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ON THE STRUCTURE OF SPERM WHALE MYOGLOBIN. II. THE TRYPTIC
HYDROLYSIS OF THE DENATURED PROTEIN.¹

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SUMMARY

The tryptic hydrolysis of heat-denatured sperm whale myoglobin (components IV + V) gives rise to two groups of peptides, distinguishable on the basis of their solubility below pH 8, which may be shown to account for all the amino acid residues in the parent protein molecule. The insoluble peptides, as yet unfractionated, account for four of the 23 basic amino acid residues and for 46 of the total of 153 residues in the protein. The soluble products of tryptic hydrolysis consist of 20 peptides and free lysine, and were separated on a preparative scale by chromatography on columns of IRC-50 and Dowex 50-X2. The final products isolated were sufficiently pure to permit their use in amino acid sequence determinations. Some of the factors affecting the yields in which the peptides were formed were studied and have been discussed.

1. INTRODUCTION

In the previous communication (Edmundson and Hirs, 1962a) the sperm whale myoglobin molecule was shown to consist of a single polypeptide chain of 153 amino acid residues devoid of either cystine or cysteine. A first objective in determining the sequence of the amino acid residues in a polypeptide chain of this kind is the division of the sequence into a series of smaller peptides which collectively account for all the residues in the original chain.

The tryptic hydrolysis of heat-denatured sperm whale myoglobin, components IV + V (for designations, cf. Edmundson and Hirs (1962)), was studied in the experiments to be reported in this paper. The protein contains 19 residues of lysine and 4 residues of arginine, and neither of these amino acids occupies a terminus of the peptide chain. A maximum of 24 peptide fragments would therefore be expected to be formed if completely specific cleavage were attained in the tryptic hydrolysis at the carbonyl bonds of lysine and arginine residues. In actuality the hydrolysis was accompanied by the formation of an insoluble fraction that accounted for four of the basic amino acid residues; the remainder were present in soluble peptides and as free lysine, together representing the rest of the original molecule.

The products formed by the tryptic degradation of myoglobin were characterized before the 2 Å Fourier synthesis of the crystal structure of the protein was achieved (Kendrew et al., 1960; Kendrew et al., 1961). At that time experiments were initiated to characterize a sufficient number of the peptides formed by chymotryptic hydrolysis of the protein to permit the

connection in sequence of the peptides obtained on tryptic hydrolysis. Some preliminary success in this direction was attained⁴. In the meantime, however, Kendrew and his colleagues (1961) have been able to derive an almost complete amino acid sequence from a consideration of the X-ray evidence alone, and this has made possible a tentative placement in series of the tryptic peptides described in this communication. In turn, the alignment of these peptides has in many portions of the sequence facilitated the X-ray identification of the amino acid side chains by limiting the number of alternatives between which a choice must be made when the observed electron density distributions are fitted to the theoretically correct ones for each type of residue.

2. EXPERIMENTAL

(a) Materials

(1) Lyophilized sperm whale myoglobin components IV + V were prepared in 250-300 mg portions from crystalline protein by the chromatographic procedure described in the previous paper (Edmundson and Hirs, 1962a). Prior to use portions of the freshly prepared protein were routinely withheld for moisture and ash determinations; another portion was subjected to acid hydrolysis for 22 hours and the amino acid content of the hydrolysate was determined by the procedures described in the previous paper (Edmundson and Hirs, 1962a). These amino acid analyses provided a check on the homogeneity of the myoglobin preparation and furnished an accurate basis for the calculation of the yields of the peptides subsequently isolated from it.

(2) Globin was prepared from myoglobin components IV + V by the

method described in the preceding paper (Edmundson and Hirs, 1962a).

(3) The twice-crystallized trypsin used in this work was obtained from a commercial source (Worthington Biochemical Sales Corp., Lot TRSF 573-75) and was capable of catalyzing the rate of hydrolysis of N-acetyl-L-tyrosine ethyl ester at a rate that would have been equalled by 0.35 to 0.55% of the same concentration of chymotrypsin α . More recently we have had occasion to determine the esterase activity against this substrate of trypsin formed by the activation of chromatographically purified (Keller, Cohen and Neurath, 1958) trypsinogen and have obtained values in the same range for the intrinsic chymotryptic activity of the purified trypsin.

(4) Dowex 50-K2 suitable for the chromatography of peptides formed in the tryptic hydrolysis was obtained by flotation from a large single batch of the resin purchased from the manufacturer (Dow Chemical Co., Lot 4187, "through 400 mesh"). The hydraulic fractionation was achieved in an apparatus of the type described by Hamilton (1958). A separatory funnel of 2 liter capacity was used for the flotation chamber and the sodium form of the resin was fractionated in portions of 200 ml (settled volume). The resin collected at a flow rate between 150 and 280 ml/min was used. This fraction contained spheres with diameters between 40 and 80 microns and was obtained in a yield of 40%. Following flotation the resin was washed exhaustively with 4 N HCl to remove multivalent cations. It was stored in the hydrogen form at 4°.

(5) The IRC-50 used in the peptide fractionations was the resin described in the preceding paper in connection with the chromatography of myoglobin (Edmundson and Hirs, 1962a).

(b) Tryptic Hydrolysis of Myoglobin

(1) For each experiment a number of separate preparations of the protein were combined, dissolved in water, and successively dialyzed against dilute ammonium hydroxide (cf. Edmundson and Hirs (1962a)), and water at 4°. The final solution was lyophilized, and a portion of the product was removed for amino acid analysis. The moisture, ash, and total nitrogen contents (micro Kjeldahl) of the combined preparation were also determined.

(2) The tryptic hydrolysis was studied at pH 8 and at pH 9. At pH 9 the heat-denatured protein is completely solubilized; at pH 8 an insoluble residue persists at the end of the hydrolysis.

In a typical experiment at pH 8, 84.9 micromoles of myoglobin were dissolved in 13.5 ml of water in a jacketed reaction vessel. The solution was stirred while the vessel was held in a water bath at 90° for 12 minutes. The suspension formed was cooled to 40°, whereupon water maintained at 40° by a thermostat was circulated through the jacket. The denatured myoglobin was finely dispersed by vigorous magnetic stirring. The pH of the suspension was elevated to 8.0 with 0.2 N NaOH; absorption of atmospheric carbon dioxide was minimized by a blanket of nitrogen previously passed through 0.01 N NaOH. Hydrolysis was initiated by addition of 1.5 ml of a 0.5% solution of trypsin in 0.001 N HCl. The pH of the suspension was maintained constant by addition of 0.10 N NaOH. After 3.0 hours and again after 15.0 hours 0.75 ml additions of trypsin solution were made (cf. Fig. 1 (a)). The total quantity of trypsin added was 15 mg, or approximately 1% of the weight of the substrate. After 20 hours the pH of the suspension was decreased to a value of 7.0 by addition of 12 N HCl. The suspension

was centrifuged at 1500 x g for 30 minutes. The supernatant was withdrawn; the insoluble residue was resuspended in 10 ml of water; and the suspension was recentrifuged. The combined supernatants ("the soluble fraction") were used at once for the isolation of peptides; the sediment ("insoluble fraction") was kept at -10° until required.

(3) In an experiment conducted at the higher pH, 33.8 micromoles of protein in 6.0 ml of water were heat-denatured by the procedure described in paragraph (2). Standard 0.2 N NaOH was added to the suspension to raise the pH (at 40°) to a value of 9.0. Hydrolysis was initiated by addition of 0.60 ml of a 0.5% solution of trypsin in 0.001 N HCl, and the pH was maintained constant by addition of 0.2 N NaOH. Successive additions of 0.30 ml each of trypsin solution were made after 1.7, 3.6, and 16 hours (cf. Fig. 1 (b)). The total quantity of trypsin added was 7.5 mg, or approximately 1.2% of the weight of substrate. After 17 hours at 40° the dark red solution that had formed was acidified with 12 N HCl to a pH of 6.3. A red precipitate immediately appeared. It was collected by centrifugation as described in paragraph (2).

(c) Separation of the Tryptic Peptides in the Soluble Fraction

(1) Chromatography on the sodium form of IRC-50 was found to afford a useful preliminary separation of the 21 principal tryptic peptides, a number of which could not be eluted satisfactorily from Dowex 50-X2. The fractionation scheme followed is best understood by reference to the flow sheet presented in Fig. 2.

(2) The peptide mixture was first subjected to chromatography on IRC-50 either at pH 6.49, or at pH 7.04 (a and b, respectively, in Fig. 2).

Chromatography at pH 6.49 gave a fraction, C, unretarded by the column, followed by eight major zones that contained peptides Tryp 1⁵ through Tryp 9 (cf. Fig. 3). While peptide Tryp 9 was present in each of these eight zones, the main concentration was found in the last three to be eluted from the column. Rechromatography of the peptides from each zone by gradient elution from columns of Dowex 50-X2 in the sodium cycle (c, d, e, f, g, h, and i, in Fig. 2; as an example, cf. Fig. 4) furnished peptides Tryp 1 through Tryp 9 in a form sufficiently pure to permit degradative studies.

