STUDIES ON MARSUPIAL PROTEINS

IV.* AMINO ACID SEQUENCE OF MYOGLOBIN FROM THE RED KANGAROO, *MEGALEIA RUFA*

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Summary

Myoglobin isolated from skeletal muscle of *M. rufa* consists of a single component containing 153 amino acid residues. The complete amino acid sequence has been determined. Cleavage with cyanogen bromide gave four polypeptides which were further fragmented by digestion with trypsin or chymotrypsin. The amino acid sequences of the peptides obtained were determined by the "dansyl"–Edman procedure. The order of the cyanogen bromide fragments was readily deduced from terminal sequences. Digestion of maleylated myoglobin with trypsin and cleavage at histidine residues with *N*-bromosuccinimide gave some overlapping sequences. Amino acid sequences in myoglobins are more conservative than in the β-chains of haemoglobin previously studied (Air and Thompson 1969) but the red kangaroo myoglobin shows more variation in amino acid sequence than has been found in myoglobins from other species.

I. Introduction

Since the determination of the amino acid sequence of sperm whale myoglobin by Edmundson (1965), myoglobins from several other species have been examined. The amino acid sequences of horse myoglobin (Dautrevaux, Boulanger, Han, and Biserte 1969), seal myoglobin (Bradshaw and Gurd 1969), and porpoise myoglobin (Bradshaw and Gurd 1969) have been completed, while partial sequences for human myoglobin (Hill *et al.* 1969), dolphin myoglobin (Genov, Shopova, and Karadzova 1968), bovine myoglobin (Dautrevaux, Han, Chaila, and Biserte 1969), and humpback whale myoglobin (Edman and Begg 1967) have been published. Following completion of the amino acid sequences of the β-chain of haemoglobin from the grey kangaroo (Air and Thompson 1969) it was of interest to investigate kangaroo myoglobin. The red kangaroo, *Megaleia rufa*, was used for this work. The β-chain of the haemoglobin of this species shows only one change from the sequence determined for the grey kangaroo (Air and Thompson, unpublished data). Myoglobin was prepared from skeletal muscle by an adaptation of the method of Hapner *et al.* (1968). Amino acid analysis of the pure material indicated three methionine residues, and after treatment

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with cyanogen bromide four fragments were isolated by gel filtration and peptide mapping. The *N*-terminal fragment (55 residues) was further degraded by trypsin, whereas chymotrypsin proved more satisfactory for the largest fragment (76 residues). The two small fragments each contained 11 amino acid residues. The arrangement of the cyanogen bromide fragments was readily deduced and the amino acid sequences of the fragments, or peptides derived from them, were determined by the "dansyl"–Edman procedure. Several overlapping peptides were obtained, but kangaroo myoglobin is so similar to myoglobins of other species that the order of peptides in the cyanogen bromide fragments could be deduced from homology with these myoglobins.

II. Materials and Methods

(*a*) *Preparation of Red Kangaroo Myoglobin*

Myoglobin was prepared by the method of Hapner *et al.* (1968). Each 500 g of kangaroo meat was partially thawed, cut up, and homogenized in 375 ml of 70% saturated ammonium sulphate in 0·1M sodium phosphate buffer containing 1 mM EDTA, pH 6·5. The homogenate was centrifuged at 11,700 *g* for 1 hr at 4°C, the supernatant filtered through Whatman No. 1 paper, and 10 mg $K_3Fe(CN)_6$ added per 100 ml filtrate. After stirring at 4°C for 15 min the oxidized filtrate was dialysed against water and concentrated by pressure dialysis (Berggard 1961). The concentrated material was dialysed against phosphate buffer of ionic strength 0·1, pH 6·4, containing a small amount of $K_3Fe(CN)_6$, centrifuged, loaded on a 3·5 by 50 cm column of SE-Sephadex (C50), and eluted with the same buffer. Fractions were read at 280 and 503 nm, and the myoglobin-containing fractions concentrated by pressure dialysis. The column was regenerated by removing the top portion of adsorbent, washing this with 0·1N NaOH, adding back to the column, and passing through more 0·1N NaOH before re-equilibrating with the pH 6·4 phosphate buffer.

Apomyoglobin was prepared by acid–acetone precipitation at −20°C (Anson and Mirsky 1930) using 1·5 ml 10N HCl per 100 ml acetone. The apomyoglobin was washed with cold acetone, dialysed against water, freeze-dried, and stored at −20°C.

For amino acid analysis, apomyoglobin was further purified by chromatography on a 1·8 by 12 cm column of CM-cellulose (Bio-Rad Laboratories) in 8M urea buffers (Clegg, Naughton, and Weatherall 1965) as used for separating globin chains of kangaroo haemoglobin (Thompson, Hosken, and Air 1969). The linear gradient used was 250 ml each of 0·01M Na^+ phosphate and 0·07M Na^+ phosphate in 8M urea containing disodium EDTA (0·001M) and mercaptoethanol (0·05M). The column was equilibrated at the lower salt concentration. The fractions obtained were dialysed against water and freeze-dried. Starch-gel electrophoresis of myoglobin and apomyoglobin fractions at pH 8·6 and 1·9 was as previously described (Thompson, Hosken, and Air 1969).

(*b*) *Cyanogen Bromide Cleavage*

Reaction with cyanogen bromide (Gross and Witkop 1962) was carried out as previously described (Thompson, Hosken, and Air 1969). The freeze-dried product was dissolved in cold 90% formic acid, diluted with water to 50% formic acid, and separated on a 2·8 by 140 cm column of Sephadex G50 in 50% formic acid run at 4°C. Tubes were read at 280 nm and the pooled fractions concentrated by rotary evaporation then freeze-dried.

(*c*) *Enzyme Digestions and Purification of Peptides*

Digestion of cyanogen bromide fragments with trypsin and chymotrypsin was as previously described (Thompson, Hosken, and Air 1969; Air and Thompson 1969). On completion of digestion, the mixture was loaded directly on a 1 by 120 cm column of Sephadex G25 in 0·01M NH_3. Tubes were read at 280 and 230 nm and appropriate fractions pooled. Individual peptides were isolated by a combination of paper ionophoresis at pH 6·4 and 1·75 and chromatography in n-butanol–acetic acid–water–pyridine (15 : 3 : 12 : 10 v/v) as used previously. Guide strips for each dimension were stained with ninhydrin in ethanol followed by chlorination and reaction with

tolidine–KI (Reindel and Hoppe 1954). Peptides were recovered by cutting the paper into 2 by 5 mm strips, compressing in a 2-ml hypodermic syringe, eluting with 3 ml pyridine–acetate buffer, pH 6·4, followed by 3 ml water, and freeze-dried.

(*d*) *Amino Acid Sequence Determination*

Amino acid sequences were determined by the dansyl–Edman method (Gray 1967) previously described (Air and Thompson 1969) except that after coupling with phenylisothiocyanate the reaction mixture was extracted three times with 1·5 ml redistilled benzene. The phenylthiohydantoin(PTH)-amino acid derived from the thiazolinone in the butyl acetate extract could then be examined by the method of Jeppsson and Sjöquist (1967) to identify asparagine and glutamine residues when necessary. However, in most cases the ionophoretic mobility at pH 6·4 was used, with the net charge calculated by the method of Offord (1966). Dansyl(DNS)-alanine was separated from dansylamide by running the polyamide sheet again in the second dimension in a third solvent, ethyl acetate–methanol–acetic acid (10 : 1 : 1 v/v) (Crowshaw, Jessup, and Ramwell 1967). DNS-homoserine was found to run just behind DNS-threonine in the benzene–acetic acid solvent. The dansyl derivatives of serine, threonine, and homoserine were well separated on rerunning the chromatogram in benzene–acetic acid. As previously reported (Air and Thompson 1969), the DNS-histidine derivative as mapped by Woods and Wang (1967) was not obtained. Magnusson (private communication) has found that mono-DNS-histidine runs near arginine, separating from it in the third solvent, but in our hands the presence of ϵ-DNS-lysine in this region coupled with streaking of the mono-DNS-histidine in the third solvent made the identification doubtful. However, definite results for all other amino acids in each peptide confirmed the position of the histidine residues.

