Studies on Myoglobin from the Finback Whale (*Balaenoptera physalus*)

PREPARATION, PHYSICOCHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATION, DIFFERENTIATION FROM SPERM-WHALE MYOGLOBIN, AMINO ACID COMPOSITION AND END-TERMINAL ANALYSES

BY M. Z. ATASSI AND BARBARA J. SAPLIN

*Departments of Biochemistry and Oral Biology, Schools of Dentistry and Medicine,
State University of New York, Buffalo, N.Y. 14214, U.S.A.*

(Received 30 June 1965)

1. Crystalline myoglobin was isolated from the skeletal muscle of the finback whale and fractionated, in its cyanmet form, into nine components (I-IX) by chromatography on CM-cellulose. Also in the cyanmet form, it was resolved into six components by electrophoresis on starch gel. Correspondence between the electrophoretic and chromatographic components was determined, and inter-conversion between components revealed by chromatography and electrophoresis. 2. The chromatographic myoglobin components were homogeneous in the ultracentrifuge. Molecular weights of certain components were determined by means of sedimentation equilibrium and by gel filtration on Sephadex G-100. Values from these two methods corresponded to the minimum molecular weight calculated from the iron content. 3. The spectral properties of the chromatographic components were investigated in the visible and the ultraviolet ranges. 4. The major components of finback-whale myoglobin and sperm-whale myoglobin showed almost identical spectral, electrophoretic and chromatographic behaviours, but had different infrared spectra. The infrared spectra of the corresponding apoproteins were almost identical. 5. Rabbit antisera to sperm-whale myoglobin component X cross-reacted with finback-whale myoglobin components V, VI and VII only about 30%. 6. The major chromatographic components of finback-whale myoglobin have identical amino acid compositions. The polypeptide chain contains 151 amino acid residues and its molecular weight is 17504. 7. The *N*-terminal end of the chain is:

Amino acids released from myoglobin by the action of carboxypeptidase A at different intervals were determined.

Myoglobin of the sperm whale is the best characterized member of the myoglobin group. Its three-dimensional structure in the crystalline form has been deduced from X-ray crystallographic studies (Kendrew *et al.* 1960, 1961), and a great deal is known about its physical and chemical properties (Atassi, 1964*a*; Edmundson & Hirs, 1962*a*), its primary structure (Watson & Kendrew, 1961; Šorm, 1962; Edmundson & Hirs, 1962*a,b,c*; Edmundson, 1963, 1965) and its immunochemical properties (Atassi, 1964*a*, 1965*a*). It was therefore decided to isolate and study another myoglobin from a closely related species of whale and to determine its chemical composition and the effect of any slight

variation in composition and primary structure on the physical, chemical and immunochemical properties of the haemoprotein.

The present paper deals with the preparation of myoglobin from the finback whale. Its chromatographic and electrophoretic heterogeneities have been studied and are discussed. Its spectral properties in the visible, ultraviolet and infrared regions have been studied and its immunochemical reaction with the antibody to sperm-whale myoglobin X (Atassi, 1964*a*) has been investigated. The molecular weight, amino acid composition and end terminal groups of the chromatographic fractions have been determined. The amino acid composition

of finback-whale myoglobin is compared with the compositions of other myoglobins.

MATERIALS AND METHODS

All experiments were carried out at 0–2°, unless otherwise stated.

Preparation and chromatography of myoglobin. Myoglobin was isolated from the skeletal muscle of the finback whale by the procedure of Perkoff & Tyler (1958) and crystallized twice by the method of Luginbuhl (1960). Spectral analysis of these preparations in the range 700–330 m μ showed that the myoglobin was in the ferri- or met-form. This was also observed when similar methods were used for human myoglobin (Perkoff, Hill, Brown & Tyler, 1962) and for SPMB* (Atassi, 1964a).

To avoid complications that might arise from autoxidation of myoglobins (Snyder, 1963), and to eliminate any chromatographic heterogeneity that disappears in the presence of cyanide, as with haemoglobin (Clegg & Schroeder, 1959) and human myoglobin (Perkoff *et al.* 1962), the haemoprotein was chromatographed in its cyanmet form. For preparative work myoglobin solutions (approx. 2 g. in 25 ml.) were chromatographed on columns (3.5 cm. $\times$ 60 cm.) of CM-cellulose (Sigma Chemical Co., St Louis, Mo., U.S.A.), with gradient elution from pH 6.2 to 7.7 in 10 mm-phosphate buffer containing KCN (0.01%). One magnetically stirred mixing chamber (250 ml.) was used and the buffer in the reservoir changed manually after a peak, or a group of peaks, had emerged. Fractions (9–10 ml.) were collected at the rate of 18–20 ml./hr. The extinctions of the fractions were read at 280, 420 and 540 m μ in a Zeiss PMQ II spectrophotometer. For analytical work, CN-MetMb samples (10–20 mg. in 1–3 ml.) were chromatographed on small columns (0.9 cm. $\times$ 60 cm.) by using a 50 ml. mixing chamber and collecting 1.5–2 ml. fractions at the rate of 3–4 ml./hr. Fractions belonging to one component were combined, dialysed against 10 mm-phosphate buffer, pH 6.5, containing KCN (0.01%), and concentrated on short CM-cellulose columns (2.2 cm. $\times$ 10 cm.) by applying at pH 6.5 and then eluting with a similar buffer at pH 7.7. By this method 20–40-fold concentration of myoglobin solutions was obtained.

Analytical methods. Characterization of the chromatographic fractions was carried out by starch-gel electrophoresis, at room temperature in the discontinuous buffer system of Poulik (1957), in gels and buffer containing KCN (0.05%). Cyanide was used in the buffers, since Perkoff *et al.* (1962) showed that differences in electrophoretic mobilities between the fractions of human myoglobin disappeared when the latter were analysed in their CN-MetMb form. The gels were stained with Amido Black (Smithies, 1959) and with benzidine hydrochloride (Haut, Tudhope, Cartwright & Wintrobe, 1962). Spectral analyses in the range 230–700 m μ were carried out in a Cary model 14 spectrophotometer. Complement-fixation experiments were done according to the method of Mayer, Osler, Bier & Heidelberg