(3) When the tryptic hydrolysate was chromatographed on IRC-50 at pH 7.04 (b in Fig. 2) two relatively unretarded fractions, A and B in Fig. 2, were obtained. The principal constituents of fraction B were peptides Tryp 9 and Tryp 10. Following fraction B on the chromatogram were six well resolved zones (cf. Fig. 5) containing, in sequence of appearance, peptides Tryp 11, 12, 5, 4, 6, 8, 13, and 7. Peptides Tryp 4, 5, 6, 7, and 12 from this column were obtained in sufficiently homogeneous condition to permit their use for structural studies without need for further purification. On chromatography by gradient elution from columns of Dowex 50-X2 in the sodium form fraction B was separated (j in Fig. 2) into its principal components, peptides Tryp 9 and Tryp 10, as well as a small quantity of peptide Tryp 2. Peptide Tryp 11, the principal component of the zone immediately following fraction B on the chromatogram was further purified by chromatography over Dowex 50-X2 (k in Fig. 2). The mixture of peptides Tryp 8 and Tryp 13 was similarly separated on Dowex 50-X2 (l in Fig. 2; cf. Fig. 6).

(4) Peptides Tryp 14 through Tryp 21 were obtained by rechromatography

of fraction C. Gradient elution from Dowex 50-X2 (m in Fig. 2; cf. Fig. 7) resolved most of the components of fraction C. Peptides Tryp 14, 15, and 16, however, were only partially separated under conditions that gave satisfactory resolution of the remaining peptides in fraction C, and were completely separated with the aid of a further column of Dowex 50-X2 (n in Fig. 2; cf. Fig. 8). It was necessary to submit peptide Tryp 18 to rechromatography (o in Fig. 2) on Dowex 50-X2 to obtain it in a condition of purity comparable to that of the rest of the peptides isolated.

(d) Chromatography on the Sodium Form of IRC-50

(1) The resin was equilibrated with the appropriate buffer (cf. Table 1) by a standard procedure (cf. Hirs (1955)). The equilibrated resin was suspended in an equal volume of buffer. The columns were assembled in at least five sections by successive additions of equal portions of the resin suspension to chromatograph tubes 4.0 cm in diameter (for details of column operation, cf. Hirs (1955)). Buffer was passed through the columns at a constant rate with the aid of a controlled volume pump until the influent and effluent exhibited identical pH values; the column flow rate was routinely 3 cm/hr in this operation. Temperature control was afforded by operating the columns in a constant temperature room maintained at 22°.

(2) Prior to transfer to the column solutions of the tryptic peptides, concentrated where necessary to a total volume less than 25 ml, were adjusted to a pH approximately 0.1 unit more acid than the pH of the eluent. Elution was allowed to take place at a rate of 3 cm/hr and the effluent was collected in fractions of 8 or 16 ml. Peptides were detected

in the effluent by the alkaline hydrolysis procedure (cf. Hirs, Moore and Stein (1956)), in combination with ninhydrin analysis; aliquots of 1/160th of the fraction volume were routinely used. At the end the resin in the columns was collected in a Büchner funnel, washed with water to remove buffer, and suspended in an equal volume of water. The suspension was stirred vigorously while 2.5 N NaOH was added until the pH had been elevated to a value of about 12, at which point all residual proteins and peptides not previously eluted from the resin are removed. The suspension was filtered immediately and the resin washed with three 100 ml portions of water. The combined filtrates were at once neutralized with 12 N HCl and evaporated to dryness under reduced pressure. The residue, suspended in 10 ml of water, was transferred to an open-ended dialysis casing immersed in 0.5 liters of water. After 20 hours the diffusate was evaporated to dryness under reduced pressure and the residue was saved for quantitative amino acid analysis.

(e) Chromatography on the Sodium Form of Dowex 50-X2

(1) Formate and acetate buffers (cf. Table 1) were used as eluents. The columns were prepared and operated by procedures described previously (cf. Hirs (1960)).

(2) The mixture of peptides comprising fraction C (cf. Fig. 2), the zone at or near the break out of the solvent front in chromatograms of the type shown in Fig. 3, was further fractionated (m in Fig. 2) on a 3.9 x 150 cm column of Dowex 50-X2 equilibrated with 0.2 N sodium formate buffer at pH 3.11. The effluent from the IRC-50 chromatogram containing this zone was acidified to a pH of 2.5 with 12 N HCl (final volume 230 ml)

the
and forced into / column at a flow rate of 2.5 cm/hr. With the column maintained at 35° elution was commenced with the formate buffer, pH 3.11, at a rate of 10 cm/hr. Fractions of 32 ml were collected in tubes measuring 18 x 300 mm. After 3.2 liters of effluent had been collected, the buffer reservoir was replaced with a constant volume, magnetically stirred mixing chamber of 10.9 liter capacity, containing 0.2 N sodium formate buffer at pH 3.11, into which 1.0 N sodium acetate buffer at pH 4.92 (cf. Table 1) was permitted to flow through a capillary connection. After the collection of a total of 8.0 liters of effluent 2.0 N sodium acetate at pH 5.09 (cf. Table 1) was substituted for the 1.0 N buffer in the reservoir. Finally, after 17.6 liters had been collected, the temperature of operation was increased to 50° and the reservoir was filled with 2 N sodium acetate buffer at pH 6.6 (cf. Table 1). The experiment was discontinued after 27.2 liters in all had been accumulated. Peptides not eluted from the column at the end were removed by a method similar to that used following the chromatography of peptides on IRC-50 (cf. (d) (2)).

(3) For the remaining separations outlined in Fig. 2 columns 1.8 - 1.9 cm in diameter were employed under conditions summarized in Table 2. In the typical experiment the mixture of peptides in buffer solution from the preliminary fractionations was diluted with water to diminish the Na^+ concentration to below 0.2 N, and the pH of the diluted solution was reduced to a value between 1.9 and 2.3 by addition of 12 N HCl. The adjusted sample solution was introduced into the column at flow rates between 7 and 20 cm/hr, following which elution was permitted to take place at flow rates from 12 to 26 cm/hr. Fractions of 8 or 16 ml were collected.

(f) Combination of Effluent Fractions Containing Peptides

(1) When complete separation of peptides was attained, as for example Tryp 8 and Tryp 13 in Fig. 6, the fractions corresponding to at least 95% of the eluted material were combined. When adjacent zones in the effluent partially overlapped, as for example successive peaks marked 5, (5,9), (6,9), and (7,8,9) in Fig. 3, only those fractions estimated to be representative of a single zone were collected; these fractions were selected after the remainder of each overlapping peak on the effluent curve was sketched in (cf. Hirs et al. (1956)). In the example cited the combined fractions represented approximately 90% (zone 5), 90% (zone 5,9), and 95% (zone 7,8,9) of the total estimated quantities of peptides in the zones.

(2) All peptide solutions collected from the chromatograms were stored at -10° until required for further fractionation or desalting.

(g) Amino Acid Analyses of Peptides

(1) Aliquots representing about 4% of the total volume of the combined fractions from each zone and containing between 0.3 and 1.6 micromoles of peptide were subjected to acid hydrolysis. Inasmuch as it was known that the two tryptophan residues in myoglobin are accounted for by the tryptophan present in the insoluble fraction of the tryptic hydrolysates, special analyses for tryptophan in the peptides from the soluble fraction were unnecessary.

Acid hydrolysates were prepared from the peptides in the column eluates in sealed tubes as described previously (Hirs, Stein and Moore (1954)). A standard heating time of 22.0 hours at 110° (maintained by a bath of toluene under reflux) was employed throughout. Hydrolysates were evaporated to dryness

under reduced pressure and the residue was in each case suspended in 5 to 10 ml of 12 N HCl. After extraction for approximately 10 minutes at 5° the suspension was filtered through glass wool and quantitative transfer achieved by three rinses with cold 12 N HCl. The combined filtrate was repeatedly concentrated to dryness under reduced pressure to remove excess acid. The residue was dissolved in 0.2 N sodium citrate buffer, pH 2.2 (cf. Moore, ^{made} Spackman and Stein (1958)) and the solution/up to a volume of 5.0 ml in a standard flask. Aliquots of the solution were subjected to quantitative amino acid analysis on an automatic analyzer of the type described by Spackman, Stein and Moore (1958).

