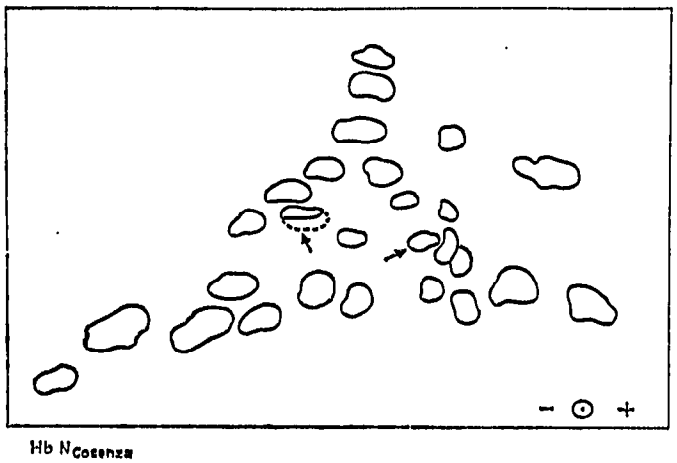
Our research work on the structural characterization of human abnormal hemoglobins involves the separation and purification by chromatographic and electrophoretic techniques of the hemoglobins studied, and also of the peptides resulting from their tryptic digests.

It was thought that it would be of some interest to report in this symposium on a problem that occurred in the course of the structural characterization of an abnormal human hemoglobin that was solved by means of gel filtration[1].

Some years ago, SILVESTRONI AND BIANCO[2,3] reported the occurrence of an abnormal fast hemoglobin in four Italian families, one of them of Sardinian origin, the others living in the outskirts of Cosenza. The hemoglobin had been classified by these authors as hemoglobin N (Hb N) on the basis of its electrophoretic mobility; in addition, as a result of hybridization and recombination experiments the hemoglobin had been proved to be an alpha chain variant. Samples of this hemoglobin have been subsequently committed to our research group for further characterization.

The abnormal Hb N was separated from hemoglobin A (Hb A), contained in the hemolysate, by starch block electrophoresis; both the purified hemoglobins A and N were subsequently submitted to digestion with trypsin[4]. The fingerprints[5] of Hb N were compared with those of Hb A (Figs. 1 and 2). One spot observed in Hb N was not present in Hb A; this abnormal spot is indicated in Fig. 1 with an arrow and was



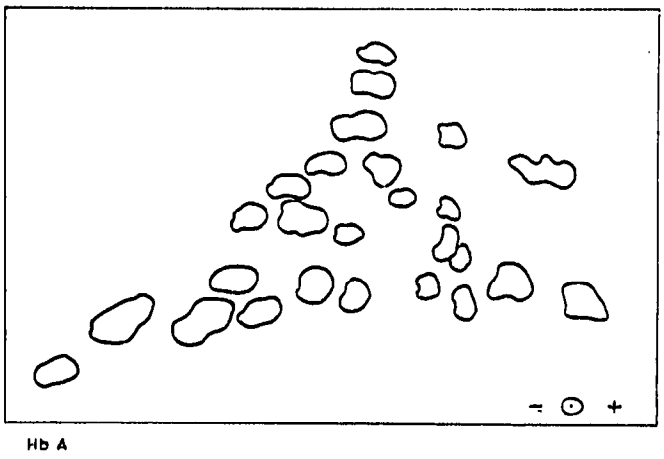


Fig. 1. Finger print tracing of Hb N. The arrow on the right indicates the abnormal peptide α^{N}TpIII. The position of the homologous normal peptide α^{A}TpIII is sketched with a dashed line and indicated by the arrow on the left.

Fig. 2. Finger print tracing of Hb A.

present in all the fingerprints from three different samples of hemoglobin N. One of the peptides normally present in Hb A, the position of which is indicated by a dashed line and the second arrow in Fig. 1, was found to be missing in Hb N; on the basis of its position this peptide has been identified as α^{A}TpIII according to the system of nomenclature recommended by GERALD AND INGRAM[6]. It was therefore possible to deduce that the abnormal spot present in the Hb N fingerprint is due to a modified peptide α^{A}TpIII and can be designated as α^{N}TpIII. This identification is also supported by the fact that this peptide, as well as α^{A}TpIII, shows a positive tryptophan reaction[7].

