HAEMOGLOBINS OF THE ADULT DOMESTIC FOWL *GALLUS DOMESTICUS*

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Summary

The haemoglobins of the adult domestic fowl were resolved by cation-exchange chromatography into two distinct components (designated Hb I and Hb II) in a ratio about 3 : 1. A third minor acidic component, Hb III, representing only 1% of the total was also present. Hb I and Hb II and each of their globin subunits (α^{I}, β^{I}; α^{II}, β^{II}) were subjected to amino acid analysis. As reported by other workers, Hb II was found to have more of the dicarboxylic amino acids and less of the dibasic amino acids than Hb I.

The two types of fowl haemoglobin are made up of four different globin chains, but amino acid composition data and tryptic peptide mapping suggest that β^{I} and β^{II} are more closely related than previously indicated by other workers. The amino-terminal sequences of β^{I} and β^{II} were Val-His-Gly- whereas in α^{I} it was Val-Leu-Ser- and in α^{II} it was the unexpected sequence Met-Leu-Thr-. The finding of α-amino groups on all globin chains is not consistent with previous reports of acetylated amino-terminal residues in fowl haemoglobin.

I. Introduction

Adult chicken haemoglobin is heterogeneous. Accumulated evidence indicates that it can be separated electrophoretically and chromatographically into two distinct components: a major one, designated Hb I,† comprising 70–80% of the total haemoglobin, and a more acidic minor component, designated Hb II,† comprising 20–30% (for recent reviews see Kabat 1968; Washburn 1968*a*).

Amino acid analyses of these two haemoglobins by Van der Helm and Huisman (1958) and Saha (1964) showed that Hb II contained more dicarboxylic amino acids and less dibasic amino acids than Hb I. This fact probably accounts for their

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† The nomenclature used here is similar to that given by many of the workers studying fowl haemoglobin. The haemoglobins are numbered in order of decreasing concentration while the globin chains are lettered according to their chromatographic elution rate. α represents the first component eluted from the column and corresponds to the slow electrophoretic subunits; β represents the second eluted component and corresponds to the faster, more cationic, electrophoretic subunits. (The *N*-terminal sequences of Hb I α- and β-chains are homologous with mammalian α- and β-chain sequences.) The superscripts indicate whether they are from Hb I or Hb II. Subscripts, e.g. β_2, were used by Saha (1964) and others to indicate the presence of two β chains in β-globin. This system has only been used in this report when comparing our data with that previously published.

differences in electrophoretic and chromatographic behaviour. There are, however, marked disparities in the amino acid composition data presented by these workers.

Starch-gel electrophoresis of globin or haemoglobin at pH 1·9 and chromatography in acidic urea solutions reveals two subunit classes (here designated α and β) in each haemoglobin (Muller 1961; D'Amelio 1966; Hashimoto and Wilt 1966). According to the criteria of immunodiffusion analysis and tryptic peptide mapping (Hashimoto and Wilt 1966; Kabat 1968) the haemoglobins appear to share many common amino acid sequences and the most extensive differences between them reside in the α subunits. It seems clear from the starch-gel electrophoretic data of these workers that the α subunits of Hb I and Hb II migrate at different rates and hence are probably structurally different. The β subunits, on the other hand, seem to migrate nearly, if not entirely, identically. On this basis conflicting conclusions have been drawn as to whether both haemoglobin components have a globin chain in common (Muller 1961; D'Amelio 1966; Hashimoto and Wilt 1966).

In more comprehensive amino acid composition studies Saha (1964) presented evidence that all the subunit classes were different, i.e. the two haemoglobins had no common globin chains.

The present studies report on the separation by column chromatography and amino acid analyses of Hb I and Hb II and each of their globin chains. Amino acid compositions, tryptic peptide maps, and *N*-terminal sequence data are discussed in relation to differences between the Hb I and Hb II component chains. The finding of *N*-terminal α-amino groups in all globin chains is not consistent with data regarding *N*-terminal acetyl masking groups, reported to be present by other workers (Satake, Sasakawa, and Maruyama 1963; Matsuda, Maekawa, and Otsubo 1965).

