ACTA CHEMICA SCANDINAVICA 23 (1969) 1137–1144

# Incorporation of $^{131}I$ into Dog Fibrinopeptides

BIRGER BLOMBÄCK and ERIC ZETTERQVIST

*Blood Coagulation Research Department, Karolinska Institutet, Fack, S-104 01 Stockholm 60, Sweden*

On iodination of dog fibrinogen iodine is incorporated in fibrinopeptide $B_1$. This peptide is released during clotting. Monosubstitution in tyrosine residues of the peptide is the main reaction. The possible substitution in tyrosine-*O*-sulphate and histidine residues is discussed.

In previous investigations the effects of coagulation promoting substances upon the elimination of $^{131}I$-labelled fibrinogen in dogs have been studied.[1,2] It was consistently observed that fibrin, formed from iodinated fibrinogen by thrombin had a considerably lower specific radioactivity than fibrinogen. This difference could not completely be accounted for on the basis of impurities present in the fibrinogen preparation used for iodination. It was assumed that the difference might at least partly be due to iodination of fibrinopeptides, split off from fibrinogen during clotting.[1,2]

Recently, Regoeczi and Walton observed the release of radioactive iodine into the clot-supernatant of labelled fibrinogen from several different species.[3] They also provided preliminary evidence for the association of the label with fibrinopeptide B from sheep.

This paper is a study on the nature of the linkage of protein bound iodine in clot-supernatants of iodinated dog fibrinogen.

## MATERIAL AND METHODS

### 1. Dog fibrinogen

Fibrinogen (fraction I-4) was usually prepared as described previously.[4] In later experiments the dimethylformamide (DMFA) method [5] was used for purification of fibrinogen. Fraction I-0 [4] was dissolved to a protein concentration of 1.5 % in 0.055 M sodium citrate, pH 6.4, 2 volumes of water were added and the mixture chilled to 0°. DMFA was slowly added to a final concentration of 4 M; pH was between 6.4 and 7.0. The precipitate formed was collected by centrifugation at 0° and dissolved in 0.055 M sodium citrate, pH 6.8, solution. The solution was eventually dialyzed against 0.055 M citrate, pH 6.8. The purity of the product, as judged by the coagulability, was for fraction I-4 between 88 and 97 % and for fibrinogen precipitated with DMFA 99 %. An ultraviolet spectrophotometric method was used for determination of coagulability.[6]

## 2. Iodination of dog fibrinogen and fibrinopeptide B

$^{131}$I-Labelled fibrinogen was prepared essentially according to the method of McFarlane [7] as described previously.[6] On iodination the coagulability of the fibrinogen decreased by, on the average, 5 %. Between 0.3 and 0.9 atoms of iodine were introduced per mole of fibrinogen. This represents between 5 and 20 % of added iodine. The radioactivity in fibrin in percent of that in fibrinogen was between 70–80.

Dog fibrinopeptide $B_1$[9,10] was iodinated according to the iodine-monochloride method of McFarlane.[11] 3 mg of peptide was used for iodination and 60 $\mu$C of $^{131}$I as sodium salt were added to the carrier iodine-monochloride solution used for iodination.[11] 0.0022 atoms of iodine were introduced per mole of peptide. This represents 7 % of added iodine. After iodination the pH was adjusted to 3.0 with formic acid and the sample applied to a Dowex 50 × 2 column (0.6 cm$^2$ × 90 cm) and chromatographed as described in section 4.

## 3. Protein determination, radioactivity measurements, electrophoresis and thin-layer chromatography

*Protein concentration* of fibrinogen and fibrin was measured in a UV-spectrophotometer (Beckman). The extinction coefficient ($E^{1\,\%}_{1\,cm} = 16.51$ at 282 m$\mu$) for bovine fibrinogen in alkaline urea was used in the calculations.[6]

*Radioactivity* in fibrinogen and fibrin was in part of the study determined in a well scintillation counter (Tracerlab) as previously described.[8] 4 ml solution were measured in polypropylene cups.

In some experiments a liquid-scintillation spectrometer (Beckman, CPM-200) was used. 2 ml of samples in aqueous solution were mixed with 10 ml of scintillation liquid (2,5-diphenyloxazole 5 g, naphthalene 100 g, dioxane up to 1000 ml). In the case of fibrin, this was first hydrolyzed in 0.1 M NaOH for 20 min on the boiling waterbath. After neutralization of the alkali with one equivalent of dilute acetic acid (0.2 M), 2 ml of the neutralized hydrolysate in a suitable dilution was mixed with the scintillation liquid.