(2) Recoveries of serine and threonine after 22 hours of hydrolysis were arbitrarily corrected by 12 and 6% respectively, the estimated losses during hydrolysis of the intact protein under comparable conditions (cf. Edmundson and Hirs (1962a)). The recoveries of isoleucine were corrected for the small amount of alloisoleucine formed by racemization. Methionine recoveries were corrected by addition of the observed content of the isomeric methionine sulfoxides present in each hydrolysate. Similarly the relatively small quantity of the chlorotyrosines found when tyrosine-containing peptides were analyzed was added to the tyrosine recoveries.

(3) Analyses for amide ammonia are not included in the present report. The use of 12 N HCl during the processing of the hydrolysates apparently introduced relatively large and variable quantities of ammonia which prevented the application of previously determined reagent corrections.

(h) Composition and Yield of Peptides

(1) The composition of each peptide was derived by a method described previously (cf. Hirs et al. (1956)). In calculating the average residue recovery, values for serine, threonine, and tyrosine were not included because of the known instability of these amino acids on hydrolysis; values for methionine were omitted because of the oxidative losses of this amino acid; and proline values were excluded because of the low color yield of this amino acid in the reaction with ninhydrin.

(2) The yield of each peptide was calculated from the average recovery per residue and from the fractional quantity removed from the sample for amino acid analysis. The yields were corrected for the amounts consumed during detection and for the quantities not collected because of admixture of peptides from partially overlapping zones. The latter quantities were estimated from the total amount of ninhydrin-reacting substances in the alkaline hydrolysates of the discarded fractions.

(i) Amino Acid Composition of the Insoluble Fraction

(1) The insoluble fractions obtained from the tryptic hydrolysates described in (b) (1) and (2) were the principal subjects of this study. Prior to amino acid analysis these samples were suspended in water and dialyzed exhaustively against distilled water to remove adhering peptides that belonged to the soluble fraction. The suspended residues were collected by centrifugation and dried in a vacuum desiccator over phosphoric anhydride. Portions of the powders were withdrawn for total nitrogen determinations (micro Kjeldahl) and for quantitative amino acid analysis by the procedures

described in the preceding paper (Edmondson and Hirs (1962a)) for the determination of the amino acid composition of the parent protein.

(2) The composition of the insoluble fraction was calculated in terms of the molar ratios of the constituent amino acids. These ratios were derived on the assumption that the insoluble fraction contained equimolar quantities of threonine, tyrosine, phenylalanine, and arginine; and the recoveries of these four amino acids were used to calculate the average molal single residue recovery.

(j) Exhaustive Treatment of the Insoluble Fraction with Trypsin

(1) Portions of the insoluble fraction (Fraction I) formed in the tryptic hydrolysis at pH 8 (cf. (b) (1)) were subjected to further exposures to trypsin, either at pH 8 in suspension, or at pH 9 in solution.

(2) Approximately 40 micromoles of Fraction I were suspended in 10 ml of 0.1 M sodium phosphate buffer at pH 8.0 that contained 2.0 mg of trypsin. No preservative was used but the suspension was maintained at 22° in a tightly stoppered flask mounted on a shaking machine. Additional portions of 1 mg each of trypsin were successively added after 16, 42, and 68 hours. After 96 hours the suspension was centrifuged, the residue was resuspended in 15 ml of water, and the suspension was dialyzed exhaustively against running distilled water. The insoluble residue was collected by centrifugation and dried in a vacuum desiccator to afford Fraction I A. A portion of the dry powder obtained was subjected to quantitative amino acid analysis.

(3) The remainder of Fraction I A was dissolved in 10.0 ml of 0.1 M tris-(hydroxymethyl)-aminomethane buffer at pH 9.0 (22°) that contained

3 mg of trypsin. Further additions of 1 mg each of enzyme to the dark red solution were made after 6 and after 24 hours. After 48 hours the solution was acidified to pH 7.0 with 12 N HCl, and the resulting brown precipitate was collected by centrifugation. The suspended residue was subjected to dialysis and was subsequently collected and dried as described in (2). A portion of the final powder, designated Fraction I B, was removed for quantitative amino acid analysis.

(4) A second portion of approximately 40 micromoles of Fraction I was subjected to the procedure described in (3) to give Fraction I C. A portion of Fraction I C was withheld for quantitative amino acid analysis while the remainder was subjected to a further treatment with trypsin (3 mg) under similar conditions for 16 hours. Portions of the final residue, designated Fraction I D, were subjected to quantitative amino acid analysis and to analyses for tryptophan. The latter were performed on starch-barium hydroxide hydrolysates of the powder by the same technique employed in connection with the tryptophan analyses of the intact protein (cf. Edmundson and Hirs (1962a)).

(k) Tryptic Hydrolysis of Globin

(1) To ascertain whether a more complete degradation of the portion of the myoglobin molecule represented by the insoluble fraction could be attained the tryptic hydrolysis of urea-denatured globin was studied. A solution of 156 mg (9.0 micromoles) of the globin prepared from myoglobin, components IV + V, in 4.0 ml of 8.0 M urea was maintained at 50° for 90 minutes, whereupon the urea was removed by dialysis against 0.01 M NaCl. The solution was transferred to a titration vessel and the denatured protein

was subjected to tryptic hydrolysis at pH 8.0, 40° by a procedure similar to that described in (b) (2). Hydrolysis was started by addition of 0.8 mg trypsin (in 0.001 N HCl) and increments of 0.2 mg each of the enzyme were added after 1.5, 3.0, 6.0, and 15 hours. The total trypsin added approximated 1% of the weight of the denatured globin. After 20 hours the pH was lowered to a value of 7.0 with 0.1 N HCl, and the insoluble precipitate that immediately separated was collected by centrifugation. After resuspension in about 10 ml of water the insoluble fraction was again centrifuged. The combined supernatants were subjected to chromatography on IRC-50 by the method described in (c) (2).

(2) The insoluble fraction was suspended in water and the suspension was dialyzed for 20 hours against running distilled water. The residue was collected by centrifugation and was dried in a vacuum desiccator over phosphoric anhydride. A portion of the powder was removed for quantitative amino acid analysis. The remainder was subjected to a further identical exposure to trypsin at pH 8.0, 40°, after which the insoluble fraction was again separated by the same procedure and a portion of it taken for amino acid analysis.

3. RESULTS

The solubility of denatured myoglobin at pH 9 may in large part be responsible for the much greater initial rate of tryptic hydrolysis observed at this pH over the rate at pH 8 (cf. Fig. 1), under which conditions the reaction takes place heterogeneously. The slight but significant augmentation in the rate of hydrolysis at pH 9 following the addition of

supplemental quantities of enzyme suggest that autolysis of trypsin at the higher pH was appreciable. Under both sets of conditions, however, the addition of trypsin at the end of the reaction failed to cause a significant increase in the rate of uptake of alkali. The total base uptake was 9.50 and 13.8 equivalents per mole at pH 8 and pH 9, respectively. Since the titration behavior of the liberated α -amino groups could not be studied readily in the heterogeneous system, it was not possible to determine the extent of proteolysis by the titration method (cf. Richards (1955)). Independent evidence based on the yields in which fragments were produced in the tryptic hydrolysis indicated that the extent of hydrolysis was essentially identical at both pH values. A very approximate estimate of the number of peptide bonds broken could therefore be arrived at by calculation from the known amino acid composition of the protein on the assumption that all the residues in the peptide mixture ultimately produced titrate normally. The following average pK_a values were assumed: ϵ -amino, 10.5; phenolic, 10.0; imidazole, 6.5. To titrate these groups from pH 8 to pH 9 in the hydrolysate would require 1.2 equivalents of base per mole of protein. Of the observed 4.3 equivalents, therefore, 3.1 equivalents would have been due to the titration of the α -amino groups in the hydrolysate. If an average pK_a of 7.5 is assigned to these groups, a difference of 3.1 equivalents per mole would correspond to 13.4 groups, or, in other words, the splitting of 12.4 bonds. Since there are 23 basic amino acid residues in the protein, the average degree of splitting at each of these residues would calculate at 54%. While this calculation is admittedly approximate

it clearly demonstrates that the tryptic hydrolysis of heat denatured myoglobin was far from complete.

Quantitative analysis of the insoluble fraction (Table 3) revealed that this fraction possessed approximately the same amino acid composition whether it was produced by hydrolysis at pH 8 or at pH 9. The composition underwent only a relatively small alteration on further exposure of the insoluble fraction to the action of trypsin. Of significance is the decrease in the lysine value recorded in Table 3, as well as an increase in the molar ratios for glutamic acid and valine. The composition attained in the stage represented by Fraction I A, however, appeared to represent a limiting composition in that further exposures to the enzyme under different conditions failed to bring about any significant alteration in this composition. The closeness in composition between Fractions I and I B demonstrates that the failure to degrade the fraction further could not have been due to the accumulation of inhibitory reaction products during proteolysis.