(1948) in buffers containing KCN (0.01%). Single diffusion in 1% agar was by Oudin's (1948) technique, and double diffusion by the method of Ouchterlony (1949). The lines were considerably sharper when gels contained KCN (0.05%). Iron content was determined by the method of Dietrich & Schmitt (1937). Sedimentation studies were made at 20° on 0.3–0.8% solutions of the proteins in 10 mm-phosphate, pH 7.2, containing KCN (0.01%), in a Spinco model E analytical centrifuge, by using a red filter and infrared film. Molecular weights were determined by means of sedimentation equilibrium (Van Holde & Baldwin, 1958) in the same buffer at 25° (density, ρ , at 25°, 0.9986 by pycnometry) and a speed of 20410 rev./min. The time at equilibrium was 16 hr. The curves obtained were scanned on a shadowgraph. The partial specific volume, $\bar{v}$, of CN-MetMb, estimated from the amino acid composition of FMB-VII, was 0.724 ml./g. Molecular-weight determination on columns (2.5 cm. $\times$ 55 cm.) of Sephadex G-100 was by a method similar to that described by Andrews (1964). In the present work, however, the above 10 mm-phosphate buffer, pH 7.2, containing KCN (0.01%), was used instead of the tris hydrochloride buffer used by Andrews (1964). The following proteins were used to calibrate the column: horse-heart cytochrome c, bovine ribonuclease, SPMB, human serum albumin, ovalbumin, chymotrypsinogen, γ -globulin and catalase.

For amino acid analysis myoglobin samples were exhaustively dialysed against water, freeze-dried and dried to a constant weight over P₂O₅ *in vacuo* at room temperature. Portions (2–3 mg.) were then dissolved in 2.0 ml. each of constant-boiling HCl (triple-distilled), flushed with N₂, evacuated (the procedure repeated twice), sealed and heated at 110° for 22 or 72 hr. The hydrolysates were freed from excess of HCl on a rotary evaporator and assayed with an amino acid analyser similar to that described by Spackman, Stein & Moore (1958). The tryptophan content was determined spectrally by the method of Goodwin & Morton (1946).

Infrared spectroscopy. For infrared analyses, the proteins were dialysed extensively against water, freeze-dried and then dried to a constant weight in a vacuum desiccator over P₂O₅. The KBr was dried and kept at 110° over P₂O₅ in an evacuated Abderhalden drier. The protein (1 mg.) was mixed with KBr (200 mg.) for 2 min. in a Clevedon amalgamator. Several proportions of protein to KBr were tried and best resolutions were obtained with the ratio 1:200. Pellets were made with an evacuable die by using 200 mg. of mixture and 20000 lb./in.² pressure for 1 min. (after evacuation for 15 min.). The pellets were scanned in a Perkin-Elmer 521 infrared spectrophotometer. A blank pellet of 200 mg. of KBr was used for the reference beam.

Preparation of globin. The apoproteins were prepared by a method similar to that described by Theorell & Akesson (1955).

Immunization. The preparation of rabbit antibodies to SPMB-X has been described by Atassi (1965a).

N-Terminal analyses by dinitrophenylation. The protein (30–40 mg.) was dissolved (or suspended) in 3% (w/v) NaHCO₃ (5 ml.), and then 2 ml. of 5% (v/v) fluorodinitrobenzene in ethanol was added. The mixture was stirred magnetically at room temperature for 2 hr. The insoluble DNP-protein was washed on the centrifuge three times each successively with water and ethanol. Excess of fluorodinitrobenzene was then extracted ten times with peroxide-free water-saturated ether and the mixture dried

* Abbreviations: SPMB, sperm-whale myoglobin; FMB, finback-whale myoglobin (when these are followed by Roman numbers, the latter indicate the number of the chromatographic component); CN-MetMb, cyanmetmyoglobin.

over P_2O_5 in a vacuum desiccator. Portions of the DNP-proteins were hydrolysed at 110° for 8, 12 and 22 hr. with 2 ml. of constant-boiling HCl in a sealed N_2 -flushed evacuated container as described above. The hydrolysates were evaporated to dryness at 30° on a rotary evaporator, excess of dinitrophenol was removed by sublimation and the ether-soluble DNP-amino acids were removed by ten extractions with water-saturated ether from N -HCl. The combined ether extracts were backwashed three times with N -HCl and finally evaporated to dryness. The residue was examined quantitatively by paper chromatography with, in the ascending dimension, the toluene-chloroethanol-pyridine-aq. NH_3 solvent of Biserte & Osteux (1951), and 1.5 M-phosphate buffer, pH 6.0, in the second descending dimension. A duplicate preparation of the ether residue was also hydrolysed with saturated $Ba(OH)_2$ (2 ml.) for 90 min. at 100° . After removal of Ba^{2+} as $BaCO_3$ the amino acids in the hydrolysates were assayed on the automatic amino acid analyser.

The aqueous phase was evaporated to dryness and hydrolysed at 110° *in vacuo* with constant-boiling HCl, to bring the total time of hydrolysis to 22 hr., and subjected to quantitative amino acid analysis as described above.

Sequential N-terminal analyses by the Edman procedure. The following modification of the Edman (1953) procedure was employed. The procedure was carried out only on FMb-VII. Myoglobin solution in water (5 ml. containing 106 mg. of Mb) was mixed with an equal volume of peroxide-free dioxan, adjusted to pH 8.7–9.0 with 0.1 N-NaOH and stirred for 2–4 hr. with 0.5 ml. of phenyl isothiocyanate. The reaction was performed in a standard-joint 50 ml. centrifuge tube. During reaction, the magnetically stirred mixture was kept at 40° and pH maintained at 8.7–9.0 by the addition of 0.1 N-NaOH. When the reaction was complete the mixture was extracted seven times with 5 ml. of thiophen-free benzene; the tubes were centrifuged to break the emulsion that usually formed. The reaction mixture was evaporated to dryness on the rotary evaporator (28°) and kept in the desiccator under vacuum for 24 hr. over NaOH pellets and paraffin shavings. For cyclization the residue was suspended in 7 ml. of acetic acid–conc. HCl (5:1, v/v) (Acher, Laurilla & Fromageot, 1956), and kept at 40° for 2–6 hr. The cyclized mixture was evaporated to dryness, suspended in 7 ml. of 10 M-N-HCl, and extracted twice with an equal volume of water-saturated ethyl acetate and five times with water-saturated ether. The combined ether and ethyl acetate extracts were backwashed three times with 5 ml. of 10 M-N-HCl and finally evaporated to dryness. The residue, containing the phenylthiohydantoin amino acids, was hydrolysed *in vacuo* in 5.7 N-HCl (2 ml.) at 140° for 20 hr., and the amino acids in the hydrolysates were determined quantitatively on the automatic amino acid analyser as described above.