For scanning of radioactivity on thin-layer plates a two-dimensional scanning equipment (Berthold, Wildbad, West Germany) with a 2 $\pi$ detector was used.

*Electrophoresis* was usually made on thin-layer (0.75 mm) cellulose (Whatman CC41) plates (20 × 20 cm) in pyridine:acetic acid:water (100:35:4865, v/v), pH 5.5, at 300 V, 21–23 mA. Around 10–15 $\mu$g each of peptide A or $B_1$ were applied in 1 $\mu$l of water. Approximately $0.5 \times 10^{-2}$ $\mu$C of radioactive material from different chromatograms were applied in 1 $\mu$l. After the run ninhydrin staining was made.[12] In a few experiments paper electrophoresis in a conventional horizontal apparatus was performed. The runs were then made in 0.1 M pyridine-acetate buffer, pH 4.1, at 300 V/cm for 4 h. For elution experiments, paper strips, 8–9 cm wide, were used. Guide strips for staining with ninhydrin reagent [12] were cut on each side of the paper. Clot-supernatant from the equivalent of 30–40 mg fibrinogen was taken up in 150 $\mu$l of water. About 10 $\mu$l/cm paper strip was applied. The peptide bands were eluted with small volumes of water and subsequently with dilute formic acid and pyridine.

*Thin-layer chromatography* was used for identification of iodo-amino acids in digests of radioactive labelled fibrinopeptide $B_1$. The same supporting medium as for thin-layer electrophoresis was used. The following solvent systems were adopted: 1) butanol:2 M acetic acid (77:23, v/v),[13,14] 2) pyridine:2 M acetic acid (80:20), v/v).[14] 1–3 $\mu$l of the digest or hydrolysate (1–2 $\mu$C × $10^{-2}$) was applied to the plate together with 1 $\mu$l of a reference mixture of thyroxine, 3-iodotyrosine, 3,5-diiodotyrosine and tyrosine (5–10 $\mu$g of each). The plate was scanned for radioactivity and afterwards stained with ninhydrin.

## 4. Isolation of fibrinopeptides from fibrinogen

*Column chromatography.* Dog fibrinopeptides were prepared essentially as described earlier.[15] After iodination of fibrinogen, the solution was adjusted to pH 7.0 and then precipitated at −3° with 53.3 % ethanol to a final concentration of 10 %. After centrifugation the precipitate was dissolved in 0.3 M ammonium acetate to a protein concentration of around 1 %. To one volume of fibrinogen solution was added one volume of water containing bovine thrombin (100−350 NIH * units/mg) at a concentration of 4 NIH units per ml. The clotted fibrinogen was left standing at room temperature for 4 to 6 h. The clot was then collected on a glass rod or on a Büchner funnel. The clot-supernatant was acidified with formic acid to pH 5.1. A small precipitate formed and was removed by centrifugation. The precipitate which may have consisted of non-clottable fibrinogen was dissolved in a small volume of urea or 0.1 M NaOH for determination of radioactivity. It contained about 2−4 % of the radioactivity of the original fibrinogen.

The clear clot-supernatant after removal of the isoelectric precipitate was freeze-dried and volatile buffer salts removed by sublimation *in vacuo* at +50°.

Freeze-dried clot-supernatants from 0.4 to 0.9 g of fibrinogen were dissolved in about 12−18 ml of 0.1 M ammonium formate buffer, pH 3.0. Some insoluble material was removed by centrifugation and dissolved in 0.1 N NaOH for assay of protein and radioactivity. This material represented as protein 3−6 % and as radioactivity about 1 % of the original fibrinogen. The soluble portion of the clot-supernatant was applied to a Dowex 50×2 column (0.6 cm²×90 cm), equilibrated with ammonium formate buffer, pH 3.0. Before equilibration the column was in the ammonium form. Step-wise elution with 0.1 M formate-formic acid buffers of varying pH was performed (Fig. 1). The flow rate was between 7 and 18 ml/h.