Peptide α^{A}TpIII actually contains five amino acid residues of the alpha chain, namely alanyl-alanyl-tryptophanyl-glycyl-lysyl, which occupy the position 12 to 16 among the 141 residues composing this chain.

A further step in the characterization of the abnormal hemoglobin was the purification on a preparative scale of the modified peptide α^{N}TpIII with a view to analysing its amino acid content and indentifying the substituent amino acids by comparison with the known amino acid composition of the corresponding peptide, α^{A}TpIII, contained in Hb A.

The purification of the peptides is generally obtained by a combination of paper electrophoresis and paper and column chromatography; however, in this particular case, it could be expected that the purification of the abnormal peptide would be troublesome since the peptide α^{N}TpIII, is positioned in a region of the fingerprint particularly rich in peptides, as already shown in Fig. 1.

Fortunately, the peptide α^{N}TpIII studied contains tryptophan as had already been stated. It is well known that the amino acids containing an aromatic ring interact with dextran gel; it would therefore be expected that gel filtration on Sephadex would give better results, since the retardation due to the relative smallness of the peptide, composed of only five residues, would be enhanced by the retardation due to the interaction of tryptophan with the dextran gel; in other words a real chromatographic process could, in this instance, augment the gel filtration.

On the basis of the foregoing considerations we developed the following procedure: 50 mg of the tryptic digest of Hb N were chromatographed on a 0.9 ×

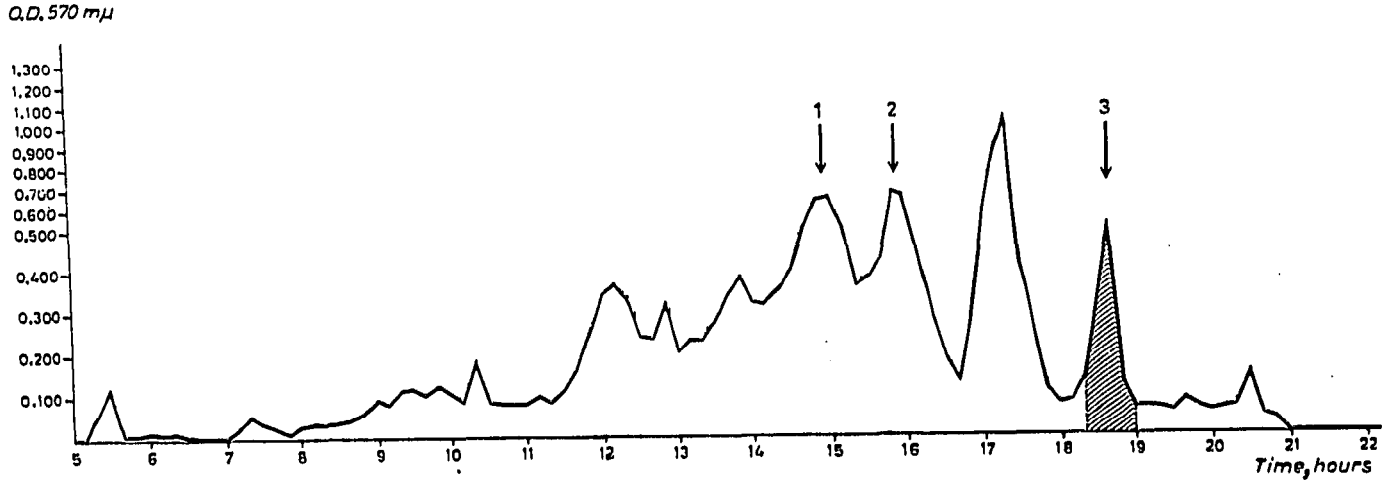


Fig. 3. Elution pattern of Hb N tryptic peptides from a Sephadex G-25 0.9 × 120 cm column (0.2 *N* acetic acid; flow rate 60 ml/h). Arrow No. 1 = β^{A}TpIV; arrow No. 2 = β^{A}TpII; arrow No. 3 = α^{N}TpIII.

120 cm column of Sephadex G-25 equilibrated with 0.2 *N* acetic acid. The separation obtained with a flow rate of 60 ml/h is shown in Fig. 3. The peptide content has been determined on an aliquot of each fraction of the effluent by reading, at 570 mμ, the color developed by YEMM AND COCKING[8]. The normal Hb A contained in the blood of the same subject was also submitted to tryptic digestion and to gel filtration on Sephadex G-25 under the same experimental conditions. The elution curve of Hb A tryptic peptides is identical with that of Hb N.