The exhaustively treated insoluble fraction, Fraction I D (cf. Table 3), was subjected to analyses for tryptophan. On the basis of the molar ratios given in Table 3, the recovery of tryptophan was equivalent to a tryptophan content of 1.69 residues. When applied to the intact protein the same method of analysis showed a tryptophan content of 1.87 residues per mole. It is therefore apparent that the two tryptophan residues in the myoglobin molecule are accounted for by the tryptophan present in the insoluble fraction.

The analysis of Fraction I D revealed molar ratios for the constituent amino acids, very few of which approximated whole numbers.

Thus, while the insoluble fraction represents one of the terminal products of tryptic hydrolysis it is undoubtedly made up of mixtures of related peptides. If it is assumed that these peptides are present in approximately equivalent quantities the composition of Fraction I D shown in Table 3 may be rounded off to the nearest integer. When this is done it becomes evident (Table 4) that the fraction represents some 50 of the 153 residues in the protein.

Since the prosthetic group of the protein remained firmly associated with the insoluble fraction an accounting of the composition of this fraction must also include the heme group. With the heme included the insoluble fraction should contain, on the basis of the composition extrapolated in Table 4, a minimum of 70 and a maximum of 77 atoms of nitrogen depending on the number of amide groups associated with the 8 aspartic acid and glutamic acid residues. Since myoglobin contains 222 nitrogen atoms per molecule the insoluble fraction accounts for between 31.5 and 34.7% of the total nitrogen in the protein. The nitrogen present in Fraction I (Table 3) was found to represent 33.7% of the nitrogen in the starting material. This result indicates that essentially complete liberation of the "statistical" insoluble fraction had been achieved in the hydrolysis with trypsin.

To determine whether the formation of an insoluble fraction from heat-denatured myoglobin might reflect some feature of the denaturation process, the hydrolysis of urea-denatured globin was investigated. Urea-denatured globin is soluble in the pH range of interest and was smoothly hydrolyzed by trypsin at pH 8 with the uptake of 7.7 equivalents of alkali

per mole, a quantity approximately 80% as great as the quantity required for heat-denatured myoglobin. At pH 7 an insoluble fraction separated from the hydrolysate. The results presented in Table 4 were obtained by quantitative amino acid analysis of this insoluble fraction. For comparison the results obtained by analysis of the insoluble fraction from heat-denatured myoglobin are also included in Table 4. While the two fractions undoubtedly exhibit differences in composition, particularly in regard to valine and proline content, it is clear that they are sufficiently similar to make inescapable the conclusion that they largely represent the same portion of the parent protein molecule. That the agreement in composition was not fortuitous is demonstrated by the observation (Table 4) that the insoluble fraction derived from the urea-denatured globin undergoes a relatively minor change in composition on further exposure to trypsin for an extended period. It is also evident that the presence of the heme group is not a factor in the protection of potentially sensitive peptide bonds in the insoluble fraction from the action of the enzyme.

The soluble fraction contained 21 principal peptides. Attempts to achieve a preliminary fractionation of the peptides on columns of the sodium form of Dowex 50-X2 were unsuccessful: several of the peptides could not be eluted below pH 7, and we wished to avoid exposure of peptides to alkaline conditions. Useful preliminary separations, however, could be attained by means of columns of IRC-50 in the sodium form. Bailey, Moore and Stein (1956), and Hirs⁶ had previously used this resin for the chromatography of peptides liberated by the action of pepsin and trypsin, respectively, from oxidized ribonuclease. More recently Ozawa, Tamai and Satake (1960),

and Sasakawa and Satake (1960) have used IRC-50 for the fractionation of tryptic and other proteolytic hydrolysates of the globin derived from horse and bovine hemoglobin.

Peptides Tryp 1 through Tryp 9 were eluted at pH 6.49 (Fig. 3). While satisfactory resolution of peptides Tryp 1 to 6 was attained, and peptides Tryp 7 and 8 migrated as a doublet, peptide Tryp 9 chromatographed poorly with extensive tailing under these conditions and was found to be a constituent of each of the zones containing peptides Tryp 1 through 8. Quantitative amino acid analysis of the fraction removed from the resin by alkaline elution on completion of the chromatogram showed that several peptides were still left on the column at that point. By contrast, when chromatography on IRC-50 at pH 7.04 was completed (cf. Fig. 5), analysis of the alkaline eluate of the resin in the chromatograph tube demonstrated that only negligible quantities of peptides remained. Since the chromatogram at pH 7.04 showed the presence of peptides Tryp 10 to 13, it is evident that these peptides were not eluted from the column at pH 6.49. Thus peptides Tryp 1 to 13 and the peptides contained in the zone moving at or near the break-out of the solvent front of the column at pH 6.49 (cf. Fig. 2, Fraction C; and Fig. 3) represented all the peptides present in the soluble fraction.

With the exception of Fraction C the mixtures of peptides arising from the preliminary separations on IRC-50 contained either two or three principal constituents. Relatively short columns of Dowex 50-X2 operated by gradient elution afforded excellent separations of these mixtures. As

examples, the separation of peptides Tryp 7, 8, and 9 is shown in Fig. 4, and the separation of peptides Tryp 8 and 13 in Fig. 6.

Fraction C contained peptides Tryp 14 through 21. These peptides were separated on Dowex 50-X2 columns as detailed in the experimental part (cf. Figs. 7 and 8). Quantitative amino acid analysis of the alkaline eluate of the resin in these columns revealed that no peptides were left on the adsorbent after chromatography had been completed.

The results obtained upon quantitative amino acid analysis of the final effluent fractions containing peptides Tryp 1 through 21 are presented in Table 5. The table also includes the yield determined for each of the peptides on the assumption that each peptide is unique and can be formed from only one portion of the sequence in the peptide chain.

The only peptide devoid of lysine or arginine that was obtained was peptide Tryp 16. This peptide was therefore considered to represent the carboxyl terminus of the peptide chain.

The fraction designated Tryp 7 was identified as free lysine by chromatography on the amino acid analyzer, and by the demonstration that following acid hydrolysis the recovery of lysine remained unchanged. The amino acid compositions of each of the pairs of peptides Tryp 9 - 12, Tryp 11 - 14, and Tryp 15 - 19, differ by one residue of lysine. In each pair the peptide containing the extra lysine residue was more strongly retained on IRC-50 or Dowex 50-X2 and was formed in the smaller yield. The existence of these pairs of peptides and the presence of free lysine in the tryptic hydrolysate demonstrates the presence in myoglobin of sequences of the type B.Lys, where B may be lysine or arginine. The recovery of the

equivalent of one molecule of lysine per molecule of myoglobin requires that there be a minimum of two such sequences. The presence of three pairs of tryptic peptides differing by only one lysine residue could be accounted for by two B.Lys sequences if two of these pairs of peptides were contiguous in the amino acid sequence in myoglobin. However, we have assumed that the peptide pairs originate from non-contiguous regions of the sequence and that, therefore, there are three B.Lys sequences in the chain. The recovery of lysine given in Table 5 is calculated on this basis.

The composition of each peptide in Table 5 is defined by the molar ratios of the constituent amino acids as determined by quantitative amino acid analysis. The homogeneity of the peptides isolated is attested to by the noteworthy closeness with which the molar ratios approximate integral values, and, in particular, by the general absence of extraneous amino acids. The most prevalent impurity encountered was serine. The highest serine contamination was found in peptide Tryp 21. Inasmuch as this pentadecapeptide chromatographed poorly and gave the least satisfactory analysis of the peptides listed in Table 5 it is likely that the serine contamination in this particular instance may in part be attributed to the presence of other peptides. The presence of as much as 10% serine in some of the smaller peptides could not be accounted for in this way. Some of these peptides were re-chromatographed with a view to removing possible serine-containing peptides present as impurities. In each instance the rechromatographed peptide still contained the serine impurity in unchanged proportion.