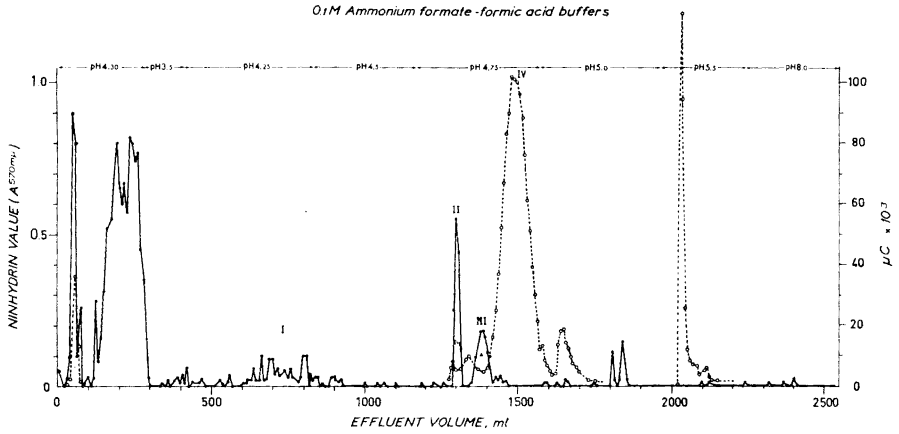


*Fig. 1.* Chromatogram of clot-supernatant. ● — ● ninhydrin; O - - - O radioactivity. For further details see Methods, sect. 4. Note: The first pH value should read 3.30 instead of 4.30.

*Gel filtration.* In some experiments the fibrinopeptides in the freeze-dried clot-supernatant were, without preliminary isoelectric precipitation, partially purified by gel filtration. The clot-supernatant from 30−40 mg of fibrinogen was dissolved in 1.5−3 ml of 0.2 M acetic acid or 0.1 M pyridine and applied to a Sephadex G-25 column (2 cm²×40 cm), which had been equilibrated with 0.2 M acetic acid or 0.1 M pyridine. The flow rate through the column was held at 60 ml/h. The effluent fractions were analyzed for

* National Institutes of Health, Bethesda, MD.

radioactivity, conductivity as well as ninhydrin positive [16,17] material (Fig. 2). The fractions containing radioactivity were pooled, freeze-dried and subjected to paper electrophoresis.

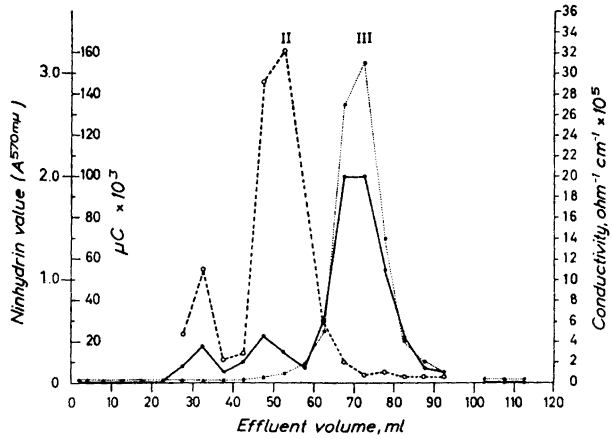


*Fig. 2.* Gel filtration pattern of clot-supernatant. ● — ● ninhydrin; ○ - - - ○ radioactivity; ● · · · ● conductivity. For further details see Methods, sect. 4.

## RESULTS

The iodine introduced into fibrinogen is to more than 99.5 % associated with thrichloroacetic acid (TCA)-precipitable material. The specific radioactivity of the fibrin, however, is considerably lower than that of fibrinogen. This is at least partly explained by the fact, that about 16 % of the iodine introduced in fibrinogen remains in the clot-supernatant.

Gel filtration on Sephadex G-25 of the clot-supernatant (Fig. 2) shows that it contains ninhydrin-positive material, which is retained by the gel. Smaller amounts of material being excluded from the gel are also present as well as low molecular weight material eluted at about the total volume ($V_t$) of the column. Most of the radioactivity (50—60 % of the original radioactivity of the clot-supernatant) was recovered in material retained by the gel (peak II). Paper electrophoresis revealed that peak II contained two main components with the same mobility as authentic peptides A and $B_1$ together with two minor components. The radioactivity was almost exclusively associated with material having the same mobility as peptide $B_1$ (Fig. 3).

