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Haemoglobins of the Shark, *Heterodontus portusjacksoni*

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Abstract

The haemoglobins of the Port Jackson shark, *H. portusjacksoni*, were resolved by cation-exchange chromatography of their carboxymethylated forms into two fractions, Hb-I and Hb-II, which were present in approximately equal proportions. A third, non-haem, acidic protein component, representing 5% of the total protein, was also resolved. This protein is apparently of high molecular weight and is present in different polymorphic forms. The haemoglobins of the shark are subject to rapid aggregation, by disulphide bond formation, following lysis of the red cells. The aggregation is reversed by treatment with thiols, and prevented by combination of the two to four 'reactive' thiol groups with iodoacetate or other thiol-blocking reagents. The molecular weights of the haemoglobins are approximately 61 000, suggesting a tetrameric molecule.

After removal of the haem, the globin from the major carboxymethylated haemoglobin fraction, Hb-I, was dissolved in 6M guanidine hydrochloride and a further six thiol groups per tetramer, previously inaccessible, could be carboxymethylated. Chromatography of the fully carboxymethylated globin on DEAE-Sephadex in 8M urea buffers at pH 8·5 gave two components. Amino acid analyses of these components were markedly different and they are thought to be the monomer subunits related to the α- and β-globin chains of mammalian haemoglobins.

Introduction

The number and nature of the variations in the primary structure of the haemoglobin molecule, from an increasing variety of animals, are being used to shed light on the process of molecular evolution. Similar data are also used to establish phylogenetic relationships within the animal kingdom (Buettner-Janusch and Hill 1965; Zuckerkandl and Pauling 1965; Fitch and Margoliash 1970; Goodman *et al.* 1971). To date, the emphasis of such research has been on the higher vertebrates, especially mammals.

Since the vertebrate line of evolution arose in an aquatic milieu (Romer 1966), it would seem reasonable to seek the ancestral respiratory pigments in primitive marine animals. The oldest extant vertebrates are the cyclostomes (Bardack and Zangerl 1968), with a fossil record extending back to mid-Pennsylvanian times. Sequence analysis of the major haemoglobin component of the lamprey, *Lampetra fluviatilis* (Braunitzer and Fujiki 1969), indicates that this pigment is a myoglobin-type monomer. Li and Riggs (1970), on the basis of their studies of the sequence of the haemoglobin of *Petromyzon marinus*, concluded that there was marked homology between this monomeric haemoglobin and both α- and β-chains of human haemoglobin. They also reported association of the non-ligated monomers. These studies clearly support Ingram's (1961) theory of gene duplication as the process by which the tetrameric haemoglobin molecule evolved.

Of the other two classes of extant fishes the sequence of the α-chain of two teleost fish, the carp (Hilse and Braunitzer 1968) and a catostomid fish (Powers and Edmundson 1972), are known. Both haemoglobins are tetrameric mammalian-type molecules. However, the cyprinoids and catostomids are 'recent' fishes and relatively highly evolved (Romer 1966). Their haemoglobins are probably well removed from the ancestral respiratory molecule.

Although the elasmobranchs arose later than the bony fishes, they are generally considered to be a primitive class of animals. The heterodontid sharks have a fossil history dating back to the mid-Jurassic (Moy-Thomas 1939) and are therefore, with the exception of the Cyclostomata, the most primitive extant vertebrates. It has been established that the haemoglobins of a number of elasmobranchs are tetrameric (Anderson *et al.* 1973). Therefore, the determination of the primary structure of the haemoglobin from a heterodontid shark could conceivably add to our knowledge of protein evolution. Accordingly this paper, the first in a series, reports on the isolation, separation and fractionation of the haemoglobin from the Port Jackson shark, *Heterodontus portusjacksoni.*

Materials and Methods

Collection and Treatment of Blood Samples

Adult Port Jackson sharks were bled regularly at Marineland, Manly, N.S.W., or Taronga Park Zoo, Mosman, N.S.W.; alternatively, blood was obtained from sharks captured by skin-diving off the New South Wales coast (McLaughlin and O'Gower 1971).