The yields in which the tryptic peptides were isolated varied widely. In some instances low recoveries were unquestionably due to the

chromatographic characteristics of the peptides involved. For example, peptide Tryp 21 contained phenylalanine and histidine, residues known (cf. Moore and Stein (1956)) to confer a high affinity for the polystyrene matrix of ion-exchangers. This peptide was eluted from Dowex 50-K2 with difficulty. The zone marked 21 ? at 17.2 effluent liters in Fig. 7 contained a peptide with the same amino acid composition as peptide Tryp 21. This suggests that Tryp 21 was undergoing extensive tailing during chromatography and that it would have been obtained in larger yield had the final solvent change in the elution sequence (cf. experimental part) been effected earlier. It is unlikely that similar chromatographic difficulties were responsible in other instances where the yields of peptides were low. The pentadecapeptide Tryp 18 was recovered in only 9% yield. It is possible that secondary proteolytic cleavages in this relatively large peptide may have reduced the yield to some extent, but a similar explanation could hardly account for the low recoveries of peptides Tryp 3 and 4, a tetra and a tripeptide, respectively. It is more likely that peptides such as Tryp 3 and 4 were formed in poor yield because of incomplete tryptic hydrolysis, to which reference has already been made in connection with the observed proton release measured in the pH-stat during hydrolysis. In order to shed some light on this question, the soluble fraction from a tryptic hydrolysate of urea-denatured globin was subjected to chromatography on IRC-50 under the same conditions as those described in Fig. 5. An identical chromatogram was obtained except that peptides Tryp 8 and 13 emerged as separate zones instead of overlapping as shown in Fig. 6. However, the yields in which the peptides were obtained were lower. For instance, the yields of Tryp 7 and 13 were 18 and 29%,

respectively, or about 60% as great as the yields shown in Table 5. Since the consumption of base in the pH-stat during the tryptic hydrolysis showed that the urea-denatured globin was less completely cleaved than heat-denatured myoglobin, the lower yield of peptides in the globin hydrolysate may be directly related to less extensive hydrolysis. The results also suggest the possibility that failure to attain complete cleavage at a particular bond may ultimately be attributed to heterogeneity of the product formed by denaturing globin or myoglobin.

The only peptide in Table 5 lacking a unique composition is Tryp 10. Since this peptide was obtained in small yield, it was at first thought to be derived by further cleavage of peptide Tryp 9, which contains two residues of lysine. When the X-ray analysis of the sequence (Kendrew et al., 1961) became available this view was no longer tenable. The proline residue in Tryp 9 corresponds to residue 86 in the sequence and was identified with certainty. For Tryp 10 to be derived from Tryp 9 this proline residue would have to be within four residues of lysine. The X-ray results show clearly that it is not. Further work is required to furnish an explanation of the origin of peptide Tryp 10.

If peptides Tryp 10, 11, 12, and 19 are omitted from consideration, the peptides in the soluble fraction of the hydrolysate account for 108 residues. The first column of Table 6 shows the distribution of these residues. The second column of the table repeats the amino acid composition of myoglobin. The third column was obtained by subtraction of the residues accounted for in the soluble fraction from the total composition, and therefore represents the theoretical composition of the insoluble fraction. The

fourth column in Table 6 presents the composition of the insoluble fraction as derived by extrapolation of the results in Table 3. The final column in Table 6 shows the difference between the third and fourth columns and therefore demonstrates the closeness with which the composition of the insoluble fraction was approximated by the extrapolation. The results permit the conclusion that a complete accounting of the residues in the parent protein molecule is possible in terms of the composition of the fragments produced upon tryptic hydrolysis.

4. DISCUSSION

The results presented raise the possibility that heat- or urea-denatured preparations of myoglobin and globin are heterogeneous and may consist of populations of variously unfolded molecules, many of which still retain elements of the native configuration. The action of trypsin on such molecules would in general be expected to be confined to those regions where the native configuration had been disrupted and to give rise to reaction products consisting of small peptides on the one hand, and on the other to larger fragments that still possessed attributes of the structure present in the parent molecule. Most of these larger fragments would undoubtedly unfold spontaneously on becoming detached from the main fabric of the protein molecule, subsequently to undergo further tryptic hydrolysis in a succession of steps involving progressive denaturation. It is possible, however, that some of the degradation products in which features of the configuration in the intact molecule are retained would be resistant to further unfolding in spite of their dissociation from an environment that may have contributed to

the stabilization of that configuration. It is also possible that some of these fragments, once liberated, could refold into other stable configurations not existing in the parent molecule, or undergo association into polymeric forms. In sum such processes would effectively reduce the number of molecules in which a particular lysine or arginine residue in the peptide chain was available to the hydrolytic action of trypsin. Evidence has been obtained that shows that larger peptides may possess an ordered structure following liberation from a protein in the process of tryptic hydrolysis. Grassmann, Hannig and Schleyer (1960) observed that some of the large peptides produced from the collagen macromolecule by the action of trypsin retained some of the structural configuration of the parent protein. In a lower range of molecular weight, Braunitzer and his colleagues⁷ showed that certain tryptic peptides from hemoglobin were resistant to chymotrypsin until "denatured" by heat treatment or solution in anhydrous formic acid.

If certain portions of the myoglobin molecule were capable of being unfolded with particular ease peptides derived from such regions would be expected to be formed in relatively higher yields. The results in Table 5 fail to provide any evidence that particularly labile regions of this kind occur. Ribonuclease A is resistant to attack by trypsin at temperatures below which the transition at 62° begins, but undergoes hydrolysis above these temperatures, in a region in which physical measurements amply demonstrate that a reversible partial unfolding of the molecule can take place. The results (Rupley and Scheraga, 1960) obtained thus far may be interpreted in terms of an unfolding of certain specific regions in the molecule while other structural features remain relatively unchanged, with

enzyme action apparently restricted to those regions where unfolding has occurred. While the unfolding of regions of the ribonuclease molecule on heating through the 62° transition is a readily reversible process, the unfolding of parts of the myoglobin molecule on denaturation is undoubtedly kinetically irreversible for all practical purposes and therefore leads to the accumulation of a large number of products as the final consequence of heat or urea treatment.

Examination of the Fourier synthesis of the electron density in myoglobin at a resolution of 2 Å has enabled Kendrew and his colleagues (1961) to deduce a tentative amino acid sequence for the protein from which the arrangement of the tryptic peptides along the polypeptide chain may be established with a high degree of certainty. Of outstanding interest in the present context is the demonstration that the insoluble fraction consists of at least two peptides⁸, derived from remote parts of the sequence that are brought together through the folding of the polypeptide chain. In conformity with commonly accepted usage, Kendrew and his colleagues have called these two peptides Core A and Core B. The first of these contains 32 residues and is represented in the structure by two α-helices, A and B, that between them take up the first 34 residues in the chain. Core B is represented by residues 101 to 113 in another α-helix, helix G, that runs very roughly at right angles to helix A. Core A contains the two tryptophan residues in the protein. The sum of the amino acid composition of Core A and Core B agrees satisfactorily with the composition of the insoluble fraction as deduced in Table 6. It must be concluded that these two regions in the molecule either become firmly associated as a consequence of denaturation

prior to hydrolysis, or become associated following their separate liberation from the protein as it undergoes degradation.

If either of the cores represented unusually stable features in the over-all configuration of the molecule, tryptic peptides corresponding to regions immediately adjacent to the cores might be formed in particularly poor yield. It may therefore be significant that peptides Tryp 3 and 4, obtained in yields of 15 and 13% respectively, immediately precede and follow Core B in the sequence.

A further feature of significance is that none of the residues in the two cores are implicated in the binding of the heme group in myoglobin. The quantitative precipitation of the heme with the insoluble fraction on tryptic hydrolysis reveals that specific heme binding is not maintained on disruption of the molecule and depends on the integrity of the structural configuration. The histidine residue at position 91 in the chain (Kendrew et al., 1961) acts as a ligand for the iron atom of the heme group. This histidine is present in peptides Tryp 9 and 12, the combined yield of which (Table 5) was relatively high. A salt linkage between one of the propionic acid side chains of the heme group and an arginine side chain is apparently one of the features of the binding of the heme group in the intact molecule. The arginine residue in question, residue 43, is located in a non-helical portion of the molecule and appears in peptide Tryp 2.

The X-ray analysis at 2 Å resolution clearly shows that peptide Tryp 16 is derived from the carboxyl-terminal end of the peptide chain. This direct proof confirms the conclusions reached from a consideration of the compositions of the tryptic peptides and from the fact that both

the intact protein and peptide Tryp 16 exhibit the same pattern of release of free amino acids upon degradation with carboxypeptidase (cf. Edmundson and Hirs (1962a)).

The amino acid sequence also makes possible an interpretation of the origin of the pairs of peptides Tryp 9 - 12, Tryp 11 - 14, and Tryp 15 - 19. Peptide Tryp 1, which contains a carboxyl-terminal arginine residue, is linked to Tryp 14 in the trypsin series through a lysine residue. The Arg.Lys sequence between the residues corresponding to these peptides may be attacked in two ways by the enzyme. When tryptic hydrolysis first takes place at the lysine residue (asymmetrical cleavage) a new carboxyl-terminal arginyllysine sequence is generated, and this may subsequently readily undergo further attack at the arginine residue with the formation of free lysine (Tryp 7). On the other hand, when tryptic hydrolysis fails to take place first at the lysine residue, but is initiated at arginine (symmetrical cleavage), the adjacent lysine residue is left as a new amino-terminal end-group, eventually found as the amino-terminus of peptide (Glu,Ala,Leu₂,Phe)Arg.Lys.(Asp,Ala₂Ileu)Lys

Tryp 11. Tryptic hydrolysis at amino-terminal lysine residues occurs extremely slowly (cf. Waley and Watson (1954)). An example is residue 1 in pancreatic ribonuclease (cf. Hirs et al. (1956)). Peptide Tryp 11 therefore in all likelihood accumulates during tryptic hydrolysis because of the

resistance to tryptic hydrolysis of the exposed amino-terminal lysine residue. The relative amount of the peptide may indeed be regarded as a rough measure of the extent to which symmetrical cleavage took place at the Arg.Lys bond.