II. Experimental Procedures

(*a*) *Animals*

Adult White Leghorn fowls weighing between 1·5 and 2 kg were purchased from the Beringa Stud Farm and Hatchery, West Pennant Hills, N.S.W.

(*b*) *Preparation of Haemoglobin Solutions*

The fowls were decapitated and their blood collected in an ice-cooled beaker to which some heparin had been added. Usually between 35 and 45 ml of blood were obtained. The blood was filtered through nylon mesh, and the plasma and most of the leucocytes were removed by centrifugation at 1000 *g* for 10 min in a Servall refrigerated centrifuge at 0–4°C. The red cell pellet was washed four times with five volumes of isotonic NKM saline (0·13M NaCl, 5 mM KCl, and 7·5 mM $MgCl_2$—Lingrel and Borsook 1963; Kabat and Attardi 1967). The washed, packed red cells were lysed by the addition of three volumes of distilled water. Clear haemoglobin solution was obtained after 1 hr by centrifugation at 20,000 *g* for 15 min. Non-haemoglobin protein was removed by adjusting the pH to 5·15 with 1M acetic acid and centrifuging off the resultant precipitate at 10,000 *g* for 5 min. The pH of the supernatant was then adjusted to 7·0 with 2N NaOH and the haemoglobins converted to the carbon monoxide form by gentle shaking under CO while cooling in ice. The carbonmonoxyhaemoglobin was fractionally precipitated with $(NH_4)_2SO_4$ between 0·45 and 0·8 saturation at 0–4°C. The haemoglobin precipitate was dissolved and dialysed overnight against 0·005M potassium phosphate buffer, pH 6·7, saturated with carbon monoxide.

Haemoglobin concentration was determined by the cyanmethaemoglobin method (Drabkin 1945; Anon. 1967).

(*c*) *Chromatographic Separation of the Haemoglobins*

Cation-exchange chromatography at 4°C on Bio-Rex 70 columns (Bio-Rad Laboratories, 200–400 mesh, 10·2 m-equiv/g; equivalent to Amberlite CG-50, type II) was used to separate the haemoglobins. Haemoglobin solutions for chromatography were treated with carbon monoxide and the carbonmonoxyhaemoglobin dialysed against 0·1M potassium phosphate, pH 6·7. All solutions were saturated with carbon monoxide. For analytical purposes dialysed carbonmonoxyhaemoglobin solution (3 ml, 50–150 mg) was loaded onto a Bio-Rex 70 column (1 by 35 cm) equilibrated with 0·1M potassium phosphate buffer, pH 6·7, and fractionated by a linear potassium phosphate gradient from 0·1 to 0·4M (200 ml in each chamber; cf. Marchis-Mouren and Lipmann 1965). Fractions of 4 ml were collected at about 20 ml/hr and measured spectrophotometrically at 540 nm. For preparative purposes, up to 2 g of carbonmonoxyhaemoglobin could be chromatographed successfully on columns 3 by 30 cm, using stepwise gradient elution at 100 ml/hr (0·1M potassium phosphate, pH 6·7, elutes Hb III; 0·15M elutes Hb II; and 0·35M elutes Hb I). The peak zones were bulked, concentrated by pressure dialysis (Berggard 1961), and dialysed against distilled water at 4°C.

(*d*) *Preparation of Globin and Conversion to Reduced Carboxymethylated Globin*

Globin was prepared from well-dialysed carbonmonoxyhaemoglobin solutions by acid acetone precipitation at −20°C similar to the procedure of Muller (1961). Traces of haem were removed by washing with cold acid acetone. The precipitated globin was washed with cold acetone then dissolved in water, dialysed, lyophilized, and stored at −20°C.

Carboxymethylation of globin reduced with 8M urea–mercaptoethanol was carried out under nitrogen in urea solution using iodoacetic acid as described by Crestfield, Moore, and Stein (1963). After completion of the reaction, the pH was adjusted to about 2 with hydrochloric acid, and urea and other reagents were removed either by exhaustive dialysis against 0·005M HCl, or by passage through a column (2 by 35 cm) of coarse Sephadex G-25 equilibrated with 0·5% formic acid. The reduced, carboxymethylated globin was lyophilized and stored at −20°C.