The aqueous layer in the reaction tube was evaporated to dryness. At this stage a portion (4 mg.) was taken for two duplicate amino acid analyses. The remainder was suspended in water (5 ml.) and dioxan (5 ml.) and the next step of the degradation procedure carried out.

Identification of C-terminal residues. This was carried out only on FMb-VII. Myoglobin (30.0 mg.), dissolved in water (24 ml.) that had been adjusted to pH 8.1, was incubated at 25° with 0.6 mg. of carboxypeptidase A (DFP-carboxypeptidase A, $3 \times$ crystallized; Worthington Biochemical Corp., Freehold, N.J., U.S.A.). The pH was maintained at

8.0 by the addition of 10 M-NaOH. Portions (4 ml. each) were withdrawn after 6, 16, 31, 60 and 120 min., the pH was adjusted immediately to 3.0 and the solutions were dialysed separately against eight 10 ml. changes of M-N-HCl. The diffusates from the digests were each combined and freeze-dried separately. The amino acids in the freeze-dried residues were determined on the amino acid analyser.

RESULTS

Chromatographic and electrophoretic behaviour. Electrophoresis on cellulose acetate of solutions of crystalline FMb, in the met form, in barbiturate buffer, pH 8.6, and room temperature showed that the preparations were completely homogeneous. Electrophoresis of CN-MetMb on starch gel revealed considerable heterogeneity. At least six bands were obtained with mobilities: A, 0.32 ± 0.03 ; B, 0.50 ± 0.03 ; C, 0.59 ± 0.02 ; D, 0.73 ± 0.02 ; E (observed only when large quantities were applied), 0.85

Fig. 1. Electrophoresis on starch gel of crystalline FMb and some chromatographic components. (a) Electrophoresis was carried out for 16 hr. at pH 8.6 (by the discontinuous buffer system of Poulik, 1957), with a potential gradient of 4.8 v/cm. The gels and buffer contained KCN (0.05%). (b) Electrophoresis was in the same buffer for 5 hr., with a potential gradient of 8.6 v/cm. The Roman numerals indicate the numbers of the chromatographic components. Electrophoresis bands are designated A–F.

± 0.01 ; *F*, 1 (9–10 cm. in 16 hr.). Bands *A–E*, all visible as red spots before staining, were haemoproteins since they all gave a positive colour with benzidine, whereas band *F* failed to stain with benzidine. The electrophoretic pattern of unchromatographed crystalline FMb is shown in Fig. 1. Chromatography on CM-cellulose resolved the preparation into nine well-defined peaks (Fig. 2). The total recovery based on the extinction at 280 m μ was 98.8%, and comprised: I, 14.2%; II, 2.31%; III, 12.3%; IV, 10.9%; V, 12.3%; VI, 6.67%; VII, 29.3%; VIII, 3.82%; IX, 6.30%.

The homogeneity of the chromatographic components was investigated by immediate electrophoresis on starch gel (Fig. 1). Components V, VI

and VII were completely homogeneous and corresponded to bands *C*, *B* and *A* respectively. Component III was extremely heterogeneous and contained all bands *A*, *B*, *C*, *D* and *E*, with *D* being the major band. Component IV showed bands *A*, *C* (major band) and *D*. The non-haem protein I showed only slight traces of myoglobin bands *C* and *D*. The heterogeneity of components III and IV was confirmed by rechromatography on CM-cellulose. Thus component III was resolved into components that were eluted like components III, IV, V, VI, VII and VIII, whereas component IV was resolved into components III, IV, VI and VII. The fractions belonging to each of these peaks were combined, concentrated on CM-cellulose and again subjected to starch-gel electrophoresis and column chromatography. Components V, VI and VII were homogeneous by electrophoresis and by immediate chromatography. Components III and IV were again heterogeneous by both methods of analysis, component III being resolved into III, IV, V, VI and VII, and component IV into III, IV, V–VI and VII. This heterogeneity of components III and IV could not therefore arise from contamination with the other components. Rather, it was most probably due to the transformation of each of these two components into all the others. This transformation was by no means confined to components III and IV, since it could easily be demonstrated in components V, VI and VII when the latter were kept at 40° in 10 mm-phosphate buffer, pH 7.0, containing cyanide (0.01%). The transformation was more effectively demonstrated by rechromatography of components V, VI and VII after dialysis against water and freeze-drying. Table 1 summarizes the results of these rechromatography experiments and suggests that the heterogeneities observed with components III and IV, and with the other components after certain physical

Fig. 2. Chromatogram of FMb on CM-cellulose. CN-MetMb was chromatographed on a column (3.5 cm. $\times$ 60 cm.) by gradient elution from pH 6.2 to 7.7 in 10 mm-phosphate buffers containing KCN and employing one mixing chamber. The buffer in the reservoir was changed at the points indicated by arrows. —, E_{420} ; ----, E_{280} .

Table 1. *Rechromatography of FMb components*

			Composition (%)								
Expt.	Material and treatment	Component ...	I	II	III	IV	V	VI	VII	VIII	IX
(a)	FMb		14.2	2.31	12.3	10.9	12.3	6.67	29.3	3.82	6.30
(b)	III from (a)		—	1.8	49.9	23.9		12.1	12.0	1.2	<1
(c)	III from (b)		—	—	46.3	26.0		11.3	10.6	<1	<1
(d)	IV from (a)		—	—	6.8	78.7	<1	1.4	15.2	<1	<1
(e)	IV from (d)		—	—	5.2	83.6		1.0	9.1	—	—
(f)	VI from (a)		—	—	—		2.4	93.0	2.1	—	—
(g)	VI from (a) after freeze-drying		—	—	<1	1.2	10.3	76.1	8.3	—	—
(h)	VII from (a)		—	—	—	—		1.1	98.8	—	—
(i)	VII from (a) after freeze-drying		—	—	—	<1	12.2	4.1	82.1	—	—

treatments, must be due to transformation of each of these components into all (or most of) the others. The possibility that this transformation was caused by alkaline and acid metmyoglobin forms at equilibrium with each other, similar to that reported for human myoglobin (Perkoff *et al.* 1962), was excluded. This was because a large number of components had to be accounted for, and both chromatography and electrophoresis were carried out in the presence of excess of cyanide (under which conditions the heterogeneity of human myoglobin disappeared).