(*e*) *Reaction with Maleic Anhydride*

Maleylation of myoglobin was by the method of Butler *et al.* (1969), with a small quantity of sodium tetraborate added to help buffer the reaction mixture during addition of the resublimed maleic anhydride. After dialysing and freeze-drying, the maleylated myoglobin was digested with TPCK-trypsin at an enzyme–substrate ratio of 5 : 100 (w/w) for 8 hr at 37°C. The digest was loaded directly on a 1 by 120 cm column of Sephadex G50 in 0·01N NH_3 and the major fractions bulked. The maleyl groups were removed by incubation in 0·2M acetic acid, pH 2·75, for 6 hr at 60°C.

(*f*) *Cleavage with* N-*Bromosuccinimide*

The absence of tyrosine and tryptophan from the CNBr2 fragment suggested that *N*-bromosuccinimide (NBS) cleavage at histidine residues might provide overlapping sequences from this fragment. The reaction was carried out as described by Ramachandran and Witkop (1967), using 18 mg CNBr2 in 2 ml pyridine–acetate buffer. After addition of the recommended amount of NBS (3 moles per mole histidine in pyridine–acetate buffer) the starch–KI test was still negative, so a small amount of solid NBS was added. After 15 min the excess NBS was destroyed by addition of solid imidazole. The tube was sealed and heated at 95°C for 1 hr. The product was recovered by freeze-drying, taken up in 50% pyridine, and loaded on a 1 by 120 cm column of Sephadex G50 eluted with 0·01N NH_3.

III. Results

As in previous papers of this series the CNBr fragments are numbered from the *N*-terminus (CNBr1 to CNBr4). Tryptic and chymotryptic peptides from each CNBr fragment are then numbered from the *N*-terminus of that fragment but using "C" instead of "CNBr" (eg. C1Tp1, C1Ch1). Residues identified as dansyl derivatives are set in *italic* fount.

(*a*) *Purification of Kangaroo Myoglobin*

In contrast to the situation in porpoise and seal muscle (Hapner *et al.* 1968), the 70% ammonium sulphate extract of kangaroo muscle contains large quantities of protein in addition to myoglobin. The separation of this mixture on SE-Sephadex is

shown in Figure 1. Several non-haem proteins are not bound by the SE-Sephadex, and the myoglobin is eluted after these. Starch-gel electrophoresis patterns of this

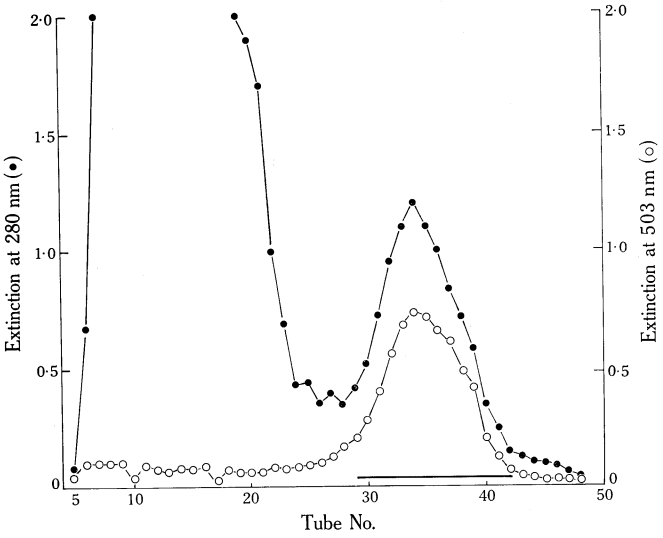


Fig. 1.—Purification of kangaroo metmyoglobin on a column of SE-Sephadex (3·5 by 50 cm). The column was equilibrated and run in a Na^+ phosphate buffer, pH 6·4, ionic strength 0·1. The material loaded was the extract from 70% saturated ammonium sulphate treatment of muscle after oxidation with $K_3Fe(CN)_6$, concentration, and dialysis against the chromatography buffer. Flow rate was 30 ml/hr, fraction size 10 ml. Fractions pooled are indicated by a bar.

myoglobin preparation at pH 8·6 and 1·9 are shown in Figure 2. In some cases two myoglobin bands were obtained with different absorption spectra, indicating incomplete oxidation. Addition of $K_3Fe(CN)_6$ to the first lot of buffer when dialysing the preparation prior to chromatography resulted in a single myoglobin peak as shown in

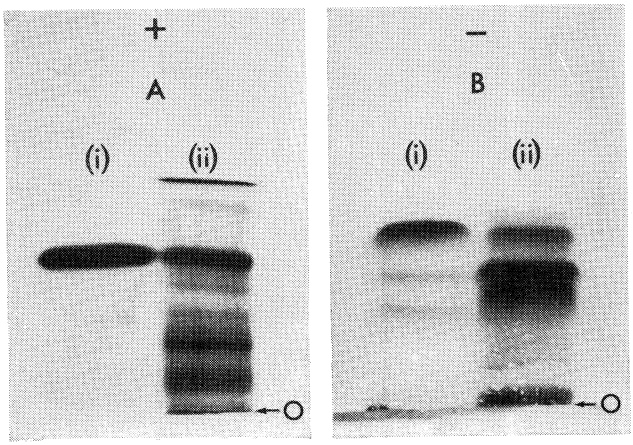


Fig. 2.—Starch-gel electrophoresis at pH 8·6 (*A*, migration towards anode) and at pH 1·9 (*B*, migration towards cathode) of kangaroo myoglobin preparations. In each case (i) is the purified fraction obtained from the SE-Sephadex column, and (ii) is the pattern given by the 70% ammonium sulphate extract of muscle. O, origin.

Figure 1. A large amount of protein, including haemoglobin and cytochrome *c*, remains bound to the top of the column under these conditions. This could be effectively eluted only by removing this section of adsorbant and washing it separately with 0·1N NaOH. Attempts to regenerate the column *in situ* with concentrated salt, 0·5M Na_2HPO_4, 8M urea, or 0·1N NaOH resulted in complete blockage of the column.

Chromatography of apomyoglobin from the SE-Sephadex fractionation on CM-cellulose in 8M urea buffers is shown in Figure 3. The amino acid analyses of the

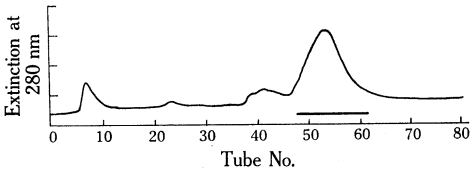


Fig. 3.—Further purification of kangaroo myoglobin. Continuous recording of the optical density at 280 nm of the effluent from a 1·8 by 12 cm column of carboxymethyl cellulose. Initial buffer was 8M urea–0·01M Na^+ phosphate–0·001M EDTA–0·05M mercaptoethanol, pH 6·7. Elution was with a linear gradient of 250 ml each of starting buffer and a limit buffer of 8M urea–0·07M Na^+ phosphate–0·001M EDTA–0·05M mercaptoethanol. 100 mg of apomyoglobin from the SE-Sephadex column was loaded. The flow rate was 96 ml/hr, fraction size 8 ml. The bar indicates the fractions bulked as myoglobin for amino acid analysis. Samples of the other peaks were also analysed but were not myoglobins.

TABLE 1

AMINO ACID COMPOSITION OF KANGAROO MYOGLOBIN

Values given are moles per mole of myoglobin, assuming a total of 153 residues. Hydrolysates were prepared in redistilled 6N HCl at 110°C under vacuum

Amino Acid	24-hr Hydrolysate	48-hr Hydrolysate	72-hr Hydrolysate	Average*	Sequence†
Lysine	20·0	20·0	22·4	21	21
Histidine	9·7	9·9	10·5	10	10
Arginine	2·1	2·1	2·2	2	2
Aspartic acid	12·6	12·6	12·8	13	12
Threonine	4·3	3·7	2·6	(5)‡	5
Serine	4·2	3·0	1·7	(6)‡	6
Glutamic acid	21·3	21·6	21·4	21	20
Proline	3·0	3·2	3·3	3	3
Glycine	14·1	14·1	15·7	14	14
Alanine	14·8	14·9	15·0	15	14
½ Cystine	0	0	0	0	0
Valine	7·5	7·5	7·6	8	6
Methionine	2·9	2·8	2·6	3	3
Isoleucine	8·6	8·9	8·9	9	9
Leucine	15·9	16·0	16·4	16	16
Tyrosine	1·2	1·1	—	1	1
Phenylalanine	8·6	8·6	8·5	9	9
Tryptophan					2
Total					153

* Average or extrapolated value.