Chromatography of clot-supernatants on Dowex 50×2 revealed several ninhydrin positive peaks (Fig. 1). The greater part of the ninhydrin positive material eluted from the column at pH 3.0 is most likely glycine and is derived from the glycine added to the fibrinogen during the iodination procedure. The main peptide peaks are II and III. On the basis of chromatographic behaviour and electrophoretic mobility in comparison with dog fibrinopeptides A and $B_1$,[9,10] peak II is identical with fibrinopeptide $B_1$ and peak III with fibrinopeptide A. The ninhydrin positive material (peak I) emerging from the column at pH 4.2 most likely represents fibrinopeptides $B_2$.[9,10]

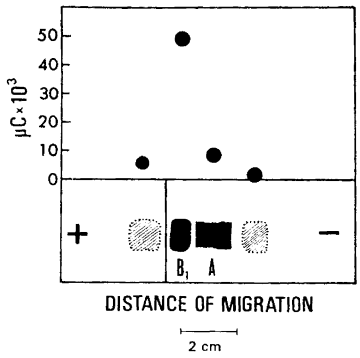


*Fig. 3.* Paper electropherogram of peptide material in clot-supernatant. The peptide material of peak II, Fig. 2, was applied to the paper. The lower diagram shows the ninhydrin staining of the paper strip. The upper diagram gives the eluted radioactivity of the ninhydrin positive material. For further details see Methods, sect. 3.

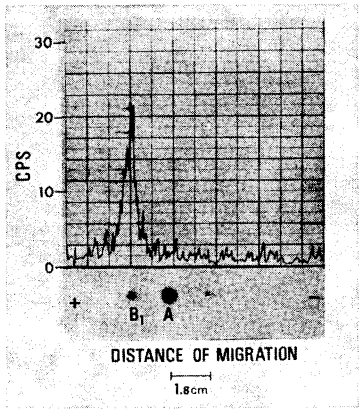


*Fig. 4.* Thin-layer electropherogram of fibrinopeptides with radioactive tracing. Material corresponding to peak IV, Fig. 1, was mixed with fibrinopeptide A and $B_1$. Lower diagram shows ninhydrin positive spots and upper diagram the radioactive tracing. CPS = counts per second. For further details see Methods, sect. 3.

The radioactive material appearing at the solvent front varied considerably in different runs. It is most likely composed of free iodide. Most of the covalently linked iodine, however, had been introduced into material, peak IV, which was slightly more retarded on the column than fibrinopeptide A (peak III) and considerably more than fibrinopeptide $B_1$ (peak II). The peak IV-fraction was only slightly ninhydrin positive. About 4 % of the radioactivity of the fibrinogen was recovered in peak IV. In addition, some other small radioactive peaks were observed in the chromatogram but they were not further studied.

After freeze-drying, the peak IV-material was taken up in a small volume of water (100 μl). Thin-layer electrophoresis of mixtures of peak IV and fibrinopeptides A and $B_1$ showed that the radioactivity completely overlapped with fibrinopeptide $B_1$ (Fig. 4).

In order to obtain further evidence for the view that peak IV contained a radioactive labelled fibrinopeptide $B_1$, an attempt was made to produce peak IV-material from fibrinopeptide $B_1$ by iodination with $^{131}I$-labelled iodine (Methods, section 2). The molar ratio of tyrosine * to iodine was about 130. This is not far from the ratio range (20—100) when whole fibrinogen was iodinated. It is evident (Fig. 5), that after iodination of fibrinopeptide $B_1$, a radioactive peak (I) appeared in the chromatogram. This peak showed about the same retardation with respect to fibrinopeptide $B_1$ as peak IV obtained from iodinated fibrinogen (*cf.* Fig. 1). The material of peak I was freeze-dried

* It is assumed that dog fibrinogen has the same number of tyrosine residues as human and bovine fibrinogen.[18]

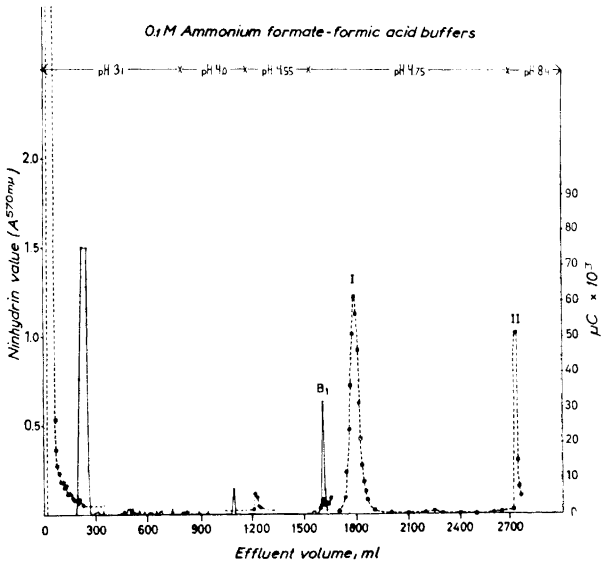


*Fig. 5.* Column chromatogram of iodinated fibrinopeptide $B_1$. ●—● ninhydrin; ○- - -○ radioactivity. For further details see Methods, sect. 4.