The pair of peptides Tryp 9 and 12 may arise in an analogous manner. The X-ray evidence indicates that the arginine peptide Tryp 18 precedes peptide Tryp 12 in the trypsin series, and that Tryp 12 possesses an amino-terminal Lys.Lys sequence. As the accompanying partial formulae show this would require that peptide Tryp 9 have an amino-terminal lysine residue:

Insert Formula 2 - Page 34

In more recent chemical work⁹ peptide Tryp 9 has in fact been shown to possess an amino-terminal glycine residue (cf. Edmundson and Hirs (1961)). Since the X-ray and chemical identification of the residue in question are equally certain the dichotomy probably indicates that the formulation for the tryptic splitting should be:

Insert Formula 3 - Page 34

This formulation requires that an additional lysine residue be accommodated in the sequence between residues 78 and 92¹⁰.

The extent of tryptic hydrolysis in the Arg.Lys.Lys cluster of myoglobin compares closely with that found (Shepherd et al., 1956; Li et al., 1955; White and Landmann, 1955) for the Lys.Lys.Arg.Arg. Pro sequence in the corticotropins: tryptic hydrolysis was most pronounced at the Lys.Lys link and took place to a considerably smaller extent at the Lys.Arg and Arg.Arg bonds.

The present interpretation of the electron density sections of

FORMULA 2

(Asp₂, Glu₂, Gly₂, Ala₂, Val₂, Leu, Ileu₂, His) Arg. Lys. Lys (Glu₃, Gly, Ala₃, Leu₂, Ser, Thr, Pro, His₂) Lys.

FORMULA 3

(Asp₂, Glu₂, Gly₂, Ala₂, Val₂, Leu, Ileu₂, His) Arg. Lys. Lys. Gly (Glu₃, Ala₃, Leu₂, Ser, Thr, Pro, His, Lys) Lys

myoglobin at a resolution of 2 Å does not provide a straightforward explanation for the origin of the pair of peptides Tryp 15 and 19. Peptide Tryp 15 derives from a helical portion of the molecule in which the residues have been identified with relatively few ambiguities, and in which no lysyllysine sequence apparently is possible. At present, therefore, it must be concluded that peptide Tryp 19 owes its formation to a trans-peptidation reaction involving peptide Tryp 15 and the free lysine formed in the course of the hydrolysis with trypsin. Further work will be required to test this hypothesis.

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FOOTNOTES

- ¹ Preliminary reports of this work have appeared (Edmundson and Hirs, 1959; 1961).
- ² Taken in part from a Thesis submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy at The Rockefeller Institute.
- ³ Research at Brookhaven National Laboratory was performed under the auspices of the United States Atomic Energy Commission.
- ⁴ A. B. E., to be published.
- ⁵ The peptides are designated with the prefix Tryp to denote that they have been isolated from a tryptic hydrolysate.
- ⁶ Unpublished results.
- ⁷ Personal communication.
- ⁸ As will be shown in an accompanying paper (Edmundson and Hirs, 1962b), valine was detected as the principal amino-terminal residue in the insoluble fraction. Until the X-ray evidence became available this observation was interpreted to define the insoluble fraction as a single, large peptide in which several potentially sensitive bonds had not been attacked by trypsin.
- ⁹ A. B. E., to be published.
- ¹⁰ It may be of interest to note that the tentative amino acid sequence of Kendrew et al. (1961) shows a "deletion" of one residue in this region of the peptide chain when the sequence is related to the amino acid sequences of the hemoglobin chains (cf. Watson and Kendrew (1961)). The tentative sequence presently accounts for 18 lysine residues.

REFERENCES

- Bailey, J. L., Moore, S., and Stein, W. H. (1956). J. Biol. Chem. 221, 143.
- Edmundson, A. B., and Hirs, C. H. W. (1959). Federation Proc. 18, 220.
- Edmundson, A. B., and Hirs, C. H. W. (1961). Nature, 190, 663.
- Edmundson, A. B., and Hirs, C. H. W. (1962a). J. Mol. Biol. 00, 000.
- Edmundson, A. B., and Hirs, C. H. W. (1962b). J. Mol. Biol. 00, 000.
- Grassmann, K., Hannig, K., and Schleyer, M. (1960). Z. physiol. Chem. 322, 71.
- Hamilton, P. B. (1958). Anal. Chem. 30, 914.
- Hirs, C. H. W. (1955). In Methods in Enzymology, ed. by S. P. Colowick and H. O. Kaplan, Vol. I, p. 113. New York: Academic Press.
- Hirs, C. H. W. (1960). J. Biol. Chem. 235, 625.
- Hirs, C. H. W., Moore, S., and Stein, W. H. (1956). J. Biol. Chem. 219, 623.
- Hirs, C. H. W., Stein, W. H., and Moore, S. (1954). J. Biol. Chem. 211, 941.
- Keller, P. J., Cohen, E., and Neurath, H. (1958). J. Biol. Chem. 233, 344.
- Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., and Phillips, D. C. (1960). Nature, 185, 422.
- Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C., and Shore, V. C. (1961). Nature, 190, 666.
- Li, C. H., Geschwind, I. I., Cole, R. D., Raacke, I. D., Harris, J. I., and Dixon, J. S. (1955). Nature, 176, 687.
- Moore, S., Speckman, D. H., and Stein, W. H. (1958). Anal. Chem. 30, 1185.
- Moore, S., and Stein, W. H. (1956). Advances in Protein Chem. 11, 191.
- Ozawa, H., Tamai, K., and Satake, K. (1960). J. Biochem. (Tokyo), 47, 244.
- Richards, F. M. (1955). Compt. rend. trav. lab. Carlsberg, Ser. chim. 29, 322.

- Rupley, J. A., and Scheraga, H. (1960). Biochim. et Biophys. Acta, 44, 191.
- Sasakawa, S., and Satake, K. (1960). J. Biochem. (Tokyo), 47, 672.
- Shepherd, R. G., Willson, S. D., Howard, K. S., Bell, P. H., Davies, D. S.,
Davis, S. B., Eigner, E. A., and Shakespeare, N. E. (1956). J. Am. Chem.
Soc. 78, 5067.
- Speckman, D. H., Stein, W. H., and Moore, S. (1958). Anal. Chem. 30, 1190.
- Waley, S. G., and Watson, J. (1954). Biochem. J. 57, 529.
- Watson, H. C., and Kendrew, J. C. (1961). Nature, 190, 670.
- White, W. F., and Landmann, W. A. (1955). J. Am. Chem. Soc. 77, 1711.

LEGENDS TO FIGURES

Fig. 1. Tryptic hydrolysis of heat-denatured myoglobin (components IV + V).

(a) 84.9 micromoles of substrate at pH 8.0, 40°; (b) 33.8 micromoles substrate at pH 9.0, 40°. For details of procedure the experimental part should be consulted.

Fig. 2. Outline of Chromatographic Fractionation of Soluble Peptides.

Each step is indicated by a lower case italicized letter; thus *a*. Column dimensions in cm are indicated for each step. Procedures *a* and *b* involved columns of IRC-50 in the sodium form at pH 6.49 and 7.04 respectively. Procedures *c* through *e* required Dowex 50-X2 columns operated in the sodium form. Capital letters A, B, and C represent mixtures of peptides. The numerals denote individual peptides and correspond with the identifications in Figs. 3 through 8; when circled such numerals denote that a peptide was isolated in that particular step in a condition sufficiently pure to permit the use of the peptide in sequence analysis. Details of the conditions of elution are given in the experimental part and in Table 2.

Fig. 3. Fractionation of the soluble peptides from a tryptic hydrolysate of 84.9 micromoles of heat-denatured myoglobin by chromatography at pH 6.49, 22°, on a 4 x 50 cm column of IRC-50 with 0.2 M sodium phosphate buffer as eluent (*cf. a* in Fig. 2). Fraction volume, 16 ml. For other details the experimental part should be consulted. The peaks on the effluent curve are numbered in accordance with the peptides present in the corresponding effluent fractions. Mixtures are indicated by a series of numbers representing the individual

peptides. The composition and yield are indicated when a peptide was isolated as the principal constituent of a given band. The peptides are shown with carboxyl-terminal arginine or lysine residues because the tryptic hydrolysis was assumed to have taken place at basic residues only.