† Total from amino acid sequence analysis.

‡ Values in parentheses corrected for decomposition.

major peak after hydrolysis under vacuum in 6N HCl at 110°C for 24, 48, and 72 hr are given in Table 1. Amino acid analyses of the minor peaks (Fig. 3) indicated they

were not myoglobins. For sequence work the preparations from SE-Sephadex chromatography were quite satisfactory. The dansyl–Edman procedure on such a preparation gave the single *N*-terminal sequence

Gly-Leu-Ser-Asx-Gly-

(*b*) *Cyanogen Bromide Cleavage of Kangaroo Myoglobin*

The amino acid analyses of kangaroo myoglobin (Table 1) show three methionine residues, hence cyanogen bromide cleavage would be expected to yield four fragments. The results of gel filtration of 500 mg apomyoglobin after treatment with 500 mg CNBr in 20 ml 70% formic acid on a Sephadex G50 column (2·8 by 140 cm) in 50% formic acid are shown in Figure 4. Five fractions were bulked as indicated. Fractions

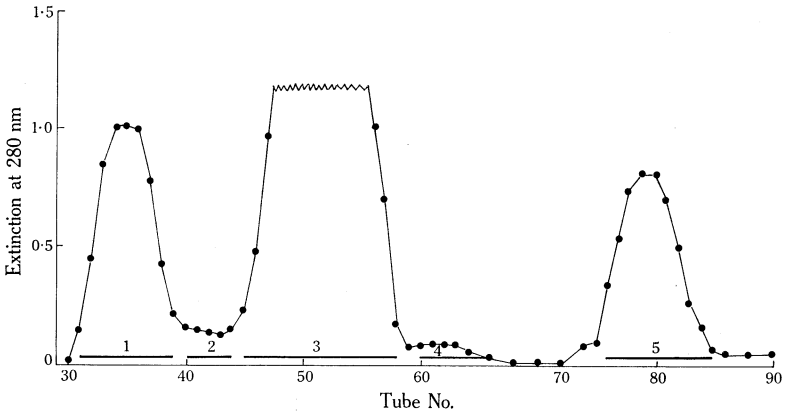


Fig. 4.—Gel filtration of a cyanogen bromide digest of kangaroo myoglobin (500 mg) on a column of Sephadex G50 (2·8 by 140 cm) run in 50% formic acid at 4°C. Flow rate 25 ml/hr, fraction size 6·3 ml. Fractions bulked are indicated by bars.

2 and 3 were taken up in 90% formic acid, diluted to 50% formic acid, and loaded on a 1 by 120 cm Sephadex G100 column equilibrated and run in 10% formic acid. The single major peak in each was freeze-dried. Amino acid analyses were carried out for each fraction after hydrolysis at 110°C for 24 hr. Fraction 1 appeared to be a mixture of aggregated material and no clear *N*-terminal sequence was obtained. Fraction 2, after re-running on Sephadex G100, had an amino acid analysis corresponding to about 76 residues. Fraction 3 after the G100 run analysed as 55 residues, including some tryptophan.

The amino acid analysis of fraction 5 showed no homoserine, hence this must be the *C*-terminal fragment. It consisted of 11 amino acid residues and was degraded completely by the dansyl–Edman method to give the sequence

Ala-Ala-Lys-Tyr-Lys-Glx-Phe-Gly-Phe-Glx-Gly

Fraction 4 contained a methionine residue and no homoserine. It appeared to be a low yield of a 22 residue fragment incorporating the *C*-terminal peptide (i.e. CNBr3+CNBr4). The Edman degradation of fraction 4 was more clear cut after

purification by ionophoresis at pH 6·4 and then at pH 1·75. DNS-lysine was obtained as the *N*-terminal residue.

The *N*-terminal sequence of fraction 3 was

Gly-Leu-Ser-Asx-Gly-Glu- -Gln-Leu-Val-Leu

The glutamic acid and glutamine residues were identified as the PTH-derivatives.

TABLE 2

AMINO ACID COMPOSITIONS OF CNBr FRAGMENTS OF KANGAROO MYOGLOBIN

The four fragments were purified as described in the text, then hydrolysed under vacuum with 6N HCl at 110°C for 24 hr. Values are given as mole per mole of each fragment, uncorrected for losses on hydrolysis or incomplete hydrolysis. Also shown are the amino acid compositions obtained from the amino acid sequence. H, hydrolysate; S, sequence

Amino Acid	CNBr1		CNBr2		CNBr3		CNBr4	
	H	S	H	S	H	S	H	S
Lysine	6·8	7	9·9	10	1·9	2	2·0	2
Histidine	3·4	3	5·7	6	1·0	1		
Arginine	0·9	1	0	0	0·9	1		
Aspartic acid	5·5	6	5·1	5	1·2	1		
Threonine	1·7	2	2·5	3				
Serine	1·6	2	2·8	4	0·8	0		
Glutamic acid*	8·3	8	9·3	9	1·9	1	2·0	2
Proline	1·1	1	2·0	2				
Glycine	6·4	7	4·9	5	0·6	0	1·9	2
Alanine	1·9†	0	10·3	11	1·2	1	2·0	2
½ Cystine	0	0	0	0				
Valine	3·1	3	2·9	3				
Isoleucine	2·8†	2	6·1	7				
Leucine	6·7	7	7·0	7	2·0	2		
Tyrosine	0	0	0	0			0·9	1
Phenylalanine	3·1	3	3·1	3	1·1	1	1·8	2
Tryptophan	+	2	0	0				
Homoserine lactone	0·5	1	‡	1	‡	1	0	0
Total		55		76		11		11

* Values for glutamic acid are high due to the elution of homoserine in the same peak.

† The CNBr1 preparation appeared to be contaminated with some CNBr2.

‡ Detected after the ammonia peak on the short column of the amino acid analyser, but could not be quantitated.

No dansyl derivative was obtained at step 7. This sequence is the same as the *N*-terminal sequence of whole kangaroo myoglobin, hence this fragment is CNBr1, with the first methionine residue at position 55 as in other myoglobins.

Fraction 2 gave the *N*-terminal sequence

Lys-Ala-Ser-Glx-Asx-Leu

and must represent CNBr2.

The three methionine residues in kangaroo myoglobin are therefore located at residues 55, 131, and 142.

No CNBr3 was obtained from the fractionation on Sephadex G50. The only aromatic residue in this 11-residue peptide is a single phenylalanine, hence the absorption at 280 nm is probably too low to detect, although it may be in the small peak just before fraction 5. Optical densities could not be read at a shorter wavelength in the 50% formic acid and a more general method such as alkaline hydrolysis followed by ninhydrin was not applied. Fractionation of the soluble part of a cyanogen bromide digest by ionophoresis at pH 6·4 gave a major arginine-containing peptide as detected by the method of Yamada and Itano (1966). Amino acid analysis of this peptide showed it to be the fragment CNBr3. The amino acid composition of each cyanogen bromide fragment is given in Table 2.

(c) *Tryptic Digestion of Maleylated Myoglobin*

Kangaroo myoglobin contains two arginine residues, one within residues 1–55, the other within residues 132–142, hence tryptic digestion of maleylated myoglobin would be expected to yield three fragments.

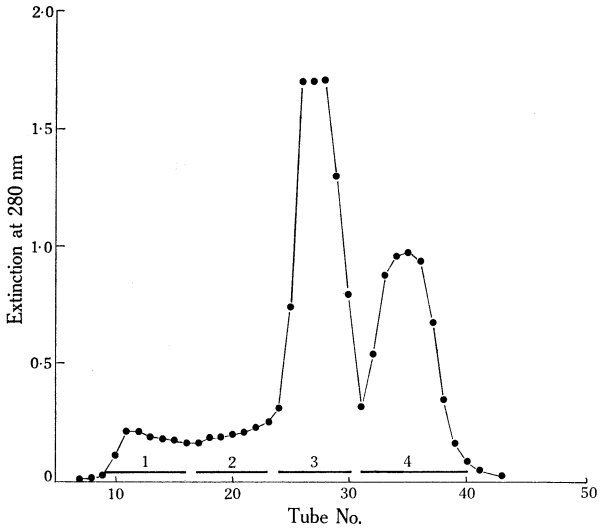


Fig. 5.—Gel filtration of a tryptic digest of maleylated myoglobin on a column of Sephadex G50 (1 by 120 cm) in 0·01N NH_3. Flow rate11 ml/hr, fraction size 2·7 ml. Fractions bulked are indicated by bars.