and taken up in a small volume of water. When mixed with fibrinopeptide A and $B_1$ (Methods, section 3), the radioactive material of the peak had the same mobility as fibrinopeptide $B_1$.

In the chromatogram (Fig. 5) there was, like in clot-supernatants of iodinated fibrinogen, a small radioactive peak (II) which was eluted at alkaline pH. This material did not have the same mobility as fibrinopeptide $B_1$. It may represent a degradation product of the peptide.

In order to localize the site of incorporation of iodine the labelled peptide (peak I, Fig. 5) was digested with pronase.[19] 50 μl (about 0.5 μC) of peak I-material in 0.15 M tris buffer, pH 8.5, was digested with 50 μg of pronase

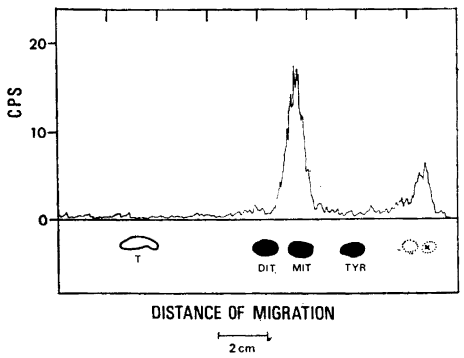


*Fig. 6.* Chromatogram of pronase digested fibrinopeptide $B_1$ with radioactive tracing. Pronase digest together with reference mixture was applied. DIT; 3,5-diiodotyrosine. MIT; 3-iodotyrosine. T; thyroxine. TYR; tyrosine. ×; point of application. Lower diagram shows ninhydrin staining and upper diagram radioactive tracing. For further details see Methods, sect. 3.

(Calbiochem, grade B) for 5 h at 37°. Thin-layer chromatography (Methods, section 3) in two solvent systems revealed that most of the radioactivity was associated with material having the same $R_F$-value as 3-iodotyrosine. The chromatogram developed with butanol:acetic acid is shown in Fig. 6.

About 0.05 $\mu$C of peak I (Fig. 5) was also subjected to alkaline hydrolysis in 50 $\mu$l of 0.1 M NaOH for 21 h at 110° in a tube filled with $N_2$. After evaporation to dryness the residue was taken up in 10 $\mu$l of 5 % ammonia and 2—3 $\mu$l used for thin-layer chromatography in the systems mentioned above. The main product in the hydrolysate seemed to be iodide but in the pyridine-acetic acid solvent, a small radioactive component (about 10 % of the total) with a lower $R_F$-value than monoiodotyrosine was observed. Traces of monoiodotyrosine might also have been present.

## DISCUSSION

The present study has shown, that on iodination of dog fibrinogen with $^{131}$I-labelled iodine, about 15 % of the isotope is bound to material, which is released during the fibrinogen-fibrin transition.

The $^{131}$I-labelled material in the clot-supernatant seems to more than 50 % be associated with peptide material retained by Sephadex G-25. The activity in this fraction constitutes roughly 9 % of the radioactivity of the original fibrinogen. Paper-electrophoresis indicates that the fraction is mainly composed of fibrinopeptides A and $B_1$ and that the radioactivity is associated with material having the same mobility as fibrinopeptide $B_1$.

Nearly half of the radioactivity in the clot-supernatants is bound to other compounds than fibrinopeptides. This iodine is most likely bound to denatured fibrinogen which is non-clottable or to other proteins present as contaminants in the fibrinogen preparation. It is possible that these proteins to a considerable degree are adsorbed to the Sephadex G-25 column as 20—30 % of the radioactivity applied to the column can not be accounted for in the eluates.

It is revealed by column chromatography on Dowex 50×2 that the iodinated fibrinopeptide $B_1$, though it has the same electrophoretic mobility, is slightly retarded on the resin as compared with the uniodinated peptide. Iodinated aromatic residues in the peptide apparently show an increased interaction with the resin.