Fig. 3 shows the results of two chromatographic experiments of FMb-VII and that of the major SPMb component (i.e. component X; Atassi, 1964a) on the same CM-cellulose column (2.5 cm. $\times$ 60 cm.). Solutions were applied in 10mm-phosphate buffer, pH 7.0, containing cyanide (0.01%),

Fig. 3. Rechromatography of FMb-VII and SPMb-X. The CN-MetMb samples were chromatographed on the same CM-cellulose column (2.5 cm. $\times$ 60 cm.). ●, FMb-VII; ○, SPMb-X. FMb-VII always tended to give sharper peaks than those given by SPMb-X. Experimental details are given in the text.

on a column that had been equilibrated with the same buffer. Gradient elution was employed, with one magnetically stirred mixing chamber containing 150 ml. of the above buffer. When application of the myoglobin solution was complete, the reservoir was filled with a similar buffer but at pH 7.32. Fig. 3 shows that SPMb-X and FMb-VII had very slightly different elution volumes, with FMb-VII being eluted first. The ratio of the volume required to elute FMb-VII over the volume required to elute SPMb-X was 0.910. Electrophoresis on starch gel showed that these two proteins had almost the same mobility, with FMb-VII occasionally moving very slightly ahead of SPMb-X.

Spectral properties. Component I [in 10mm-phosphate buffer, pH 7.2, containing cyanide (0.01%)] had an absorption maximum at 280 m μ and a small maximum at 400 m μ . The absorption at 400 m μ was most probably due to contamination with myoglobin. This was confirmed by the results of starch-gel electrophoresis, since, as mentioned above, a small amount of material migrating like bands C and D was observed. Solutions of components II–IX, when analysed spectrally in 10mm-phosphate buffer, pH 7.2, containing cyanide (0.01%), were in the CN-MetMb forms, showing maxima at 540, 424, 360–358 and 278–280 m μ . Table 2 lists the ratios of the extinctions at the various absorption maxima to the extinction at 280 m μ . The molar extinction coefficient of FMb-VII, on a dry-weight basis, was ϵ_{280} 32.0×10^3 . Component I had a very low E_{400}/E_{280} ratio (0.132), indicating that it was mostly (over 95%, assuming E_{424}/E_{280} 3.60 for 100% Mb) a non-haem protein. After rechromatography on CM-cellulose at pH 6.2, the peak at 400 m μ disappeared almost completely. Component II was also highly contaminated with

Table 2. Ratio of extinctions at the absorption maxima of FMb components

Proteins were prepared by one chromatography experiment, and their spectra determined at pH 7.20 in 10mm-phosphate buffer, containing KCN (0.01%).

Component	E_{360}/E_{280}	E_{424}/E_{280}	E_{540}/E_{280}	E_{424}/E_{540}
I	—	0.132*	—	—
II	0.488	1.28	0.186	6.88
III	0.645	2.31	0.225	10.23
IV	1.19	3.91	0.381	10.19
V	1.04	3.82	0.362	10.57
VI	0.951	3.54	0.331	10.70
VII	0.949	3.61	0.339	10.64
VIII	0.828	2.78	0.310	8.94
IX	0.617	1.77	0.170	10.38

* This value represents the E_{400}/E_{280} ratio, since component I showed a minor peak at 400 m μ only.

non-haem protein, as shown in Table 2 (E_{424}/E_{280} 1.28). Component III represented the first of the myoglobin components, but was probably contaminated with colourless protein. However, such a conclusion based on E_{424}/E_{280} could be misleading, since this ratio might actually vary for the different chromatographic components, which could all be pure myoglobins. However, rechromatography of component III showed, as pointed out above, the presence of small amounts of component II (Table 1). Components IV–VIII had constant, almost identical, extinction ratios and probably represent pure myoglobins. These myoglobin components

had identical amino acid compositions (see Table 5), thus confirming their purity. The absorption spectrum of component VIII (Fig. 4) was unique in that it showed in the ultraviolet region, in addition to a maximum at $280\text{m}\mu$, absorption maxima at 261.5 , 256 , 249 and $244\text{m}\mu$, most probably due to contamination or complex-formation with a low-molecular-weight compound. The significance of the occurrence of these maxima, in FMB-VIII only, is not understood.

Molecular-weight measurements. The minimum molecular weights calculated from the iron content of the twice-chromatographed components are shown in Table 3. The average value for the molecular weight, assuming 1 haem group/mol., was 18120. The possibility that the electrophoretic and chromatographic heterogeneities of FMB, and the intertransformation of the components, might arise from the presence of different aggregates of the monomer was investigated in the ultracentrifuge. Components II, VIII and IX were not analysed, but the other components (all chromatographed twice) sedimented as single sharp peaks. The peaks obtained with components III–VII sedimented at the same rate when the solutions had the same concentration. The sedimentation coefficient, $S_{20,w}^0$, of component VII was 1.96 s.

Table 3 also shows the results of the molecular-weight determinations of certain components by means of sedimentation equilibrium. The values for the weight-average molecular weights, M_w , and the number-average molecular weights, M_n , obtained in each case corresponded to the molecular weight of the monomer. Therefore an equilibrium involving different aggregates of the monomer was ruled out as being the cause of the electrophoretic and chromatographic heterogeneities. Molecular-weight determinations of certain components by gel filtration on Sephadex G-100 (Table 3) confirmed the results obtained with sedimentation equilibrium.

Infrared spectroscopy. The above reported differences in the chromatographic and possibly electrophoretic behaviours of SPMb-X and FMB-VII could not be considered as evidence that these two myoglobins are different. The first conclusive demonstration, by physical methods, of differences between the two proteins came from the infrared spectra of the crystalline proteins. Table 4 lists the principal absorption bands of SPMb and FMB. No significant differences at frequencies higher than 1800cm^{-1} were observed. The only differences observed in the region $2000\text{--}4000\text{cm}^{-1}$ were actually in intensity rather than position of the above group frequencies. FMB repeatedly gave spectra with bands that were not as strong or as sharp as the corresponding bands of SPMb.

Fig. 5 shows the spectra of FMB and SPMb in the range $250\text{--}2000\text{cm}^{-1}$. The amide I band (C=O

Fig. 4. Spectra of freshly prepared FMB-VII (A) and FMB-VIII (B) in the ultraviolet region. Solutions (130mg. of Mb/ml.) were in 10mM-phosphate buffer, pH 7.2, containing KCN (0.01%). In addition to a maximum at $280\text{m}\mu$ (given by both FMB-VII and FMB-VIII), FMB-VIII shows maxima at 261.5 , 256 , 249 and $244\text{m}\mu$, most probably due to contamination or complex-formation with a low-molecular-weight compound. The spectra are displaced vertically for clarity.

Table 3. *Molecular-weight determinations of FMb components*

Molecular weights were determined, on the twice-chromatographed components, by the different procedures mentioned below and are listed together to facilitate comparison.