Fig. 4. Separation of peptides Tryp 7, 8, and 9 (cf. 1 in Fig. 2) by chromatography on a 1.8 x 23 cm column of Dowex 50-X2. Fraction volume, 8 ml. For other details consult the experimental part, Fig. 3, and Table 2. Peptides Tryp 7 and 8 were separated at pH 4.9, 40°, by gradient elution with acetate buffer increasing in Na⁺ concentration from 0.2 N to 0.5 N. Subsequently peptide Tryp 9 was eluted at pH 6.6 with 2.0 N sodium acetate buffer.

Fig. 5. Fractionation of soluble peptides from a tryptic hydrolysate of 33.8 micromoles of heat-denatured myoglobin by chromatography at pH 7.04, 22°, on a 4 x 30 cm column of IRC-50 with 0.2 N sodium phosphate buffer as eluent (cf. b in Fig. 2). Fraction volume, 8 ml. For other details the experimental part should be consulted. The peptides are designated as in Fig. 3. Although the peptides represented by the peaks on the effluent curve shown here were obtained by tryptic hydrolysis at pH 9.0, an identical curve was obtained on chromatography (by the same procedure) of the peptides formed by tryptic hydrolysis at pH 8.0.

Fig. 6. Separation of peptides Tryp 8 and 13 (cf. l in Fig. 2) by chromatography on a 1.8 x 10 cm column of Dowex 50-X2. Fraction volume, 8 ml. For other details the experimental part, Fig. 3, and Table 2 should be consulted. The peptides were separated at pH 4.9, 40°, by gradient elution with acetate buffer increasing in Na⁺ concentration from 0.2 N to 1.0 N.

Fig. 7. Preliminary separation of peptides in Fraction C (cf. m in Fig. 2; and Fig. 3) by chromatography on a 3.9 x 150 cm column of Dowex 50-X2. Fraction volume, 32 ml. For other details the experimental part and Fig. 3 should be consulted. With the exception of peptide Tryp 21 the peptides were eluted at 35° by a gradient of sodium formate-acetate buffer increasing in Na⁺ concentration and in pH from 0.2 N, pH 3.1 to 2.0 N, pH 5.1. Tryp 21 was subsequently removed from the column at 50° by gradient elution with 2.0 N sodium acetate buffer increasing in pH from 5.1 to 6.6. For the significance of the zone marked 21 ?, reference should be made to the text.

Fig. 8. Separation of peptides Tryp 14, 15, and 16 (cf. n in Fig. 2) by chromatography on a 1.9 x 150 cm column of Dowex 50-X2. Fraction volume, 16 ml. For other details the experimental part, Fig. 3, and Table 2 should be consulted. The peptides were separated at 35° by gradient elution with 0.2 N sodium formate-acetate buffer increasing in pH from 3.1 to 4.9. For preparative purposes the zone containing peptides Tryp 14, 15, and 16 in chromatogram n of Fig. 2

(cf. Fig. 7) was cut at two points to afford three fractions, each of which was chromatographed by the same procedure (cf. Table 2). The chromatogram shown here was obtained from the center cut.

NINHYDRIN COLOR VALUE AFTER ALKALINE HYDROLYSIS
(LEUCINE EQUIVALENTS, mM CONC)

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(LEUCINE EQUIVALENTS, mM CONC)

TABLE 1

Preparation of Buffers Utilized in the Chromatography of Peptides in the Soluble Fraction

All chemicals are reagent grade. The pH values are those measured with a glass electrode at 22°.

pH	Anion	Concentration	Components	Volume
6.49	Phosphate	0.2 <u>N</u>	331 gm $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 227 gm Na_2HPO_4	20 liters
7.04	Phosphate	0.2 <u>N</u>	156 gm $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 409 gm Na_2HPO_4	20 liters
3.11	Formate	0.2 <u>N</u>	250 ml 99% formic acid, 200 ml 12 <u>N</u> HCl, 4000 ml <u>N</u> NaOH	20 liters
5.09	Acetate	2.0 <u>N</u>	5444 gm sodium acetate trihydrate, 1140 ml glacial acetic acid	20 liters
4.92	Acetate	1.0 <u>N</u>	Dilution of 2 <u>N</u> buffer	
4.90	Acetate	0.5 <u>N</u>	Dilution of 2 <u>N</u> buffer	
4.89	Acetate	0.2 <u>N</u>	Dilution of 2 <u>N</u> buffer	
6.6	Acetate	2.0 <u>N</u>	2722 gm sodium acetate trihydrate, 25 ml glacial acetic acid	10 liters

TABLE 2

Procedures Employed in the Rechromatography of Peptides on Columns of Dowex 50-X2

The zone containing peptides Tryp 14, 15, and 16 obtained upon chromatography of Fraction C (cf. m in Fig. 2; and Fig. 7) was divided into three portions: the first portion contained peptides Tryp 14 and 15; the second, peptides Tryp 14, 15, and 16; and the third, peptides Tryp 15 and 16. The same 1.9 x 150 cm column could be used for the separation of the three fractions thus obtained. For procedural details the experimental part should be consulted.

Zones	Step							Buffers Employed			
Containing	in				pH	Temperature					
Peptides	Fig.	Column	Volume of:		of	of	0.2 N, pH 4.89	0.5 N, pH 4.90	1.0 N, pH 4.92	2.0 N, pH 6.6	
Numbered:	2	Size	Sample	Fraction	Sample	Column	Sodium Acetate	Sodium Acetate	Sodium Acetate	Sodium Acetate	
							Buffer added at	Buffers added to mixing	Buffer added at	Buffer added at	
							fraction number	chamber at fraction number	fraction number	fraction number	
I. 270 ml chamber											
1,9	c	1.8 x 20	130	8	2.0	40°	1	3	52	92	
2,9	d	1.8 x 20	260	8	2.0	40°	1	5	50	-	
3,9	e	1.8 x 20	280	8	2.0	40°	1	10	60	110	
4,9	f	1.8 x 20	250	8	2.0	40°	1	4	44	118	
8,13	g	1.8 x 10	310	8	1.9	40°	1	5	50	-	
9,10	h	1.8 x 30	90	8	2.0	40°	1	10	88	150	
11	i	1.8 x 5	25	8	2.1	22°	1	2	30	-	
18	j	1.8 x 20	600	8	2.0	40°	1	5	50	125	
II. 540 ml chamber											
5,9	k	1.8 x 25	360	8	2.2	40°	1	38	182	282	
6,9	l	1.8 x 23	400	8	2.0	40°	1	36	236	271	
7,8,9	m	1.8 x 23	400	8	2.3	40°	1	25	-	225	
0.2 N, pH 3.11											
Sodium Formate											
0.2 N, pH 4.89											
Sodium Acetate											
III. 2460 ml chamber											
14,15	n	1.9 x 150	900	16	1.9	35°		1		100	
14,15,16	o	1.9 x 150	600	16	2.1	35°		1		100	
15,16	p	1.9 x 150	700	16	2.1	35°		1		100	

TABLE 3

Amino Acid Composition of Insoluble Fraction Obtained in Tryptic Hydrolysis of Heat-denatured Myxobolbin

The compositions are expressed in terms of the molar ratios of the constituent amino acids. Fraction I was obtained following the tryptic hydrolysis of 84.9 micromoles of protein for 20 hours at pH 8.0 (cf. Fig. 1 (a)). Fraction II was obtained from 33.8 micromoles of protein after tryptic hydrolysis for 17 hours at pH 9.0 (cf. Fig. 1 (b)). Fraction I A remained after further exposure of Fraction I to the action of trypsin at pH 8.0 for 96 hours. On further exposure for 48 hours at pH 9.0 Fraction I A gave Fraction I B. Fraction I C was obtained by exposure of Fraction I to the enzyme at pH 9.0 for 48 hours; and Fraction I D was the final product remaining after further exposure for 16 hours of Fraction I C to trypsin at pH 9.0. For details of procedure and analysis the experimental part should be consulted.

Conditions in Tryptic Hydrolysis	Fraction I	Fraction II	Fraction I A	Fraction I B	Fraction I C	Fraction I D
	20 hr, pH 8.0	17 hr, pH 9.0	<u>cf.</u> I,	<u>cf.</u> I, I A,	<u>cf.</u> I,	<u>cf.</u> I, I C,
	40°	40°	96 hr, pH 8.0	48 hr, pH 9.0	48 hr, pH 9.0	16 hr, pH 9.0
			25°	25°	25°	25°
Time of Hydrolysis						
Amino acid	22 hr	22 hr	24 hr	22 hr	70 hr	22 hr
Aspartic acid	2.19	1.96	1.92	2.00	1.92	1.92
Glutamic acid	4.17	5.03	6.28	6.43	6.36	6.50
Glycine	3.86	3.82	4.22	4.22	4.22	4.18
Alanine	4.76	4.98	4.88	4.81	4.86	4.89
Valine	5.16	4.65	6.32	6.16	6.57	6.38
Leucine	6.51	6.28	7.02	6.99	7.19	7.20
Isoleucine*	4.45	3.93	4.31	3.81	4.46	4.28
Serine [†]	2.35	2.22	2.51	3.07	2.35	2.53
Threonine [†]	1.09	1.41	1.12	1.26	1.07	1.20
Methionine*	0.27	0.41	0.11	0.0	0.0	0.08
Proline	0.74	0.93	0.60	0.64	0.62	0.42
Phenylalanine	1.26	1.14	0.96	1.01	0.90	0.94
Tyrosine*	0.72	0.70	0.97	0.92	0.67	0.77
Histidine	3.93	4.13	4.01	4.00	4.06	4.09
Lysine	3.25	4.14	2.64	2.73	2.80	2.60
Arginine	1.39	1.17	1.42	1.29	1.28	1.43

* Isoleucine values corrected for transformation to alloisoleucine; methionine for transformation to sulfoxides; and tyrosine for transformation to chlorotyrosines.