Blood was taken from the subcaudal vein of the sharks with a glass syringe and 17-gauge needle. The volume removed varied with the size of the animal, 20 ml being the maximum taken from adult females about 1·5 m in length. The blood was stored in heparinized sample tubes which were packed in ice during transit to the laboratory. The plasma and leucocytes were removed after centrifugation at 1250 *g*. Isotonic saline solution (1·5% NaCl) was used to wash the red cells three times. The packed red cells were lysed by the addition of an equal volume of distilled water and 0·1 volume of CCl_4. After vigorous shaking the lysed cells were centrifuged at 20000 *g* for 15 min on a Sorvall RC2B refrigerated centrifuge at 0–4°C. The clear haemoglobin solution was removed and the cell debris washed and recentrifuged to obtain residual haemoglobin. Haemoglobin concentrations were determined by the cyanmethaemoglobin method (International Committee for Standardisation in Hematology 1967). In later experiments, when it was realized that oxidation of thiol groups was occurring, 0·1 ml of mercaptoethanol per 100 ml of haemolysate was added at this stage and the mixture was reacted for 1 h at room temperature. Reactive thiol groups on the intact haemoglobin molecule, and the excess mercaptoethanol, were carboxymethylated with 268 mg of iodoacetic acid at pH 8·5 in 0·15M tris buffer.

Chromatography of Carboxymethylated Haemoglobin

Cation-exchange chromatography at 4°C on Bio-Rex 70 (Biorad Laboratories, California) was used to separate the carboxymethylated haemoglobins. A column, 17 by 2·8 cm, was packed with the resin as described by Thompson *et al.* (1969) and equilibrated with the starting buffer: 0·05M NaH_2PO_4–0·01M KCN–0·001M EDTA, titrated to pH 6·35 with phosphoric acid if necessary. The carboxymethylated haemoglobin solution was dialysed against four changes of the starting buffer. After the haemoglobin solution had been loaded, 100 ml of starting buffer was passed through the column followed by a linear NaCl gradient from 0 to 0·5M, using 400 ml buffer in each chamber and a flow rate of 180 ml/h. Approximately 10-ml fractions were collected and the relative protein and haemoglobin concentrations in each tube were determined spectrophotometrically at 280 and 540 nm respectively. The peak tubes were bulked and pressure-dialysed to small volumes (Berggard 1961).

Preparation of Globin and Conversion to Reduced Carboxymethylated Globin

Globin was prepared by acid–acetone precipitation at −20°C (Anson and Mirsky 1930) of the concentrated haemoglobin solutions. The precipitated globin was centrifuged at 3000 *g* for 2 min

and the supernatant poured off. Ice-cold acetone was used to wash the globin three times, or until only traces of haem remained. After the final washing and centrifugation the globin was dissolved in 6M guanidine hydrochloride and carboxymethylated by the variation of the method of Crestfield *et al.* (1963) used by Moon and Thompson (1969). On completion of the reaction the globin was dialysed against several changes of the chromatography buffer, then frozen and stored at −20°C to minimize carbamoylation.

Chromatography of S-*carboxymethylglobin*

The fully carboxymethylated globin was fractionated on DEAE-Sephadex A25 (Pharmacia, Uppsala, Sweden) using a modification of the method of O'Donnell (1973). Deionized 8M urea was made 0·025M in tris and 0·1% in mercaptoethanol and the pH was adjusted to 8·5 with 10N HCl. After equilibration of the column (1·8 by 15 cm) a sample of the frozen globin solution, containing approx. 100 mg protein, was thawed and loaded. After the protein had been washed into the resin 100 ml of starting buffer were pumped through the column at 66 ml/h using a Beckman Accu-Flo pump. A further 150 ml of buffer, made 0·3M with NaCl, were then pumped through the column. Fractions of 4·0 ml were collected and assayed for protein at 280 nm. The peak tubes were bulked, dialysed exhaustively against tap water and freeze-dried.

Electrophoresis

Electrophoresis was carried out using Cellogel (Chemetron, Milano, Italy) according to the method of Pabis *et al.* (1968). Haemoglobins were separated at room temperature at a current of 0·75 mA/cm and a bridge gap of 10 cm. The pH of the buffer was 8·6. For the separation of the globin chains the same conditions and tank buffer were used but the Cellogel strips were pre-soaked for 1 h in a solution of the buffer made 8M with urea. Gels were stained with Amido Black and destained in the normal manner (Kohn 1968).