The results of gel filtration of the trypsin digest of maleylated myoglobin on Sephadex G50 in 0·01N NH_3 are shown in Figure 5. Four fractions were bulked as indicated. Acid hydrolysis of an aliquot from each fraction indicated that fractions 1 and 2 were similar, and mainly the large middle maleylated fragment. Fraction 3 appeared to be a mixture. Fraction 4 contained the phenol from the digestion mixture but no peptide material.

Separation of fraction 3 on a 1 by 15 cm column of DEAE-cellulose (Bio-Rad Laboratories) is shown in Figure 6. The column was equilibrated in 0·005M NH_4HCO_3 and run with a linear gradient of 100 ml each of 0·005M NH_4HCO_3 and 0·5M NH_4HCO_3, pH 8·5, followed by 50 ml of 0·5M NH_4HCO_3, at 48 ml/hr. The optical density was

recorded at 254 nm. Fractions were bulked as indicated, demaleylated, and samples taken for hydrolysis and amino acid analysis. It was clear that the Sephadex G50

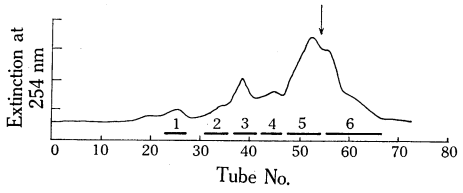


Fig. 6.—Fractionation of tryptic peptides from maleylated kangaroo myoglobin. Continuous recording of optical density at 254 nm of the effluent from a 1 by 15 cm column of DEAE-cellulose loaded with fraction 3 from Figure 5. The initial buffer was 0·005M NH_4HCO_3, pH 8·5. A linear gradient was used consisting of 100 ml each of starting buffer and 0·5M NH_4HCO_3, pH 8·5, followed by 50 ml of 0·5M NH_4HCO_3. Flow rate 48 ml/hr, fraction size 4 ml. Fractions bulked are indicated by bars. Arrow indicates end of gradient.

fraction 3 (Fig. 5) was a fairly complex mixture, and that many chymotryptic hydrolyses had occurred during the prolonged tryptic digestion, although under normal milder conditions chymotryptic activity in this sample of TPCK-trypsin was not detectable.

TABLE 3

AMINO ACID COMPOSITIONS OF SOME PEPTIDES OBTAINED FROM TRYPTIC DIGESTION OF MALEYLATED MYOGLOBIN AFTER FRACTIONATION ON SEPHADEX G50 AND DEAE-CELLULOSE

Amino Acid	DEAE 2A*	DEAE 5A*	DEAE 3
Lysine	1·9 (2)	2·0 (2)	2·0
Histidine		0·9 (1)	1·1
Arginine	0·9 (1)		1·4
Aspartic acid	0·3	1·1 (1)	3·6
Threonine			0·9
Serine	0·3		1·4
Glutamic acid	1·1 (1)	2·1 (2)	3·7
Proline			
Glycine	0·4	1·9 (2)	4·6
Alanine	1·2 (1)	2·0 (2)	2·2
Valine			3·0
Methionine		0·6 (1)	
Isoleucine			1·9
Leucine	1·9 (2)		1·9
Tyrosine		0·9 (1)	
Phenylalanine	0·9 (1)	1·8 (2)	1·0
Tryptophan			

* Further purified by ionophoresis at pH 6·4 after demaleylation.

The amino acid composition of some peptides obtained from the DEAE column are given in Table 3. The amino acid sequences determined were

DEAE 2A: *Lys-Lys-Ala-Leu-Glu-Leu-Phe-Arg*

The ionophoretic mobility at pH 6·4 indicated glutamic acid at residue 5.

DEAE 5A: His-*Asp-Met-Ala-Ala-Lys-*

This is the *C*-terminal maleyl tryptic peptide, which overlaps CNBr3 and CNBr4. The ionophoretic mobility at pH 6·4 is the same as that of CNBr4, hence the second residue is aspartic acid.

DEAE 3: This appeared to be the *N*-terminal maleyl tryptic peptide, residues 1–31, although insolubility prevented further purification.

(*d*) *Isolation of Tryptic Peptides from CNBr1*

The fractionation of a tryptic digest of 50 mg CNBr1 on a 1 by 120 cm column of Sephadex G25 in 0·01N NH_3 is shown in Figure 7. The four fractions indicated were freeze-dried, taken up in pyridine–acetate buffer, pH 6·4, centrifuged, and the soluble peptides separated by ionophoresis at pH 6·4. In some cases ionophoresis at pH 1·75 or chromatography in n-butanol–acetic acid–water–pyridine followed by ionophoresis at pH 1·75 was necessary for purification of peptides. Peptide C1Tp1 is insoluble, but was obtained sufficiently pure as the insoluble part of fraction 2 from the G25 column. The peptides in each fraction are listed in the legend to Figure 7. The amino acid compositions of the major peptides isolated are shown in Table 4, together with the electrophoretic mobility at pH 6·4 with respect to lysine, R_{Lys}.

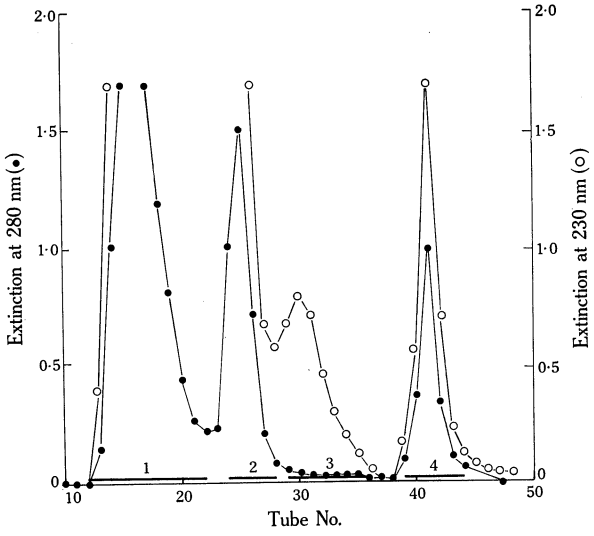


Fig. 7.—Gel filtration of a tryptic digest of CNBr1 from kangaroo myoglobin on a column of Sephadex G25 (1 by 120 cm) in 0·01N NH_3. Flow rate 6 ml/hr, fraction size 2 ml. Fractions pooled are indicated by bars. The peptides present in each fraction were as follows (fraction 4 was mainly phenol):

Fraction 1	Fraction 2	Fraction 3
C1Tp1	C1Tp1	C1Tp4
C1Tp2	C1Tp2	C1Tp6
C1Tp2+3	C1Tp3	C1Tp7
C1Tp5	C1Tp5	C1Tp6+7
C1Tp9	C1Tp9	C1Tp8

(*e*) *Amino Acid Sequences of Tryptic Peptides from CNBr1*

(i) *C1Tp1*

The amino acid sequence of the first 11 residues of this peptide was obtained from Edman degradation of whole CNBr1, except for a gap at residue 7. The sequence was continued using peptides isolated from a chymotryptic digest of C1Tp1. The first was a heptapeptide with an R_{Lys} value of −0·59, indicating two acidic groups, and was shown by Ehlich reagent to contain tryptophan. The amino acid composition was Asp 1·1, Ser 1·5, Glu 1·1, Gly 2·0, Leu 0·8. Hence the *N*-terminal part of C1Tp1 is Gly-Leu-Ser-Asp-Gly-Glu-Trp. Another chymotryptic peptide contained only glycine and lysine, and was placed at the *C*-terminus of C1Tp1. The sequence was

completed by isolation of a neutral peptide from chymotryptic digestion of whole CNBr1 with the amino acid sequence *Asn-Ile*-Trp. Since only C1Tp1 contains tryptophan in kangaroo myoglobin, the complete sequence is