Human hemoglobin contains three tryptophanyl residues, one of them present in the alpha chain, the others in the beta chain. In the elution curve the tryptophan-containing peptides are indicated by the arrows Nos. 1, 2 and 3 and appear among the last peaks as was to be expected owing to the interaction between tryptophan and dextran gel. The tryptophan-containing peptides have been identified by submitting aliquots of the effluent to the same chromatographic and electrophoretic procedure followed in the development of the fingerprint of the whole tryptic digest, and, of course, to the specific staining for tryptophan. The peak marked with arrow No. 1 contains β^{A}TpIV peptide constituting ten amino acid residues. In the next peak, marked with arrow No. 2, peptide β^{A}TpII, containing nine residues, is eluted; the dashed peak, marked with arrow No. 3, contains the abnormal α^{N}TpIII peptide, or alternatively the normal peptide α^{A}TpIII when the tryptic digest of Hb A is submitted to gel filtration. This was again to be expected since both peptides contain five residues including the tryptophanyl one and therefore should emerge from the column with the same K_d.

As it is evident, the tryptophan-containing peptides emerge from the Sephadex column with a K_d which increases according to the decrease in their molecular size. This affords further evidence that in this experiment the molecular sieving and a real chromatographic process augment each other.

In this instance the technique of gel filtration has thus proved to be extremely useful in simplifying the preparation and purification of the peptide studied. This concludes our report on the work directly concerning gel filtration, however it could be of some interest to mention the results of the experiments we spoke of.

Both the abnormal peptide α^{N}TpIII and the normal one, α^{A}TpIII, were subjected to acid hydrolysis with 6 *N* hydrochloric acid at 110° for 24 h. The quantitative amino acid analysis[9] of the acid hydrolysate gave the results reported in Table I.

As is well known, tryptophan undergoes almost complete destruction in the course of acid hydrolysis.

TABLE I

AMINO ACID ANALYSIS OF α^{A}TpIII AND α^{N}TpIII PEPTIDES (μMOLES/μMOLE PEPTIDE)

	α^{A}TpIII	*α^{N}TpIII*
Ala	1.9 μmoles	1.8 μ moles
Gly	1.1 μmoles	0.1 μmoles
Asp	0.1 μmoles	1.0 μmoles
Lys	1.1 μmoles	1.0 μmoles
Try	+	+

These results prove that in the abnormal peptide an aspartyl residue is present instead of the glycyl residue normally occurring in α^{A}TpIII peptide. It is most likely that the aspartyl residue occupies position 15 in the amino acid sequence of the Hb N alpha chain, which is normally occupied by a glycyl residue in the corresponding chain of Hb A.

As it is well known that asparagine is quantitatively converted into aspartic acid at the beginning of acid hydrolysis, it was necessary to establish whether the substituent amino acid in the abnormal peptide was originally an aspartyl or an asparagyl residue. The peptide was therefore digested first with carboxypeptidase B and then with carboxypeptidase A.

Carboxypeptidase B liberated one residue of lysine per mole of the peptide, but carboxypeptidase A did not act any further. It is known[10] that carboxypeptidase A does not liberate acidic amino acids under the experimental conditions which have been used in the present work. An aspartyl residue should therefore occupy position 15 immediately preceding that of the carboxyl-terminal lysyl residue of the abnormal peptide. Had it, on the other hand, been an asparagyl residue, which is not an acidic amino acid, it should have been liberated by the action of carboxypeptidase A.

The abnormality present in the Hb N studied can therefore be identified as the substitution of aspartic acid for glycine 15 which normally occurs in the amino acid sequence of the Hb A alpha chain. An identical structural abnormality has been already reported[11] under the name of hemoglobin J_{Oxford} in the hemoglobin present in the blood of some subjects in an English family.

SUMMARY

Gel filtration has proved most useful in the separation on a preparative scale of the abnormal peptide α^{N}TpIII present in the human hemoglobin variant $\alpha_2^{15Asp}\beta_2$.

The abnormal peptide contains a tryptophanyl residue whose aromatic ring interacts with dextran gel; in this instance, therefore, a real chromatographic process augments the separation due to gel filtration.

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