Ultracentrifugation

Sedimentation velocity experiments were carried out in a Beckman model E analytical ultracentrifuge using a single-sector cell with quartz windows and Kel-F centrepiece. A 4° wedge cell was used in place of the Schlieren counterbalance. Experiments were carried out at 59 780 rev/min and the temperature was regulated and maintained at 20°C. The automatic camera was set for 8-min intervals, the first photo being taken when the peak was visible. Eastman Kodak type 1N (red sensitive) plates, containing five exposures, were used and the plates were later read on a Nikon comparator. A shark haemoglobin solution 48 h old was used for the first run. The same solution was treated with mercaptoethanol (0·5 μl/mg protein) and maintained at 4°C overnight before being used for the second run.

Amino Acid Analyses

These were carried out, using a Beckman model 120C amino acid analyser, on solutions that had been hydrolysed in sealed evacuated tubes (Crestfield *et al.* 1963) with redistilled 6N HCl containing 0·1 mg phenol/ml at 110°C for 24, 48 and 72 h. Calculations of the number of residues were based on the chains being similar in length to human globin chains and used the content of the amino acids stable to hydrolysis to estimate the contribution in residues per mole. Extrapolation of the amounts of amino acids destroyed or liberated with time followed the standard procedure (Hill 1965).

Estimation of Sulphydryl Groups

Sulphydryl groups were determined by the method of Ellman (1959) on globin made from *S*-carboxymethylated haemoglobin and also on globin prepared from untreated haemoglobin in the following way: about 5 mg of protein was accurately weighed and dissolved in 1 ml of water. To this was added 1·5 ml of 0·2M tris–HCl–0·02M EDTA buffer (pH 8·2), with mixing, followed by 0·5 ml of 5% sodium dodecyl sulphate in water then 0·2 ml of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) solution (containing 0·8 mg DTNB) in 0·2M potassium phosphate buffer (pH 7·0). After a few minutes 6·8 ml of water were added and the extinction of the mixture was read at 412 nm against a reagent blank within 30 min. A value of 13 600 was taken as the molar extinction coefficient of DTNB (Habeeb 1972), although other workers have reported values ranging from 11 400 to 13 000 (Guidotti 1967; Robyt *et al.* 1971; Brauner and Ullrich 1972).

Results

Ultracentrifugation

When centrifuged the untreated shark haemoglobin was resolved into a major peak which developed a leading and a trailing shoulder. The major peak and the leading shoulder both sedimented at a faster rate than the human haemoglobin standard, while the trailing shoulder appeared to sediment at approximately the same rate as the standard (Fig. 1). After treatment with mercaptoethanol the shark haemoglobin was resolved into a symmetrical peak sedimenting at approximately the same rate as the human standard (Table 1). Towards the end of the run this peak developed a minor leading shoulder. The simplest explanation for these observations is that the lighter species of shark haemoglobin is formed, on reduction, at the expense of the major and minor heavier species. Since no species lighter than 61 000 daltons was observed, this appears to indicate the presence *in vivo* of a tetrameric haemoglobin molecule with some exposed reactive thiol groups that readily undergo oxidation to disulphide bonds, causing polymerization.

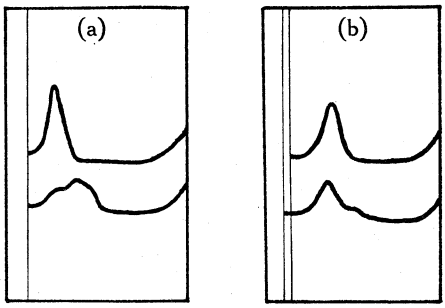

Fig. 1. Tracings of sedimentation velocity patterns, obtained at 20°C and 59 780 rev/min, of (*a*) untreated shark haemoglobin and (*b*) shark haemoglobin incubated overnight with mercaptoethanol. The upper curve in each case was formed by the human haemoglobin standard. Protein concentration was 0·35% in 0·1M phosphate buffer, pH 7·0. Five exposures were taken at 8-min intervals, the first being taken when the peaks appeared. The curves illustrated were taken from the final exposure in each run.

Separation of Haemoglobins

Ion-exchange chromatography resolved the carboxymethylated haemoglobin solution into three major components (Fig. 2). The first component, eluted with the buffer front, is a non-haem protein of high molecular weight which is polymorphic in *H. portusjacksoni* (Nash and O'Gower, unpublished data). This protein migrates more slowly than the haemoglobin on Cellogel. There are two carboxymethylated haemoglobin components eluted with the salt gradient. The major peak (operationally defined as Hb-I) represents approximately 55% of the total haemoglobin while the more positively charged component (Hb-II) represents approximately 41% of the total. A further minor peak can be eluted with strong salt and is probably polymerized material. The carboxymethylated fractions Hb-I and Hb-II are difficult to resolve electrophoretically at pH 8·6, whereas they were readily separated at pH 6·35 on the cation-exchange column.