Gly-Leu-Ser-Asp-Gly-Glu-Trp-*Gln-Leu-Val-Leu-Asn-Ile-Trp*-Gly-Lys

TABLE 4

AMINO ACID COMPOSITIONS OF CNBr1 TRYPTIC PEPTIDES FROM KANGAROO MYOGLOBIN

Peptides were purified by gel filtration, paper chromatography in butanol–acetic acid–water–pyridine, and paper ionophoresis at pH 6·4 and 1·75. Hydrolysates were prepared with 6N HCl at 110°C for 24 hr. Values are not corrected for losses during hydrolysis and are given as moles per mole of protein with values from sequence data in parentheses

Amino Acid	C1Tp1*	C1Tp2	C1Tp3	C1Tp4	C1Tp5	C1Tp6+7	C1Tp8†	C1Tp9	Total
Lys	1·1(1)	0·9(1)		1·0(1)	1·0(1)	1·9(2)	1·5(1)		7
His		0·9(1)			0·8(1)		0·9(1)		3
Arg			0·8(1)						1
Asp	2·0(2)	1·2(1)	0·7(1)			1·0(1)		1·0(1)	6
Thr		1·0(1)			1·0(1)				2
Ser	1·0(1)				0·3			1·1(1)	2
Glu	2·1(2)	1·6(2)			2·2(2)			2·4(2)§	8
Pro					1·0(1)				1
Gly	2·9(3)	3·0(3)			1·0(1)			0·3	7
Ala									0
Val	1·0(1)	1·0(1)	1·2(1)						3
Ile	0·9(1)		1·2(1)						2
Leu	2·9(3)		1·1(1)	0·9(1)	1·0(1)		0·8(1)		7
Tyr									0
Phe				1·1(1)		1·8(2)	0·4		3
Hser lactone								+(1)	1
Trp‡	1·1(2)								2
Total	16	10	5	3	8	5	3	5	55
R_{Lys}	Insol.	−0·25	0	+0·53	+0·04	+0·36	+0·65	−1·04	

* Insoluble at pH 6·4. Purified by washing the precipitate obtained when Sephadex G25 fraction 2 (Fig. 7) was suspended in pH 6·4 buffer.

† Contaminated with C1Tp7.

‡ Partially destroyed by acid hydrolysis.

§ Glutamic acid+homoserine.

(ii) *C1Tp2*

This peptide is acidic at pH 6·4, therefore must contain two glutamic acid residues and one aspartic acid, since one residue each of lysine and histidine is present. The amino acid sequence by the dansyl–Edman method is

Val-Glu-Thr-Asp-Glu-Gly-Gly-His-*Gly-Lys*

Very little dansyl derivative was obtained from residue 5 onwards, indicating blocking of the Edman degradation. This was probably due to formation of a β-aspartyl link at

residue 4 (Naughton *et al.* 1960; Smyth, Stein, and Moore, 1963). It is of interest that Weeds and Hartley (1968) reported formation of the α,β-imide from the sequence Thr-Asn-Glu- in myosin.

This peptide was obtained only in low yield, probably due to slow tryptic hydrolysis of the Lys-Asp bond, resulting in an insoluble double peptide.

(iii) *C1Tp3*

This peptide is neutral at pH 6·4, hence contains one acidic residue. The amino acid sequence is

Asp-Val-Leu-Ile-Arg

(iv) *C1Tp4*

The amino acid sequence of C1Tp4 is

Leu-Phe-Lys

(v) *C1Tp5*

The dansyl–Edman procedure gave the sequence

Gly-His-*Pro-Glu-Thr-Leu-Glu-Lys*

The peptide is neutral at pH 6·4, indicating two glutamic acid residues.

(vi) *C1Tp6+7*

This peptide has a mobility at pH 6·4 corresponding to one net basic group. The amino acid sequence is

Phe-Asp-Lys-Phe-Lys

The presence of an Asp-Lys sequence inhibits tryptic hydrolysis, resulting in the double peptide as the major product.

(vii) *C1Tp8*

Although the amino acid analysis of this peptide shows some contamination, the sequence was clearly obtained as

His-*Leu-Lys*

(viii) *C1Tp9*

The amino acid sequence of C1Tp9 is

Ser-Glu-Asp-Glu-Hser

The evidence for three acidic residues in this peptide is from the R_{Lys} value and was supported by isolation of a neutral chymotryptic peptide with amino acid composition corresponding to the nine *C*-terminal residues of CNBr1 which included three basic amino acid residues.

(*f*) *Ordering of the CNBr1 Tryptic Peptides*

The amino acid sequence of peptide C1Tp1 corresponds to the *N*-terminal sequence of whole myoglobin, hence this is the *N*-terminal peptide. Peptide C1Tp9 contains homoserine therefore is *C*-terminal in the CNBr fragment.

A fragment isolated from tryptic digestion of maleylated myoglobin (DEAE 3, Table 3) had an amino acid composition corresponding to C1Tp1+C1Tp2+C1Tp3.

Since arginine must be *C*-terminal, the order of the first three tryptic peptides from CNBr1 is established.

The rest of the tryptic peptides were placed in order from peptides isolated from a chymotryptic digest of CNBr1 which are listed below:

(1) *Ile-Arg-Leu-Phe* (linking C1Tp3 to C1Tp4);
(2) (Lys,Gly,His,Pro,Glu,Thr,Leu,Gln,Lys)Phe;
(3) (Lys,Gly,His,Pro,Glu,Thr,Leu,Gln,Lys,Phe,Asp,Lys)Phe (linking C1Tp5-C1Tp6-C1Tp7);
(4) *Lys*(His,Leu,Lys,Ser,Glu,Asp,Glu)Hser (linking C1Tp8-C1Tp9).

(*g*) *Isolation of Chymotryptic Peptides from CNBr2*

Examination of the region corresponding to CNBr2 in myoglobins from other species (Dayhoff 1969) suggested that tryptic digestion of this fragment would give only very short or very long peptides. Hence chymotryptic digestion was used as the principal method of obtaining peptides suitable for sequence work.

The fractionation of a chymotryptic digest of 25 mg of CNBr2 on a 1 by 120 cm column of Sephadex G25 in 0·01N NH_3 is shown in Figure 8. Peptides were further

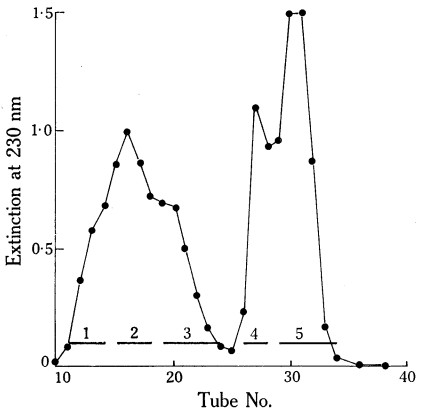


Fig. 8.—Gel filtration of a chymotryptic digest of CNBr2 from kangaroo myoglobin on a column of Sephadex G25 (1 by 120 cm) in 0·01N NH_3. Flow rate 12 ml/hr, fraction size 3 ml. Fractions pooled are indicated by bars. The peptides present in each fraction (fractions 4 and 5 were mainly phenol) were:

Fraction 1	Fraction 2	Fraction 3
C2Ch1	C2Ch2	C2Ch2
C2Ch9	C2Ch3	C2Ch5
C2Ch11	C2Ch4	C2Ch6
C2Ch1+2	C2Ch9	C2Ch7
	C2Ch10	C2Ch8
	C2Ch1+2	

purified by ionophoresis at pH 6·4 and 1·75 and chromatography in butanol–acetic acid–water–pyridine. Due to the small amount of material used for this digest (approximately 3 μmoles), conclusive amino acid analyses of peptides were difficult to obtain, mainly due to contamination with additional glycine and serine. However, if the peptide was completely degraded by the dansyl–Edman procedure to give a clear sequence, further purification on a larger scale was considered unnecessary. Only where difficulties occurred in the sequence determination was the preparation repeated to give an unambiguous amino acid analysis. The amino acid compositions of the major peptides isolated are given in Table 5.

(*h*) *Amino Acid Sequences of Chymotryptic Peptides from CNBr2*

(i) *C2Ch1*

The amino acid sequence of this peptide was

Lys-Ala-Ser-Glu-Asp-Leu

The peptide was acidic at pH 6·4, hence contains glutamic acid and aspartic acid.