Component	Procedure of molecular-weight determination				
	Iron content		Sedimentation equilibrium		Sephadex G-100
	Iron (%)	M	M_w	M_n	
III	0.298	(18742)	(18817)	(18628)	—
IV	0.307	18192	17634	18220	—
V	0.306	18250	18000	17920	17800
VI	0.308	18133	18140	18500	18000
VII	0.312	17900	18040	18170	17800
Average M^* ...		18120	17950	18200	17870

* Values in parentheses are not included in the averages.

Table 4. *Principal absorption bands of SPMb and FMb*

—, Band absent; (s), strong; (m), medium; (w), weak.

Band position (cm. ⁻¹)	
SPMb	FMb
3300 (s)	3300 (s)
3070 (w)	3070 (w)
2960 (c), 2928 (s), 2870 (w)	2960 (m), 2928 (m), 2870 (w)
1650 (vs)*	1650 (vs)*
1534, 1517, 1500* (vs)	1534, 1517, 1502 (vs)*
1468 (vw)	1468 (vw)
1450 (s)	1450 (vw)
1390 (s)	1391 (s)
1310 (vw)	1310 (s)
1260, 1235 (s)	—
1150 (m), 1105 (s)	—, 1095 (vs)
1075 (m)	—
1025–1045 (s)	—
—	980 (m)
930 (m)	915 (w)
—	848 (m)
695 (w), 660 (w), 615 (m)	698, 663 almost absent
—	532 (vs)

* Band showed splitting.

stretching) appeared in both proteins at 1650 cm.⁻¹. The group of bands at 1534, 1517 and 1500 cm.⁻¹ was due to the amide II band. The splitting observed in amide I and II vibrations was most probably due to vibrational coupling between neighbouring peptides (Miyazawa, 1960). The weak band at 1468 cm.⁻¹ was probably due to the CH₃ group frequency of alanine (Fraser, 1961), and the bands of 1450 and 1390 cm.⁻¹ were probably due to the C–CH₃ asymmetric and –C(CH₃)₂ symmetric deformation frequencies (Fraser, 1961). The five-membered ring stretching vibration at about 1400 cm.⁻¹ (strong in haemin) could also contribute

Fig. 5. Infrared spectra of FMb-VII (a) and SPMb-X (b). The spectra are given in the range 2000–250 cm.⁻¹. Above 2000 cm.⁻¹ the spectra of the two proteins were almost identical and are therefore not shown.

to the band at 1390 cm.⁻¹. The pyrrolic C–N stretching frequency (Schwartz, Berg, Bossenmaier & Dinsmore, 1960) at 1340 cm.⁻¹, which was a very weak band in SPMb, disappeared completely in FMb. The first major change in the spectra was in the remarkable difference in the intensity of the band at 1310 cm.⁻¹ (CH₂ and CH₃ deformation frequencies; Fraser, 1961). This band was only a minor band in SPMb and appeared as a strong major one in FMb. The bands at 1260 and 1235 cm.⁻¹ in SPMb might be due to the amide III vibration. Also, at 1270–1230 cm.⁻¹ the C–O stretching frequency of acid groups on porphyrins appears (Schwartz *et al.* 1960). SPMb showed a distinct sharp band at 1150 cm.⁻¹ (CH₃ group frequency of alanine; Fraser, 1961; phenolic C–OH stretch; Jones, 1959) and another yet stronger band at 1105 cm.⁻¹ (bending and deformation of pyrrolic

NH; Schwartz *et al.* 1960). In FMb the 1150cm^{-1} band appeared only as a shoulder on the high-frequency side of the 1095cm^{-1} band. The next two bands at 1075 and 1035cm^{-1} (C—OH stretching; Zeiss & Tsutsui, 1953) were present in SPMb, but absent from FMb. A sharp band at 980cm^{-1} appeared in FMb and was absent from SPMb. This might be due to the =CH (or vinyl groups) bending and deformation that usually appears in porphyrins at 990cm^{-1} (Schwartz *et al.* 1960). FMb had a unique band of medium intensity at 848cm^{-1} , probably due to CH out-of-plane vibrations as shown by pyrroles at 838cm^{-1} (Katritzky, 1959). Amides V and IV at 695 and 660cm^{-1} respectively were much weaker in FMb. The amide VI vibration (C=O out-of-plane bending) at 615cm^{-1} disappeared completely in FMb, and a unique strong and sharp band appeared at 532cm^{-1} . This new band might be due to a tremendous shift of amide VI. The spectra of the two apoproteins, although different from their parent myoglobins, were almost identical.

Immunochemical studies. On double diffusion in agar of FMb components V, VI and VII, together with SPMb-X (Atassi, 1964a), against rabbit antiserum to SPMb-X (Atassi, 1965a), the FMb components gave very faint lines as compared with that given by SPMb-X. In each case, a spur was formed, as a continuation of the SPMb-X line, indicating a partial similarity between SPMb and FMb. The extent of reaction of FMb components V, VI and VII with the antibody to SPMb-X was determined quantitatively by complement fixation. Although all myoglobins showed maximum complement fixation at the same antigen concentration (Fig. 6), the FMb components fixed at that maximum no more than one-third the amount of complement fixed by SPMb-X. The partial cross-reaction of FMb with anti-SPMb was reported qualitatively by Kendrew, Parrish, Marrack & Orlans (1954), who used gel diffusion methods.

Fig. 6. Reactions of antibody to SPMbX with: ○, SPMb-X; ●, FMb-IV; ▲, FMb-V; ■, FMb-VII. Complement fixed (C'H₅₀ units) is plotted against the amount of antigen nitrogen.

Amino acid composition. Chromatographic components III, IV, VI, VII, VIII and IX of FMb, obtained after one chromatographic procedure, were subjected to amino acid analysis. Values were calculated on an ash-free and moisture-free basis. Amino acid contents are expressed as molar proportions of the stable amino acids: valine, glycine, aspartic acid, glutamic acid, leucine, alanine and lysine. The average molar proportions of these seven amino acids were (to the closest integer): 6:11:12:17:17:18:20. Treatment of the results is described in the footnotes of Table 5.

Table 5 shows the amino acid compositions of FMb components. The composition of FMb-III appeared to be slightly different from the composition of components IV–VII, the latter probably having identical amino acid compositions. The differences in the amino acid composition of FMb-III were probably due to contamination of this protein with some non-myoglobin protein. This was discussed above (one preliminary analysis indicated that the amino acid composition of component I was completely different from that of myoglobin). Slight differences in composition were also observed with components VIII and IX. This might have also been due to contamination. Although no differences in amino acid compositions could be observed with components IV–VII, there is some degree of uncertainty in some amide ammonia values with components that were subjected only to one hydrolysis time (e.g. FMb-IV). The molecular weight of components IV–VII, based on the amino acid composition and the nitrogen recovery, together with the contribution of the haem, was 17504.