Separation of the Globin Chains from Carboxymethylated Fraction Hb-I

Chromatography of the shark globin resulted in the separation of the protein into three components (Fig. 3). The first two peaks were eluted close to the buffer

Table 1. Sedimentation coefficients and approximate molecular weights of shark haemoglobins

Sedimentation coefficients were calculated for both shark and human haemoglobins by the method of Schachman (1957). Approximate molecular weights (W) were calculated using the formula $W_s = W_h \times (S_s/S_h)^{3/2}$, where S is the sedimentation coefficient and the subscripts s and h designate shark and human haemoglobins respectively. The molecular weight of human haemoglobin was taken to be 64 500

	Sedimentation coefficient	Molecular weight
Human haemoglobin	3·7	—
Untreated shark haemoglobin	6·5	151 000
Shark haemoglobin after mercaptoethanol treatment	3·6	61 000

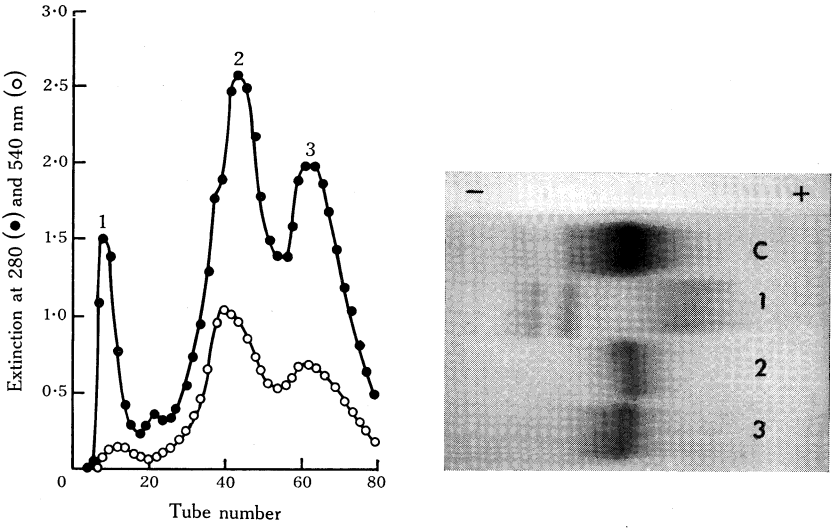

Fig. 2. Separation of haemoglobins of shark red cell haemolysate on a column of Bio-Rex 70 (2·8 by 17 cm) by linear gradient elution at 4°C after 100 ml of the starting buffer (0·05M NaH_2PO_4–0·01M KCN–0·001M EDTA, pH 6·35) had been passed through the column. The limit buffer contained 0·5M NaCl and there were 400 ml of buffer in each chamber. Fraction size 10 ml; flow rate 180 ml/h. The non-haem component is eluted first. Diagram at right shows the electrophoretic pattern obtained when the separated components were run on Cellogel at pH 8·6. The origin was at the left edge of the photograph. C = unfractionated carboxymethylated haemolysate.

front while 0·3M NaCl was required to elute the third peak. Of the three peaks the first is u.v.-light-absorbing, non-protein material; the second and third peaks appear to represent the globin chains. A peptide map of a tryptic digest of the more acidic

protein yielded a peptide tyrosylarginine, which has been *C*-terminal in all α-chains so far examined (Dayhoff 1972). The order of elution of the shark globin chains from the anion-exchange resin was therefore assumed to be β then α.