TABLE 5

AMINO ACID COMPOSITION OF CNBr2 CHYMOTRYPTIC PEPTIDES FROM KANGAROO MYOGLOBIN

Peptides were purified by gel filtration, paper ionophoresis at pH 6·4 and 1·75, and paper chromatography in butanol–acetic acid–water–pyridine. Hydrolysates were prepared with 6N HCl for 24 hr at 110°C. Values are not corrected for losses during hydrolysis or incomplete hydrolysis. Values from amino acid sequence data are given in parentheses

Amino Acid	C2Ch1	C2Ch2	C2Ch3	C2Ch4	C2Ch5	C2Ch6	C2Ch7	C2Ch8	C2Ch9	C2Ch10	C2Ch11	Total
Lysine	0·9 (1)	1·5 (2)		2·9 (3)	1·0 (1)	1·0 (1)	1·0 (1)		0·5	1·0 (1)		10
Histidine		1·1 (1)		1·6 (2)		1·9 (2)				0·8 (1)		6
Arginine												0
Aspartic acid	1·1 (1)		1·0 (1)	0·4					1·1 (1)	0·8 (1)	1·0 (1)	5
Threonine		0·7 (1)	0·7 (1)			1·0 (1)						3
Serine*	1·9 (1)		0·5	0·6		1·3 (1)		0·4	1·0 (1)	1·0 (1)		4
Glutamic acid	1·1 (1)		0·3	2·0 (2)		1·1 (1)	1·0 (1)	1·1 (1)	2·0 (2)	0·5	1·4 (1)†	9
Proline					1·1 (1)		1·1 (1)					2
Glycine*	0·9	1·5 (1)	1·3 (1)	1·0 (1)				0·4	0·4	1·2 (1)	0·8 (1)	5
Alanine	1·1 (1)		1·5 (1)	1·3 (1)		2·0 (2)			1·2 (1)	1·1 (1)	3·8 (4)	11
Valine		0·6 (1)					1·1 (1)		0·8 (1)			3
Isoleucine		1·2 (1)	1·0 (1)				0·9 (1)		3·0 (4)			7
Leucine	0·7 (1)	1·1 (1)	1·7 (2)	1·1 (1)	1·0 (1)			1·0 (1)	0·5			7
Tyrosine												0
Phenylalanine							1·0 (1)	1·0 (1)		0·7 (1)		3
Homoserine lactone											+ (1)	1
Total	6	8	7	10	3	8	6	3	10	7	8	76
R_{Lys}	−0·26	+0·78	0	+0·65	+0·68	+0·90	+0·39	−0·49	Insol.	+0·38	−0·41	

* Serine and glycine values tend to be relatively high when very small amounts of material are used. † Contains homoserine.

(ii) *C2Ch2*

This peptide was particularly difficult to purify due to incomplete hydrolysis resulting in a significant amount of the double peptide C1Ch1+2 and contamination with other basic peptides from CNBr2. The amino acid sequence by the dansyl–Edman method was

Lys-*Lys*-His-*Gly-Ile-Thr-Val-Leu*

The first dansyl reaction resulted in several derivatives, but subsequent steps gave clear results. The *N*-terminal lysine residue was confirmed by a tryptic peptide from CNBr2 which included two lysine residues with the amino acid composition: lysine 2·1, aspartic acid 1·0, serine 1·1, glutamic acid 1·1, alanine 1·0, leucine 1·0.

(iii) *C2Ch3*

The dansyl–Edman procedure on this peptide gave the sequence

Thr-Ala-Leu-Gly-Asn-Ile-Leu

The peptide is neutral at pH 6·4, indicating asparagine at the fifth residue.

(iv) *C2Ch4*

The amino acid sequence of this peptide is

Lys-Lys-Lys-Gly-His-His-*Glu-Ala-Glu-Leu*

No clear-cut results were obtained at positions 5 and 6, corresponding to the two histidine residues. The ionophoretic mobility at pH 6·4 corresponds to three net positive charges, hence there are two glutamic acid residues present.

(v) *C2Ch5*

The amino acid sequence of this peptide is

Lys-Pro-Leu

(vi) *C2Ch6*

The amino acid sequence of this peptide is

Ala-Gln-Ser-His-*Ala-Thr-Lys*-His

The ionophoretic mobility at pH 6·4 was used to identify the glutamine residue.

(vii) *C2Ch7*

The amino acid sequence of this peptide, basic at pH 6·4, is

Lys-Ile-Pro-Val-Gln-Phe

(viii) *C2Ch8*

The dansyl–Edman method on this acidic peptide gave the sequence

Leu-Glu-Phe

(ix) *C2Ch9*

This was the only insoluble peptide in the chymotryptic digest of CNBr2, and was obtained in sufficient purity for sequence work by washing the precipitate in fraction 1

from the Sephadex G25 column and using this preparation for amino acid analysis and sequence work. The amino acid sequence is

Ile-Ser-Asp-Ala-Ile-Ile-Gln-Val-Ile-Gln

The aspartic acid and glutamine residues were identified as the PTH-derivatives. Although the result for the *C*-terminal glutamine residue was not absolutely clear, due to the low yield obtained by the tenth step, glutamine is a much more likely *C*-terminal residue from chymotryptic hydrolysis than glutamic acid (Heller and Smith 1966).

(x) *C2Ch10*

The amino acid sequence of the peptide is

Ser-Lys-His-*Ala-Gly-Asn-Phe*

The R_{Lys} value at pH 6·4 indicated two net positive charges, hence asparagine is at position 6.

(xi) *C1Ch11*

The dansyl–Edman procedure applied to this peptide gave the sequence

Gly-Ala-Asx-Ala-Glx-Ala-Ala-Hser

The ionophoretic mobility at pH 6·4 indicated one acidic residue. After three steps of the Edman degradation the remaining peptide was neutral. Hence the amino acid sequence of C2Ch11 is

Gly-Ala-Asp-Ala-Gln-Ala-Ala-Hser

(*i*) *Ordering of CNBr2 Chymotryptic Peptides*

The homology between kangaroo myoglobin and myoglobins of other species (Edmundson 1965; Bradshaw and Gurd 1969; Dautrevaux, Boulanger, Han, and Biserte 1969; Dautrevaux, Han, Chaila, and Biserte 1969; and Hill *et al.* 1969) is so marked that there is little doubt of the order of CNBr2 chymotryptic peptides. However, digestion of CNBr2 with trypsin and with NBS was used to obtain some overlapping sequences. The products were fractionated by gel filtration in 0·01N NH_3 and by peptide mapping by ionophoresis at pH 6·4 and chromatography in butanol–acetic acid–water–pyridine. The peptide maps were dipped in dilute ninhydrin (0·02% in ethanol) and peptides eluted with 6N HCl after removal of excess ninhydrin by washing in acetone (Clegg, Naughton, and Weatherall 1965). After hydrolysis and amino acid analysis, tryptic peptides isolated in sufficient yield for amino acid analysis were as follows:

C2Tpa—lysine 2·1, aspartic acid 1·0, serine 1·1, glutamic acid 1·1, alanine 1·0, leucine 1·0 (residues 56–62);

C2Tpb—lysine 2·7, aspartic acid 1·0, serine 1·0, glutamic acid 1·3, alanine 1·1, leucine 1·0 (residues 56–63);

C2Tpc—lysine 2·9, histidine 2·9, threonine 1·0, serine 1·0, glutamic acid 3·3, proline 1·2, glycine 1·0, alanine 2·8, leucine 2·0 (residues 79–96);

C2Tpd—lysine 1·9, histidine 2·5, threonine 0·9, serine 1·0, glutamic acid 3·2, proline 1·2, glycine 0·9, alanine 3·1, leucine 2·0 (residues 80–96);

C2Tpe—histidine 0·8, aspartic acid 2·1, glutamic acid+homoserine 1·8, glycine 2·0, alanine 5·0, phenylalanine 1·0, homoserine lactone+(residues 119–131);

C2Tpf—lysine.

Other tryptic peptides, representing residues 64–77 and 97–118, are insoluble at pH 6·4.