Identification of the N-terminal residues. The N-terminus was determined for components I–VII by dinitrophenylation. Components III–VII, by quantitative paper chromatography of the ether-soluble fractions, gave DNP-valine as the major spot with recoveries 0.6–0.8 mol. of valine/mol. of Mb. Amino acid analysis of the ether-soluble fraction of FMb-VII, after hydrolysis with barium hydroxide, revealed the presence of valine and leucine, with recoveries (in mol. of amino acid/mol. of Mb) 0.790 and 0.085 respectively. Amino acid analyses of the aqueous phases from the acid hydrolysates of the DNP-Mb components III–VII revealed in every case a decrease only in the valine value (as compared with the parent fraction) by 1 mol./mol. of Mb. It was therefore concluded that the N-terminus for all components III–VII was valine. Qualitative paper chromatography revealed only glycine as the N-terminus of component I.

Edman degradation was carried out only on FMb-VII. Amino acids in the ether-ethyl acetate phase from the first cyclization step were (in mol. of amino acid/mol. of Mb): valine, 0.830; alanine, 0.238; glycine, 0.164; leucine, 0.092; aspartic acid,

Table 5. *Amino acid compositions of FMb components*

Values for FMb-VIII and FMb-IX were obtained from one analysis and are not therefore corrected. Values for FMb-III and FMb-IV are averages from two determinations of two 22hr. hydrolysates each. Values for FMb-VI are averages from two determinations each of 22, 24 and 72hr. hydrolysates (i.e. a total of six determinations). Values for FMb-VII are averages from five 22hr., one 24hr. and three 72hr. hydrolysates (i.e. a total of nine determinations). N.D., Not determined. Values in parentheses were not included in the averages.

Amino acid	Amino acid composition (residues/mol.)									
			FMb-III		FMb-IV		FMb-VI		FMb-VII	
	FMb-VIII	FMb-IX	Average	Nearest integer	Average	Nearest integer	Average	Nearest integer	Average	Nearest integer
Asp	12.4	12.9	13.5	14	12.1	12	12.15	12	12.20	12
Thr	6.10	4.74	5.25	5	5.01	5	5.38*	5	5.14*	5
Ser	6.29	6.06	4.78	5	4.92	5	5.14*	5	5.42*	5
Glu	16.6	17.1	16.2	16	16.6	17	16.57	17	17.05	17
Pro	4.38	5.37	5.34	5	4.09	4	3.77	4	4.00	4
Gly	11.1	11.6	11.2	11	11.2	11	10.87	11	11.07	11
Ala	16.8	16.3	13.7	14	18.0	18	18.04	18	17.94	18
Val	7.44	7.19	8.00	8	6.15	6	6.16	6	5.98	6
Met¶	1.34	0.593	2.15	2	1.52	2	1.85	2	1.87	2
Ile†	8.40†	8.74†	7.59†	8	8.71†	9	9.07†	9	9.35†	9
Leu	17.6	17.0	16.0	16	17.3	17	16.94	17	17.19	17
Tyr	2.47	2.27	2.53	3	2.00	2	1.84*	2	1.86*	2
Phe	6.87	7.11	6.85	7	6.85	7	6.78	7	6.62	7
Lys	17.4	16.3	17.2	17	20.1	20	19.74	20	20.02	20
His	9.54	8.34	8.90	9	10.9	11	10.93	11	10.97	11
NH ₃	(11.6)	(32.4)	(14.1)	(14)	(14.3)	(14)	(13.52)*‡	(14)	(12.78)*‡	(13)
Arg	3.06	3.54	3.69	4	2.83	3	2.59	3	3.00	3
Trp§	N.D.	N.D.	N.D.	—	N.D.	—	1.82§	2	1.76§	2
Total				144		149		151		151

* Values obtained by extrapolation to zero hydrolysis time.

† Isoleucine was corrected for the formation of alloisoleucine.

‡ Ammonia was corrected for the ammonia formed in the decomposition of serine and threonine.

§ Values of tryptophan represent the averages of two independent determinations.

|| Value was not included in total.

¶ Methionine sulphone and sulphoxides were not formed and no correction was necessary.

0.065. Amino acid analysis of the phenyl isothiocyanate-treated protein in the aqueous phase showed a decrease in valine from 5.98 to 5.04 mol./mol. of Mb. The ether phase from the second cyclization step contained (in mol./mol. of Mb): tyrosine, 0.672; glycine, 0.260; leucine, 0.200; serine, 0.178; aspartic acid, 0.173; alanine, 0.135. The water phase showed a decrease only in the tyrosine content from 1.90 to 1.16 mol./mol. of Mb. In the third cyclization step the ether extract contained (in mol./mol. of Mb): glycine, 0.552; valine, 0.109; alanine, 0.105; leucine, 0.097; isoleucine, 0.087; glutamic acid, 0.070; aspartic acid, 0.058. This suggested to us a glycine residue in position 3. However, amino acid analysis of the protein in the aqueous phase revealed no detectable decrease in the glycine content, but, instead, a decrease in the threonine content equivalent to 1 mol./mol. of Mb was obtained. The discrepancy might have been

caused by the hydrolysis of some of the more labile bonds that accompany slow cyclizations. The cyclization of the *N*-terminal glycine was carried out for 6hr. Margoliash (1962) reported that it is about 80% complete in 10–12hr. On the other hand, the glycine in the ether extract of the phenylthiohydantoin amino acids could have been from the decomposition of phenylthiohydantoin tryptophan that takes place on acid hydrolysis (Fraenkel-Conrat, Harris & Levy, 1955). Also, phenylthiohydantoin threonine undergoes decomposition on acid hydrolysis, which would account for the discrepancy between the amino acid contents of the ether and water phases. The identity of the residue in position 3 therefore remains uncertain. In the fourth cyclization the ether extract contained (in mol./mol. of Mb): leucine, 0.570; glycine, 0.362; alanine, 0.192; glutamic acid, 0.139; aspartic acid, 0.131; phenylalanine, 0.061. For the protein in the

Fig. 7. Rate of release of amino acids from FMB-VII by digestion with carboxypeptidase A. $\circ$, Serine; Δ , glycine; $\square$, lysine; $\bullet$, alanine; $\blacktriangle$, aspartic acid. Experimental details are given in the text.

aqueous phase, the only change was observed in the leucine content, which decreased from 16.65 to 15.57 mol./mol. of Mb. The amino acid in position 4 was most probably a leucine residue. In conclusion, the *N*-terminal sequence of FMB was:

Valine was also reported as the *N*-terminus of FMB by Schmid (1949).