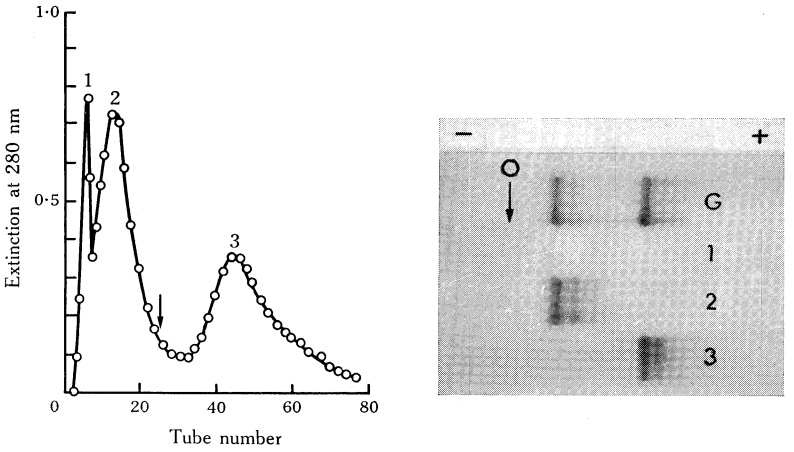

Fig. 3. Separation of the globin chains from shark haemoglobin-I on a column of DEAE-Sephadex A25 (1·8 by 15 cm) at room temperature. The first two components were eluted with 100 ml of the starting buffer (8M urea–0·025M tris–HCl–0·1% mercaptoethanol, pH 8·5), while the third component was eluted with the starting buffer made 0·3M with respect to NaCl. Fraction size 4 ml; flow rate 66 ml/h. Arrow indicates point at which starting buffer was changed to 0·3M NaCl. Diagram at right shows separation of the components by electrophoresis at pH 8·6 on Cellogel soaked in 8M urea buffer (O = origin). The fainter bands of faster mobility are carbamoylated components. G = whole globin.

Amino Acid Analysis of Isolated Chains

Table 2 shows the most probable amino acid composition of the two chains. These values are based on the results that have been extrapolated for losses on hydrolysis, or incomplete hydrolysis. The results indicate marked differences between the content of many amino acids such as lysine, glutamic acid, alanine, valine and isoleucine. The totals of the most probable values are similar to those normally found for α- and β-globin chains of mammalian species. A more precise estimate of the amino acid content of the chains will be given by the amino acid sequences of the chains when these have been determined. The approximate values provide valuable data on which to base fragmentation studies, e.g. with cyanogen bromide.

Sulphydryl Group Determination

Globin made from *S*-carboxymethylated haemoglobin analysed for six free sulphydryl groups per tetramer, while untreated globin appeared to have eight to ten such groups. It was therefore assumed that two to four reactive cysteine residues were present on the surface of the native protein. The total number of sulphydryl groups expected for a tetrameric molecule, containing four subunits, two of each of the globin chains (Table 2), would by analysis be eight to ten.

Discussion

Multiple haemoglobins appear to be the rule, rather than the exception, amongst the lower vertebrates, especially marine animals (Hashimoto *et al.* 1960; Riggs 1970; Binotti *et al.* 1971). Although little work has been done on the blood of elasmobranchs, Manwell (1963) and Anderson *et al.* (1973) both reported the presence of multiple haemoglobins in the haemolysates from several species of sharks. In a study parallel to the one being reported, Martin (1972) demonstrated that the apparent heterogeneity of the haemoglobin from the dogfish, *Scylliorhinus canicula*, was due to aggregation of a single haemoglobin species. This aggregation could be reversed by reduction of the disulphide bridges formed between reactive cysteine residues, as has been demonstrated in the present work.

Table 2. Amino acid composition of the globin chains from shark haemoglobin
Values are moles per mole of globin calculated from the number of μmoles recovered from the column, and converted to moles per mole by reference to phenylalanine, which is stable to hydrolysis. Hydrolysis was for 24–72 h at 110°C in 6N HCl, with corrections for losses on hydrolysis or incomplete hydrolysis

Amino acid	α-Globin: Mean or extrapolated value	α-Globin: Most probable value	β-Globin: Mean or extrapolated value	β-Globin: Most probable value
Lysine	10·1	10	14·6	15
Histidine	6·9	7	7·3	7
Arginine	3·7	4	2·4	2–3
SCM-cysteine	3·2	3	1·5	1–2
Aspartic acid	11·6	12	9·9	10
Threonine	(10)	10	(9)	9
Serine	(11)	11	(9)	9
Glutamic acid	10·5	10	15·0	15
Proline	3·2	3	2·3	2
Glycine	3·8	4	8·7	9
Alanine	24·5	24	13·0	13
Valine	8·0	8	14·0	14
Methionine	0·9	1	1·6	2
Isoleucine	1·5	2	6·6	7
Leucine	16·4	16	13·4	13
Tyrosine	6·6	7	5·6	6
Phenylalanine	8·0	8	8·0	8
Tryptophan[A]	1·3	1	3·2	3
Total		141		145–7

[A] Estimated by the method of Beavan and Holiday (1952).