The only peptides obtained from NBS cleavage at histidine residues were:

(1) lysine 3·3, aspartic acid 1·1, serine 1·5, glutamic acid 1·3, glycine 1·0, alanine 1·4, leucine 0·9 (residues 56–64, plus glycine);

(2) aspartic acid 1·8, serine 0·5, glutamic acid+homoserine 1·7, glycine 2·4, alanine 5·0, phenylalanine 1·0 (residues 120–131).

The yield of these peptides was approximately 10% of the weight of CNBr2 used for the reaction. Some other peptides were present, but in yields too low for amino acid analysis. One of these appeared to be residues 83–93.

Combining these results the order of chymotryptic peptides is established to some extent. The amino acid sequence of C2Ch1 is the same as the *N*-terminal sequence of CNBr2, hence this peptide must be *N*-terminal. The tryptic peptide C2Tpb links it to either C2Ch2 or C2Ch4. Isolation of the double chymotryptic peptide C2Ch1+2 confirms the former order as correct. Tryptic peptide C2Tpc links chymotryptic peptides C2Ch4-C2Ch5-C2Ch6, while tryptic peptide C2Tpe and NBS peptide (2) link the chymotryptic peptides C2Ch10-C2Ch11. The presence of homoserine in C2Ch11 places this as the *C*-terminal peptide. Although overlapping sequences were not obtained to place peptides C2Ch3, C2Ch7, C2Ch8, and C2Ch9, the similarity between the order proposed and the amino acid sequences of myoglobins from other species suggests that this order is correct.

(*j*) *Amino Acid Sequence of CNBr3*

The amino acid sequence of this fragment was given by two peptides from tryptic digestion of maleylated myoglobin described above. One (DEAE 2A) had the amino acid sequence

Lys-Lys-Ala-Leu-Glu-Leu-Phe-Arg

The other peptide (DEAE 5A) was

His-*Asp-Met-Ala-Ala-Lys*(Tyr,Lys,Glu,Phe,Gly,Phe,Gln,Gly)

Hence the amino acid sequence of CNBr3, taking the amino acid composition into account, is

Lys-Lys-Ala-Leu-Glu-Leu-Phe-Arg-His-*Asp-Hser*

(*k*) *Amino Acid Sequence of CNBr4*

The electrophoretic mobility of CNBr4 at pH 6·4 indicated one net positive charge, hence one glutamic acid and one glutamine residue are present. Digestion with trypsin gave an acidic peptide of amino acid composition $Glu_2\ Gly_2\ Phe_2$ and sequence

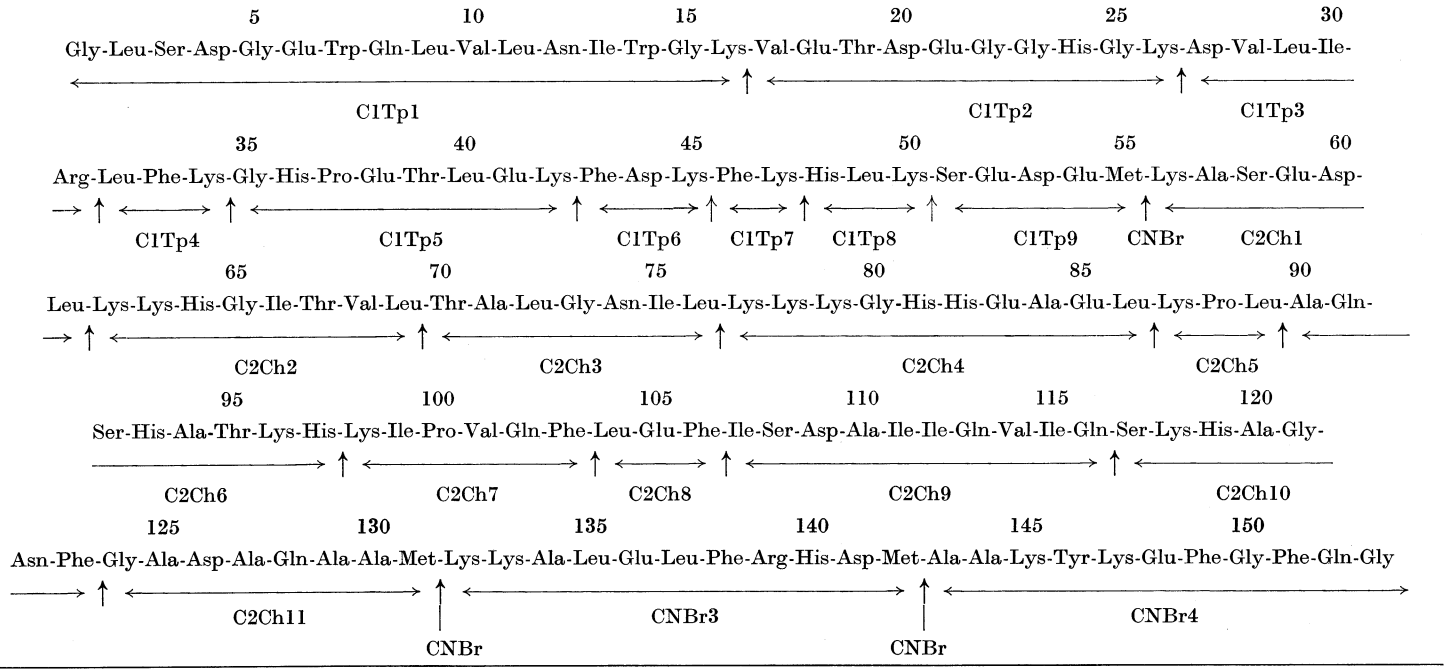
Glx-Phe-Gly-Phe-Glx-Gly

Digestion of this peptide with chymotrypsin gave an acidic peptide of amino acid composition Glu,Phe, and two neutral peptides, one of composition Gly,Phe, and the

TABLE 6

AMINO ACID SEQUENCE OF KANGAROO MYOGLOBIN

Residues are numbered from the *N*-terminal residue and the positions of cleavage by trypsin or chymotrypsin are indicated by vertical arrows
Positions of cleavage by CNBr are also indicated



other Glx,Gly. Hence the glutamine residue is second from the *C*-terminus of CNBr4, the complete sequence being

Ala-Ala-Lys-Tyr-Lys-Glu-Phe-Gly-Phe-Gln-Gly

The complete amino acid sequence of myoglobin from the red kangaroo is shown in Table 6. The bonds cleaved by trypsin or chymotrypsin giving rise to the major peptides used for sequence work are indicated.

IV. Discussion

The myoglobin isolated from several animal species has been found to be heterogeneous to varying degrees. Atassi (1964) separated sperm whale myoglobin into 12 components by chromatography on carboxymethylcellulose. No differences in amino acid composition could be detected, and Edmundson (1965) concluded that the minor components were due to partial loss of amide groups from glutamine residues. This appears also to be a major cause of heterogeneity in bovine myoglobin (Satterlee, Lillard, and Snyder 1969). In human metmyoglobin preparations, Perkoff *et al.* (1962) found that the minor components differed in absorption spectrum due to changes in the haem moiety. Hapner *et al.* (1968) found several minor components in sea and porpoise myoglobins which were not characterized, while the camel myoglobin investigated by Awad and Kotite (1966) contained only 2% of minor myoglobins. Most investigators have concluded that the heterogeneity arises during the preparation and is not significant *in vivo*. No such heterogeneity was detected in the preparations of red kangaroo skeletal muscle myoglobin by chromatography of metmyoglobin on SE-Sephadex and of apomyoglobin on CM-cellulose in 8M urea, starch-gel electrophoresis, or amino acid sequence. The minor components seen during CM-cellulose chromatography and starch-gel electrophoresis were non-myoglobin proteins.

Determination of amino acid sequences of myoglobins has been hindered by large amounts of insoluble material in tryptic and chymotryptic digests. Edmundson and Hirs (1962) found in tryptic digests of sperm whale myoglobin that 30% of the amino acid sequence was insoluble, and Dautrevaux, Han, Chaila, and Biserte (1969) reported a similar value for bovine myoglobin. Complete carboxymethylation of histidine residues in sperm whale myoglobin resulted in all tryptic peptides being solubilized (Edmundson 1965), but when applied to seal and porpoise myoglobins some peptides remained insoluble (Bradshaw, Garner, and Gurd 1969).