Amino acids liberated by the action of carboxypeptidase. Fig. 7 shows the rate of release of amino acids from FMB-VII by digestion with carboxypeptidase. Only the first five amino acids are shown. These were released in the order: serine > glycine > lysine > alanine > aspartic acid. Since more than 1 mol. of serine/mol. is released, this amino acid is most probably present in more than one position in the *C*-terminal sequence. Lysyl peptide bonds are not hydrolysed by carboxypeptidase A to a significant extent. The results therefore suggest that the carboxypeptidase A preparation was appreciably contaminated with carboxypeptidase B.

DISCUSSION

The chromatographic resolution of SPMb, in its cyanmet form, into at least 12 components has been reported (Atassi, 1964a). These components showed a tendency towards transformation of each com-

ponent into some, or most, of the others, as revealed by both chromatography and electrophoresis. The extent of conversion in a given time was dependent on the particular component. The situation with FMB, reported in the present paper, seems to be similar to that of SPMb. Also, Atassi (1964b) reported that the two forms of normal adult haemoglobin, A_1 and A_0 (in their met, cyanmet or oxy forms), were interconverted freely in aqueous solution. As with SPMb and adult haemoglobin, the heterogeneity of FMB does not seem to be the result of different degrees of aggregation of the protein. The existence of chromatographic components of myoglobin that possess identical amino acid compositions and different electrophoretic charges, but are interconverted in solution, might be the result of conformational changes in the protein. No explanation can be offered at the moment for the nature of these changes, which did not seem to affect the immunochemical properties of the protein. Therefore the chromatographic fractions of SPMb (Atassi, 1964a) and those of adult haemoglobin (Atassi, Brown & McEwan, 1965) had identical complement-fixing and antibody-precipitating efficiency when treated with antibodies to the major components of SPMb and adult haemoglobin respectively. The heterogeneities reported for some other myoglobins by various workers have been discussed by Atassi (1964a).

Since only very slight differences could be observed in the chromatographic and electrophoretic properties of the major components of SPMb and FMB, it is likely that these two haemoproteins are not too different in primary structure. The first evidence that they are not identical came from the infrared spectroscopic studies. This was unexpected, since proteins with the folded-chain configuration usually show almost identical spectra and this technique cannot, as a rule, be used for the identification or differentiation of individual proteins (Halford, 1957). Conformational factors, rather than alterations in primary structure, were responsible for the differences in the infrared spectra of SPMb and FMB. This was confirmed by the finding that the spectra of the globins were identical.

In addition to conformational differences that were responsible for the variations in the infrared spectra of FMB and SPMb, the two proteins probably had differences in their primary structures. These primary structural differences could not have been so drastic as to make the proteins completely dissimilar. This is revealed from the partial antigenic similarity of the two proteins. It is probable, however, that conformational differences might account for some of the lack of the antigenic efficiency of FMB when treated with the antibody to SPMb.

Table 6. *Amino acid composition of myoglobins from various species*

References: (a) Konosu *et al.* (1958); (b) Hirs & Olcott (1964); (c) Timmer, van der Helm & Huisman (1957); (d) Karadzova, Nedkov, Atanasov & Keil (1964); (e) Akeson & Theorell (1960); (f) Perkoff *et al.* (1962); (g) Rumen (1960); (h) Timmer *et al.* (1957); (i) this paper; (j) Edmundson & Hirs (1962a). N.D., Not determined.

Amino acid	Amino acid composition of myoglobin (residues/mol.)									
	Pisces		Mammals							
	Blue-fin tuna (a)	Yellow-fin tuna (b)	Ox* (c)	Dolphin (d)	Horse (e)	Human (f)	Seal (g)	Sheep* (h)	Finback whale (i)	Sperm whale (j)
Asp	12	12	14	12	11	12	11	12	12	8
Thr	7	7	5	5	7	4	5	5	5	5
Ser	4	5	5	5-6	5	8	7	5	5	6
Glu	11	11	17	17	19	21	16	16	17	19
Pro	6	5	4	4	4	6	4	3	4	4
Gly	14	14	12	14	16	15	12	14	11	11
Ala	21	21	16	18	16	11	14	17	18	17
Cys (half)	1	1	0	0	0	0	0	0	0	0
Val	9	8	9	6	7	8	6	9	6	8
Met	2	3	2	2	2	3	2	3	2	2
Ile	9	10	5	8	9	7	7	6	9	9
Leu	16	17	18	20	18	16	18	19	17	18
Tyr	2	2	2	2	2	2	2	2	2	3
Phe	6	6	6	8	7	7	7	9	7	6
Lys	15	15	19	21	19	19	18	19	20	19
His	5	6	13	12	11	9	12	11	11	12
Arg	2	2	3	3	2	3	5	2	3	4
Trp	1	N.D.	N.D.	2	N.D.	3	N.D.	N.D.	2	2

* Values were calculated from the authors' data (expressed as g./100g.) assuming mol.wt. 18000.

The amino acid composition of FMb is compared (in Table 6) with the compositions of some mammalian and fish myoglobins. It is apparent that FMb is very similar to SPMB in composition. This similarity might explain the partial reaction of FMb with anti-SPMB.* In fact, only small differences in composition exist between the various mammalian myoglobins. The two tuna myoglobins (Table 6) have been shown to contain cysteine residues (Konosu, Hashimoto & Matsuura, 1958; Hirs & Olcott, 1964). Brown, Martinez, Johnstone & Olcott (1962) have found, in addition to the above tuna haemoproteins, that myoglobins from Albacore tuna, Shipjack tuna and Chinook salmon also contained cysteine. It would be significant to determine whether this is a general property of fish myoglobins. Table 6 shows that no mammalian myoglobin so far investigated contains cysteine. The contents of mammalian myoglobins in the basic amino acids (lysine plus histidine plus arginine) appear to be remarkably constant if expressed in 'residues per cent', i.e. (no. of residues of lysine + histidine + arginine) $\times$ 100/(total no. of residues/mol.). The mean value of this content for the eight myoglobins in Table 6 is 22.15 ± 1.78

residues per cent. The basic amino acid content of the two tuna myoglobins appears to be considerably lower (blue-fin tuna, 15.38; yellow-fin tuna, 15.75 residues per cent). The two fish myoglobins also have a characteristically higher non-polar amino acid (alanine plus valine plus isoleucine plus leucine) content than the mammalian myoglobins (mammalia, 30.6 ± 3.4 ; blue-fin tuna, 38.5; yellow-fin tuna, 38.4 residues per cent). We have shown that constant differences in composition also exist between mammalian and fish haemoglobins. The significance of these composition characteristics has also been discussed in detail (Atassi, 1965b) in relation to structure, function and evolution of these haemoproteins. In conclusion, it is sufficient to point out here that FMb, with contents 22.36 residues per cent in basic amino acids and 32.9 residues per cent in the non-polar amino acids, can be classified as an average mammalian myoglobin.