Fractionation of the haemoglobins of *H. portusjacksoni* was impossible without prior carboxymethylation of the reactive sulphydryl groups on the surface of the molecule. The ultracentrifugation studies clearly demonstrated that aggregation of the tetrameric molecular species occurs and that the thiol mercaptoethanol causes reversion to a molecule of approximately 61 000 daltons. This aggregation can occur within 2 h of lysis, the degree of polymerization being time-dependent. However,

fractionation of the carboxymethylated haemolysate consistently gave two haemoglobin fractions and solutions of these two proteins were indefinitely stable at 4°C, as judged by electrophoretic studies. After taking precautions to carboxymethylate reactive thiol groups, fractionation of shark haemolysates on ion-exchange columns always gave two haemoglobin fractions. It is therefore probable that *H. portus-jacksoni* possesses two haemoglobins in the red cell in approximately equal proportions, but until the Hb-II fraction has been characterized by amino acid analysis, peptide mapping, and amino acid sequence studies, its relationship to Hb-I is unresolved. It has not so far been demonstrated to be a true component as distinct from a separation due to interaction effects or non-uniform carboxymethylation.

Similar reactivity of exposed thiol groups in haemoglobins has been reported previously for a variety of species, e.g. reptiles and amphibians (Riggs *et al.* 1964), monkeys (Barnicot *et al.* 1966) and humans and mice (Bonaventura and Riggs 1967).

Using Ellman's method (1959) two to four free sulphydryl groups were calculated, by difference between the sulphydryl content of carboxymethylated haemoglobin and fully carboxymethylated globins, to be present on the surface of the native haemoglobin molecule. Other thiol-blocking reagents such as iodoacetamide or *N*-ethylmaleimide could be used to prevent aggregation by disulphide bond formation. Compounds that form disulphide bonds with the reactive thiol groups, e.g. DTNB, were less satisfactory due to disulphide interchange reactions (Baptist, Nash and Thompson, unpublished data).

The minor protein peak eluted in the void volume of the cation-exchange column contained traces of haemoglobin. However, electrophoresis strips stained for haemoglobin by the method of Smithies (1962) showed no traces of peroxidase activity in the vicinity of this protein. It constitutes approximately 5% of the total protein, is polymorphic, and preliminary gel-filtration studies indicate that it has a molecular weight in excess of 5×10^5 daltons. Studies on the distribution of this protein, not reported here, show that its expression is determined by a triple allelic system and the gene frequencies of the phenotypes appear to be geographically dependent (Nash and O'Gower, unpublished data).

Total amino acid analyses reveal several major variations between shark α- and β-chains in their complements of individual amino acids. The amounts of hydrophilic residues, noticeably lysine and glutamic acid, are markedly different, as are the relative proportions of the hydrophobic residues such as alanine, isoleucine and valine. A comparison of the complement of amino acids in shark α-chain and that from *Catostomus clarkii* (Powers and Edmundson 1972) reveals similar variations, the teleost having many more proline residues, while the shark has greater numbers of cysteine and alanine residues. The low number of proline residues in both shark subunits may prove to have interesting effects on the secondary structure of the intact proteins.

Acknowledgments

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References

Anderson, M. E., Olson, J. S., Gibson, Q. H., and Caret, F. G. (1973). *J. Biol. Chem.* **248**, 331.

Anson, M. L., and Mirsky, A. E. (1930). *J. Gen. Physiol.* **13**, 469.

Bardack, D., and Zangerl, R. (1968). *Science* (*Wash. D.C.*) **162**, 1265.

Barnicot, N. A., Huehns, E. R., and Jolley, C. J. (1966). *Proc. R. Soc. Lond. B* **165**, 224.

Beavan, G. H., and Holiday, E. R. (1952). *Adv. Protein Chem.* **7**, 319.

Berggard, I. (1961). *Ark. Kemi* **18**, 291.

Binotti, I., Giovenco, B., Giardina, B., Antonini, E., Brunori, M., and Wyman, J. (1971). *Arch. Biochem. Biophys.* **142**, 274.