To reduce the complexity of the enzyme digests, Edmundson (1963) used cyanogen bromide to cleave sperm whale myoglobin into three fragments. Han *et al.* (1966) used this reagent as the first step in determining the amino acid sequence of horse myoglobin. This cleavage of methionyl bonds was also found very useful in the study of kangaroo myoglobin. As found by Edmundson (1963) for sperm whale myoglobin, trypsin gave very few soluble peptides from the largest fragment of kangaroo myoglobin (CNBr2), whereas chymotryptic digestion enabled the determination of the amino acid sequence in this region. The problems of tryptic activity in chymotrypsin reported by Han *et al.* (1969) were not encountered in our work.

Of the other methods of obtaining large fragments used in this study, tryptic digestion of the maleylated protein was useful in cleaving at arginine residues. The

significant chymotryptic splitting encountered may have been reduced by carrying out the tryptic digestion under milder conditions. Although treatment of CNBr2 with NBS resulted in very low yields of peptides resulting from cleavage of histidine residues, no non-specific cleavage was detected.

As found in previous work (Air and Thompson 1969), the dansyl–Edman procedure gave clear, reliable results for all amino acid residues except histidine and tryptophan. The Edman degradation gave a low yield of the aspartic acid residue at position 20, presumably due to formation of a β-aspartyl link (Naughton *et al.* 1960; Smyth, Stein, and Moore 1963; Weeds and Hartley 1968).

Consideration of the amino acid sequence of kangaroo myoglobin in relation to the three-dimensional structure of sperm whale myoglobin shows no substitutions which would be expected to interfere with the structure. Unlike the detailed structure–function relationships available for horse oxyhaemoglobin (Perutz *et al.* 1968), the X-ray diffraction structure of sperm whale myoglobin at 2Å resolution has not been discussed in detail. However, the amino acid residues which appear to be involved in the folding of the chain and interaction with the heme (Watson 1966, 1969) are either identical in kangaroo myoglobin or replaced by a very similar residue, the most striking substitution being the replacement of valine 21 of sperm whale myoglobin by glutamic acid in the kangaroo.

TABLE 7

TOTAL AMINO ACID SEQUENCE DIFFERENCES IN MYOGLOBINS

The amino acid sequences are from Edmundson 1965 (sperm whale), Bradshaw and Gurd 1969 (seal and porpoise), Dautrevaux, Boulanger, Han, and Biserte 1969 (horse). Partial amino acid sequences have been published for other myoglobins (see Introduction), but it is difficult to estimate the number of differences from incomplete data

	Horse	Seal	Porpoise	Whale	Kangaroo
Horse	0	20	16	19	25
Seal	20	0	17	26	27
Porpoise	16	17	0	15	28
Whale	19	26	15	0	31
Kangaroo	25	27	28	31	0

Comparison of the amino acid sequence of kangaroo myoglobin with myoglobins from other species confirms this conservation of structure. The total numbers of amino acid differences between all myoglobins of known amino acid sequence are shown in Table 7. It is clear that kangaroo myoglobin is the most different of this group, as observed also for the β-chain of kangaroo haeomoglobin (Air and Thompson 1969), but the number of changes between species is not as great. There are 46 differences between β-haemoglobin chains of kangaroo and horse, compared with 25 differences in the myoglobins. The amino acid substitutions involved appear to be very conservative in all the myoglobins examined so far.

V. Acknowledgments

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VI. References

Air, G. M., and Thompson, E. O. P. (1969).—*Aust. J. biol. Sci.* **22**, 1437.
Anson, M. L., and Mirsky, A. E. (1930).—*J. gen. Physiol.* **13**, 469.
Atassi, M. Z. (1964).—*Nature, Lond.* **202**, 496.
Awad, E. S., and Kotite, L. (1966).—*Biochem. J.* **98**, 909.
Berggard, I. (1961).—*Ark. Kemi* **18**, 291.
Bradshaw, R. A., Garner, W. H., and Gurd, F. R. N. (1969).—*J. biol. Chem.* **244**, 2149.
Bradshaw, R. A., and Gurd, F. R. N. (1969).—*J. biol. Chem.* **244**, 2167.
Butler, P. J. G., Harris, J. I., Hartley, B. S., and Leberman, R. (1969).—*Biochem. J.* **112**, 679.
Clegg, J. B., Naughton, M. A., and Weatherall, D. J. (1965).—*Nature, Lond.* **207**, 945.
Crowshaw, K., Jessup, S. J., and Ramwell, P. W. (1967).—*Biochem. J.* **103**, 79.
Dautrevaux, M., Boulanger, Y., Han, K., and Biserte, G. (1969).—*Eur. J. Biochem.* **11**, 267.
Dautrevaux, M., Han, K., Chaila, X., and Biserte, G. (1969).—*Bull. Soc. Chim. biol.* **51**, 989.
Dayhoff, M. O. (1969).—"Atlas of Protein Sequence and Structure." (National Biomedical Research Foundation: Silver Spring.)
Edman, P., and Begg, G. (1967).—*Eur. J. Biochem.* **1**, 80.
Edmundson, A. B. (1963).—*Nature, Lond.* **198**, 354.
Edmundson, A. B. (1965).—*Nature, Lond.* **205**, 883.
Edmundson, A. B., and Hirs, C. H. W. (1962).—*J. molec. Biol.* **5**, 683.
Genov, N., Shopova, M., and Karadzova, M. (1968).—*FEBS Letters* **1**, 108.
Gray, W. R. (1967).—In "Methods in Enzymology". (Ed. C. H. W. Hirs.) Vol. 11. p. 469. (Academic Press, Inc: New York.)
Gross, E., and Witkop, B. (1962).—*J. biol. Chem.* **237**, 1856.
Han, K., Dautrevaux, M., Boulanger, Y., and Biserte, G. (1969).—*FEBS Letters* **3**, 141.
Han, K., Dautrevaux, M., Boulanger, Y., Moschetto, Y., and Biserte, G. (1966).—*Bull. soc. Chim. biol.* **48**, 371.
Hapner, K. D., Bradshaw, R. A., Hartzell, C. R., and Gurd, F. R. N. (1968).—*J. biol. Chem.* **243**, 683.
Heller, J., and Smith, E. L. (1966).—*J. biol. Chem.* **241**, 3165.
Hill, R. L., Harris, C. M., Naylor, J. F., and Sams, W. M. (1969).—*J. biol. Chem.* **244**, 2182.
Jeppsson, J-O., and Sjöquist, J. (1967).—*Analyt. Biochem.* **18**, 264.
Naughton, M. A., Sanger, F., Hartley, B. S., and Shaw, D. C. (1960).—*Biochem. J.* **77**, 149.
Offord, R. E. (1966).—*Nature, Lond.* **211**, 591.
Perkoff, G. T., Hill, R. L., Brown, D. M., and Tyler, F. H. (1962).—*J. biol. Chem.* **237**, 2820.
Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968).—*Nature, Lond.* **219**, 131.
Ramachandran, L. K., and Witkop, B. (1967).—In "Methods in Enzymology". (Ed. C. H. W. Hirs.) Vol. 11. p. 283. (Academic Press, Inc: New York.)
Reindel, F., and Hoppe, W. (1954).—*Chem. Ber.* **87**, 1103.
Satterlee, L. D., Lillard, H. S., and Snyder, H. E. (1969).—*Life Sci.* **8**, 871.
Smyth, D. G., Stein, W. H., and Moore, S. (1963).—*J. biol. Chem.* **238**, 227.
Thompson, E. O. P., Hosken, R., and Air, G. M. (1969).—*Aust. J. biol. Sci.* **22**, 449.
Watson, H. C. (1966).—In "Hemes and Hemoproteins". (Eds. B. Chance, R. W. Estabrook, and T. Yonetani.) (Academic Press, Inc: New York.)
Watson, H. C. (1969).—In "Progress in Stereochemistry". (Eds. B. J. Aylett and M. M. Harris.) Vol. 4. p. 299. (Butterworths Scientific Publications: London.)
Weeds, A. G., and Hartley, B. S. (1968).—*Biochem. J.* **107**, 531.
Woods, K. R., and Wang, K-T. (1967).—*Biochim. biophys. Acta* **133**, 369.
Yamada, S., and Itano, H. A. (1966).—*Biochim. biophys. Acta* **130**, 538.