[†] Corrected for hydrolytic losses. cf. the experimental part.

TABLE 4

Amino Acid Composition of Insoluble Fraction Obtained in the Tryptic
Hydrolysis of Urea-denatured Globin

The compositions are expressed in terms of the molar ratios of the constituent amino acids. Fraction III was obtained by tryptic hydrolysis of 9.0 micromoles of protein at pH 8.0 for 20 hours. Fraction III A was obtained by further exposure of Fraction III to the action of trypsin at pH 8.0. For details of procedure and analysis the experimental part should be consulted. For comparison the composition of Fraction I B obtained from heat-denatured myoglobin is included in this table; cf. Table III.

Conditions in Tryptic Hydrolysis	Fraction III	Fraction III A	Fraction I B
	20 hr, pH 8.0 40°	cf. III, 20 hr, pH 8.0, 40°; then additional 20 hr, pH 8.0, 40°	cf. Table III
Time of Hydrolysis			
Amino acid	22 hr	24 hr	24 hr
Aspartic acid	2.55	2.50	2.00
Glutamic acid	6.18	6.32	6.43
Glycine	3.90	4.16	4.22
Alanine	5.00	5.02	4.81
Valine	4.41	4.84	6.16
Leucine	5.98	5.90	6.99
Isoleucine*	4.04	4.17	3.81
Serine'	2.58	3.11	3.07
Threonine'	1.00	0.90	1.26
Methionine*	0.38	0.21	0.0
Proline	1.31	1.17	0.64
Phenylalanine	1.45	1.36	1.01
Tyrosine*	0.82	0.73	0.92
Histidine	4.60	3.95	4.00
Lysine	3.86	3.20	2.73
Arginine	1.45	1.28	1.29

* ' Have the same significance as in Table 3.

TABLE 5

Amino Acid Composition of the Peptides in the Soluble Fraction of Tryptic Hydrolysates of Heat-denatured Myoglobin

The compositions are expressed in terms of the molar ratios of the constituent amino acids. Values for the principal constituents are shown in bold-faced type. Values smaller than 0.01 residue are omitted. Numbers in parentheses are not included in the totals.

Amino acid	Peptide																					Total Number of Residues from Peptides
	Tryp 1	Tryp 2	Tryp 3	Tryp 4	Tryp 5	Tryp 6	Tryp 7	Tryp 8	Tryp 9	Tryp 10	Tryp 11	Tryp 12	Tryp 13	Tryp 14	Tryp 15	Tryp 16	Tryp 17	Tryp 18	Tryp 19	Tryp 20	Tryp 21	
Aspartic acid	-	<u>0.99</u>	-	-	-	-	-	-	0.01	0.03	<u>0.91</u>	0.08	-	<u>0.98</u>	<u>0.97</u>	-	-	<u>1.93</u>	<u>0.98</u>	0.08	<u>2.86</u>	8
Glutamic acid	<u>0.98</u>	-	-	-	-	-	-	-	<u>3.03</u>	0.22	0.13	<u>3.00</u>	-	-	<u>1.00</u>	<u>1.96</u>	<u>1.98</u>	<u>2.00</u>	<u>1.04</u>	<u>1.99</u>	<u>1.16</u>	14
Glycine	0.01	-	-	0.03	-	-	-	-	<u>0.95</u>	0.06	0.17	<u>1.14</u>	0.04	0.06	-	<u>2.04</u>	0.02	<u>2.10</u>	0.20	0.09	<u>3.04</u>	8
Alanine	<u>1.01</u>	-	0.06	<u>1.01</u>	-	-	-	-	<u>2.99</u>	<u>0.98</u>	<u>1.94</u>	<u>3.05</u>	-	<u>2.00</u>	<u>1.03</u>	-	<u>1.02</u>	<u>2.02</u>	<u>1.09</u>	0.07	<u>2.85</u>	14
Valine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<u>2.11</u>	-	-	-	2
Leucine	<u>2.01</u>	-	-	-	<u>0.96</u>	-	-	-	<u>1.98</u>	<u>1.00</u>	0.06	<u>2.05</u>	<u>1.02</u>	-	<u>1.01</u>	<u>1.00</u>	-	<u>1.02</u>	<u>0.98</u>	<u>1.01</u>	0.12	10
Isoleucine*	-	-	<u>1.95</u>	-	-	-	-	-	-	-	<u>1.02</u>	0.06	-	<u>0.97</u>	-	-	-	<u>1.98</u>	0.04	-	0.04	5
Serine'	0.03	-	0.06	0.08	0.12	0.04	-	-	<u>0.98</u>	0.13	0.21	<u>0.98</u>	0.05	0.10	<u>0.92</u>	-	-	0.14	<u>0.96</u>	<u>0.97</u>	0.36	3
Threonine'	-	-	-	<u>1.01</u>	-	-	-	-	<u>1.00</u>	-	0.04	<u>0.98</u>	-	-	-	-	<u>0.98</u>	-	-	<u>0.98</u>	0.03	4
Methionine*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<u>0.89</u>	-	-	-	<u>0.96</u>	2
Proline	-	-	<u>1.03</u>	-	-	-	-	-	<u>1.04</u>	<u>0.96</u>	-	<u>1.13</u>	-	-	-	-	-	-	-	<u>1.03</u>	<u>0.99</u>	4
Phenylalanine	<u>1.03</u>	<u>1.00</u>	-	-	<u>0.98</u>	<u>0.95</u>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<u>0.95</u>	5
Tyrosine*	-	-	-	-	-	-	-	<u>0.88</u>	-	-	-	-	-	-	-	<u>0.96</u>	-	-	-	-	-	2
Histidine	-	-	-	-	-	-	-	-	<u>2.97</u>	-	0.10	<u>2.94</u>	<u>1.00</u>	-	-	-	-	<u>0.85</u>	-	<u>1.00</u>	<u>1.11</u>	7
Lysine	0.02	-	<u>1.02</u>	<u>0.98</u>	<u>1.07</u>	<u>1.05</u>	<u>3.00</u>	<u>1.00</u>	<u>2.07</u>	<u>1.06</u>	<u>2.03</u>	<u>2.92</u>	<u>0.98</u>	<u>1.03</u>	<u>0.99</u>	-	<u>1.00</u>	0.06	<u>1.90</u>	<u>1.03</u>	<u>1.05</u>	16
Arginine	<u>0.96</u>	<u>1.01</u>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<u>0.94</u>	-	-	-	3
Total Number of Residues	6	3	4	3	3	2	3	2	17	(4)	(6)	(18)	3	5	6	6	6	15	(7)	8	15	107
Yield in %	50	42	15	13	42	82	33	45	35	7	20	10	49	62	33	50	72	9	10	56	17	

* ' Have the same significance as in Table 3.

— Signifies bold-faced type.

TABLE 6

Amino Acid Residues Identified in the Products of Tryptic
Hydrolysis of Heat-denatured Myoglobin

The values are based on the results presented in Tables 3 and 5. The amino acid composition is that found in Edmundson and Hirs (1962a).

Amino acid	I	II	III	IV	V
	Number of Residues in Soluble Peptides	Number of Residues in Parent Protein	II - I	Number of Residues in Insoluble Fraction, Extrapolated	IV - III
Aspartic acid	8	8	0	2	+2
Glutamic acid	14	19	5	6	+1
Glycine	8	11	3	4	+1
Alanine	14	17	3	5	+2
Valine	2	8	6	6	0
Leucine	10	18	8	7	-1
Isoleucine	5	9	4	4	0
Serine	3	6	3	3	0
Threonine	4	5	1	1	0
Methionine	2	2	0	0	0
Proline	4	4	0	0	0
Phenylalanine	5	6	1	1	0
Tyrosine	2	3	1	1	0
Histidine	7	12	5	4	-1
Lysine	16	19	3	3	0
Arginine	3	4	1	1	0
Tryptophan		2	2	2	0
Total	107	153	46	50	