Bonaventura, J., and Riggs, A. (1967). *Science* (*Wash. D.C.*) **158**, 800.

Brauner, T., and Ullrich, J. (1972). *Z. Physiol. Chem.* **353**, 825.

Braunitzer, G., and Fujiki, H. (1969). *Naturwissenschaften* **56**, 322.

Buettner-Janusch, J., and Hill, R. L. (1965). *Science* (*Wash. D.C.*) **147**, 836.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963). *J. Biol. Chem.* **238**, 622.

Dayhoff, M. O. (1972). 'Atlas of Protein Sequence and Structure.' (National Biomedical Research Foundation: Silver Spring.)

Ellman, G. L. (1959). *Arch. Biochem. Biophys.* **82**, 70.

Fitch, W. M., and Margoliash, E. (1970). In 'Evolutionary Biology'. (Eds T. Dobzhansky, M. K. Hecht and W. C. Steere.) Vol. 4, p. 67. (Appleton Century-Crofts: New York.)

Goodman, M., Barnabas, J., Matsuda, G., and Moore, G. W. (1971). *Nature* (*Lond.*) **233**, 604.

Guidotti, G. (1967). *J. Biol. Chem.* **242**, 3673.

Habeeb, A. F. S. A. (1972). In 'Methods in Enzymology'. (Eds C. H. Hirs and S. N. Timasheff.) Vol. XXV, Part B, p. 457. (Academic Press: New York.)

Hashimoto, K., Yamaguchi, Y., and Matsuura, F. (1960). *Bull. Jap. Soc. Sci. Fish.* **26**, 827.

Hill, R. L. (1965). *Adv. Protein Chem.* **20**, 37.

Hilse, K., and Braunitzer, G. (1968). *Hoppe-Seylers Z. Physiol. Chem.* **349**, 433.

Ingram, V. M. (1961). *Nature* (*Lond.*) **189**, 704.

International Committee for Standardisation in Hematology (1967). *Med. J. Aust.* **1967**(2), 84.

Kohn, J. (1968). In 'Chromatography and Electrophoretic Techniques'. (Ed. I. Smith.) Vol. 2, p. 84. (William Heinemann: London.)

Li, S. L., and Riggs, A. (1970). *J. Biol. Chem.* **245**, 6149.

Manwell, C. (1963). *Arch. Biochem. Biophys.* **101**, 504.

Martin, F. (1972). *Biochemie* **54**, 25.

McLaughlin, R. H., and O'Gower, A. K. (1971). *Ecological Monographs* **41**, 271.

Moon, K. E., and Thompson, E. O. P. (1969). *Aust. J. Biol. Sci.* **22**, 463.

Moy-Thomas, J. A. (1939). *Biol. Rev.* **14**, 1.

O'Donnell, I. J. (1973). *Aust. J. Biol. Sci.* **26**, 401.

Pabis, A., Sulis, E., Alessio, L., and Mannucci, P. M. (1968). *Clin. Chim. Acta* **20**, 449.

Powers, D. A., and Edmundson, A. B. (1972). *J. Biol. Chem.* **247**, 6694.

Riggs, A. F. (1970). In 'Physiology of Fishes'. (Eds W. S. Hoar and D. J. Randall.) Vol. 4, p. 209. (Academic Press: New York.)

Riggs, A., Sullivan, B., and Agee, J. R. (1964). *Proc. Natl Acad. Sci. U.S.A.* **51**, 1127.

Robyt, J. F., Ackerman, R. J., and Chittenden, C. G. (1971). *Arch. Biochem. Biophys.* **147**, 262.

Romer, A. S. (1966). 'Vertebrate Paleontology.' 3rd Ed. p. 12. (University of Chicago Press: New York and London.)

Schachman, U. K. (1957). In 'Methods in Enzymology'. (Eds S. P. Colowick and N. O. Kaplan.) Vol. 4, p. 32. (Academic Press: New York.)

Smithies, O. (1962). *Arch. Biochem.* Suppl. 1, p. 125.

Thompson, E. O. P., Hosken, R., and Air, G. M. (1969). *Aust. J. Biol. Sci.* **22**, 449.

Zuckerkandl, E., and Pauling, L. (1965). In 'Evolving Genes and Proteins'. (Eds V. Bryson and H. J. Vogel.) p. 97. (Academic Press: New York.)

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