Dog fibrinopeptide $B_1$ contains 19 amino acid residues.[10] Among these are two tyrosine residues and one N-terminal histidine residue. In peptide $B_1$ one of the tyrosine residues is present as tyrosine-*O*-sulphate.[9] The peptide variant $B_2$[9,10] has basically the same amino acid sequence as peptide $B_1$ with the exception that both tyrosine residues are present as tyrosine-*O*-sulphate. No iodinated derivative of fibrinopeptide $B_2$ has been identified in this study. This might indicate that esterification of the hydroxyl group of tyrosine hampers the substitution at the 3 or 5 positions in the phenolic ring. It should, however, be borne in mind that the yield of peptide $B_2$ in a clot-supernatant is low which means that an iodinated derivative might have escaped attention with the methods used.

Pronase digestion of the peptide $B_1$ gave strong evidence for the conclusion that under the conditions of the iodination mainly monosubstituted iodotyrosine is formed. The nature of the smaller radioactive "peak" (Fig. 6) in the chromatograms of the digest is not yet clear. It may be completely or partially undigested peptide or iodotyrosine-*O*-sulphate. No derivative with the expected $R_F$-value of monoiodohistidine can be observed in the chromatogram. If it is present in the original peptide one would expect it to be released by pronase as the N-terminal sequence in the peptide is His-Tyr-Tyr-, unless iodination of the imidazole ring makes the His-Tyr bond unsusceptible to pronase action.

In the alkaline hydrolysates of the peptide mainly iodide but also a component with $R_F$-value lower than monoiodotyrosine was found. The $R_F$-value was similar to that of monoiodohistidine.[19] It is therefore possible that histidine residues are to some degree iodinated in the peptide.

*Acknowledgement.* This work was supported by grants from *U.S. National Institutes of Health* (HE 07379—05), the *Swedish Medical Research Council* (B 69-19X-520-05A, B 69-13X-2475-02A) and *Greta and Harald Jeanssons Stiftelse.*

We thank Miss Eivor Holmberg and Miss Bodil Jönsson for their most skilful cooperation.

## REFERENCES

1. Nordström, S. and Zetterqvist, E. *Acta Physiol. Scand.* **72** (1968) 85.
2. Nordström, S., Blombäck, B., Blombäck, M., Olsson, P. and Zetterqvist, E. *Ann. N. Y. Acad. Sci.* **146** (1968) 701.
3. Regoeczi, E. and Walton, P. L. *Thromb. Diath. Haemorrhag.* **17** (1967) 237.
4. Blombäck, B. and Blombäck, M. *Arkiv Kemi* **10** (1956) 415.
5. Blombäck, B., Blombäck, M. and Holmberg, E. *Acta Chem. Scand.* **20** (1966) 2317.
6. Blombäck, B. *Arkiv Kemi* **12** (1958) 99.
7. McFarlane, A. S. *Biochem. J.* **62** (1956) 135.
8. Blombäck, B., Carlson, L. A., Franzén, S. and Zetterqvist, E. *Acta Med. Scand.* **179** (1966) 557.
9. Krajewski, T. and Blombäck, B. *Acta Chem. Scand.* **22** (1968) 1339.
10. Blombäck, B., Blombäck, M., Gröndahl, N. J. and Holmberg, E. *Arkiv Kemi* **25** (1966) 411.
11. McFarlane, A. S. *Nature* **182** (1958) 53.
12. Levy, A. L. and Chung, D. *Anal. Chem.* **25** (1953) 396.
13. Roche, J., Lissitzky, S., Michel, O. and Michel, R. *Biochim. Biophys. Acta* **7** (1951) 439.
14. Covelli, I. and Wolff, J. *Biochemistry* **5** (1966) 860.
15. Blombäck, B., Blombäck, M., Edman, P. and Hessel, B. *Biochim. Biophys. Acta* **115** (1966) 371.
16. Moore, S. and Stein, W. H. *J. Biol. Chem.* **211** (1954) 907.
17. Sjöquist, J., Blombäck, B. and Wallén, P. *Arkiv Kemi* **16** (1960) 425.
18. Henschen, A. and Blombäck, B. *Arkiv Kemi* **22** (1964) 347.
19. Covelli, I. and Wolff, J. *J. Biol. Chem.* **242** (1967) 881.

Received July 26, 1968.