We thank Dr Ray K. Brown for valuable discussions. This work was supported by the Office of Naval Research, Biochemistry Branch, Contract no. Nonr-4564(00), and by a grant (no. AM08804-01) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service.

REFERENCES

- Acher, R., Laurilla, U.-R. & Fromageot, C. (1956). *Biochim. biophys. Acta*, **19**, 97.
- Akeson, A. & Theorell, H. (1960). *Arch. Biochem. Biophys.* **91**, 319.
- Andrews, P. (1964). *Biochem. J.* **91**, 222.
- Atassi, M. Z. (1964a). *Nature, Lond.*, **202**, 696.
- Atassi, M. Z. (1964b). *Biochem. J.* **93**, 189.
- Atassi, M. Z. (1965a). *Nature, Lond.*, (in the Press).
- Atassi, M. Z. (1965b). *J. theoret. Biol.* (in the Press).
- Atassi, M. Z., Brown, R. K. & McEwan, M. (1965). *Immunochemistry*, (in the Press).
- Biserte, G. & Osteux, R. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 50.
- Brown, W. D., Martinez, M., Johnstone, M. & Olcott, H. S. (1962). *J. biol. Chem.* **237**, 81.
- Clegg, M. D. & Schroeder, W. A. (1959). *J. Amer. chem. Soc.* **81**, 6065.
- Dietrich, K. & Schmitt, K. (1937). *Z. anal. Chem.* **111**, 176.
- Edman, P. (1953). *Acta chem. scand.* **7**, 700.
- Edmundson, A. B. (1963). *Nature, Lond.*, **198**, 354.
- Edmundson, A. B. (1965). *Nature, Lond.*, **205**, 883.
- Edmundson, A. B. & Hirs, C. H. W. (1962a). *J. molec. Biol.* **5**, 663.
- Edmundson, A. B. & Hirs, C. H. W. (1962b). *J. molec. Biol.* **5**, 683.
- Edmundson, A. B. & Hirs, C. H. W. (1962c). *J. molec. Biol.* **5**, 706.
- Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955). *Meth. biochem. Anal.* **2**, 359.
- Fraser, R. D. B. (1961). In *Analytical Methods of Protein Chemistry*, vol. 2, p. 285. Ed. by Alexander, P. & Bloch, R. J. New York: Pergamon Press.
- Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, 630.
- Halford, R. S. (1957). *Ann. N.Y. Acad. Sci.* **69**, 63.
- Haut, A., Tudhope, G. R., Cartwright, G. B. & Wintrobe, M. M. (1962). *J. clin. Invest.* **41**, 579.
- Hirs, C. H. W. & Olcott, H. S. (1964). *Biochim. biophys. Acta*, **82**, 178.
- Jones, R. N. (1959). *Bull. nat. Res. Coun., Ottawa*, no. 6: *Infrared Spectra of Organic Compounds: Summary Charts of Principal Group Frequencies*.
- Karadzova, M., Nedkov, P. T., Atanasov, B. & Keil, B. (1964). *Coll. Czech. chem. Commun.* **29**, 551.
- Katritzky, A. R. (1959). *Quart. Rev. chem. Soc., Lond.*, **4**, 353.
- Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C. & Shore, V. C. (1960). *Nature, Lond.*, **185**, 422.
- Kendrew, J. C., Parrish, R. G., Marrack, J. R. & Orlans, E. S. (1954). *Nature, Lond.*, **174**, 946.
- Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C. & Shore, V. C. (1961). *Nature, Lond.*, **190**, 666.
- Konosu, S., Hashimoto, K. & Matsuura, F. (1958). *Bull. Jap. Soc. sci. Fish.* **24**, 513.
- Luginbuhl, W. H. (1960). *Proc. Soc. exp. Biol., N.Y.*, **105**, 504.
- Margoliash, E. (1962). *J. biol. Chem.* **237**, 2161.
- Mayer, M. M., Osler, A. G., Bier, O. G. & Heidelberger, M. (1948). *J. Immunol.* **59**, 195.
- Miyazawa, T. (1960). *J. chem. Phys.* **32**, 1647.
- Oudin, J. (1948). *Ann. Inst. Pasteur*, **75**, 30.
- Ouchterlony, O. (1949). *Acta path. microbiol. scand.* **26**, 507.
- Perkoff, G. T., Hill, R. L., Brown, D. M. & Tyler, F. H. (1962). *J. biol. Chem.* **237**, 2820.
- Perkoff, G. T. & Tyler, F. H. (1958). *Metabolism*, **7**, 751.
- Poulik, M. D. (1957). *Nature, Lond.*, **180**, 1477.
- Rumen, N. M. (1960). *Acta chem. scand.* **14**, 1217.
- Schmid, K. (1949). *Helv. chim. Acta*, **32**, 1198.
- Schwartz, S., Berg, M. H., Bossenmaier, I. & Dinsmore, H. (1960). *Meth. biochem. Anal.* **8**, 221.
- Smithies, O. (1959). *Advanc. Protein Chem.* **14**, 141.
- Snyder, H. E. (1963). *Biochim. biophys. Acta*, **69**, 200.
- Šorm, F. (1962). *Coll. Czech. chem. Commun.* **27**, 994.
- Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
- Theorell, H. & Akeson, A. (1955). *Ann. Acad. Sci. fenn.* **2A**, **60**, 303.
- Timmer, R., van der Helm, H. J. & Huisman, T. H. J. (1957). *Nature, Lond.*, **180**, 240.
- Van Holde, K. E. & Baldwin, R. L. (1958). *J. Amer. chem. Soc.* **62**, 734.
- Watson, H. C. & Kendrew, J. C. (1961). *Nature, Lond.*, **190**, 670.
- Zeiss, H. H. & Tsutsui, M. (1953). *J. Amer. chem. Soc.* **75**, 897.