(*e*) *Chromatographic Separation of the Globin Chains*

A slight modification of the procedure of Muller (1961) was adopted. Bio-Rex 70 was equilibrated at room temperature with 10% formic acid solution and a resin bed 1·5 by 10 cm was poured. Reduced, carboxymethylated globin (SCM-globin, 30–100 mg, in 3–5 ml 10% formic acid) was applied to the column and washed in with about 5 ml of 10% formic acid, followed by about 50 ml of 2M urea, pH 1·9. Separation of the globin chains was achieved at room temperature by convex gradient elution (type A, Morris and Morris 1964) with solutions of urea titrated to pH 1·9 with 6N hydrochloric acid. Elution was started by running 300 ml of 7M urea (pH 1·9) from the reservoir into the mixing chamber containing 75 ml of 2M urea (pH 1·9), followed by 300 ml of 9M urea (pH 1·9). The flow rate was 60 ml/hr and 5-ml fractions were collected. The effluent was monitored continuously at 280 nm with an Isco ultraviolet recorder or the extinction at 280 nm of each fraction was measured in a Beckman DB spectrophotometer. The peak zones were pooled, dialysed thoroughly against several changes of water at 4°C, followed by 0·005M HCl solution, then lyophilized and stored at −20°C.

The urea solutions were prepared from 10M urea deionized by passage through a mixed-bed ion-exchange resin at 30°C.

(*f*) *Gel Electrophoresis of Haemoglobins and Globins*

(i) *Starch-gel Electrophoresis.*—Horizontal starch-gel electrophoresis (Smithies 1955) of the haemoglobins in gels containing 2M urea (Saifer, Robin, and Ventrica 1961), 0·01M KCN, and 0·01M EDTA was employed. Electrophoresis was performed at 4°C and pH 8·6 with the discontinuous buffer system of Poulik (1957) for about 16 hr at 8 V/cm and 10 mA. The gels were stained for protein with amido black in methanol–acetic acid–water (45 : 10 : 45 v/v) and for haemoglobin with benzidine–hydrogen peroxide.

Electrophoresis of globin solutions in starch gels were carried out at pH 1·9 [1·4M formate, ionic strength = 0·02 (Muller 1961)] for 6 hr at 8 V/cm and then stained with nigrosine (β subunits tend to fade after staining with amido black).

(ii) *Analytical Polyacrylamide-gel Electrophoresis.*—The haemoglobins were examined by the Davis (1964) and Ornstein (1964) disc-electrophoresis method, using the modification of Moss and Ingram (1968). The reservoir buffers contained 6·32 g Tris, 3·94 g glycine, and 0·10 g KCN per litre and electrophoresis was carried out at 2°C. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine were from Eastman Organic Chemicals, New York.

Haemoglobin samples were adjusted to a concentration of 2·5 mg/ml by the addition of 0·1 ml of a solution 2% in $K_3Fe(CN)_6$, 0·5% in KCN, and 1% in $NaHCO_3$; 0·5 ml of 40% sucrose; and water to 1 ml final volume. The cyanmethaemoglobin (10–20 μl) was layered over the concentrating gel and electrophoresis commenced by maintaining a current of 0·5 mA/tube for 15 min followed by 2 mA/tube for 1 hr. The gels, after removal from the glass tubes, were stained for 15 min by immersion in 1% amido black in 7% acetic acid. Excess stain was removed by several washes with 7% acetic acid.

(*g*) *Amino Acid Analysis*

Hydrolysis of SCM-globin (1–5 mg) was carried out at 110°C in 1 ml of redistilled 6N HCl containing 0·1 mg phenol (Sanger and Thompson 1963) in evacuated, sealed tubes for 24, 48, and 72 hr. Amino acid analyses were performed by the accelerated procedure of Spackman (1964) with a Beckman Model 120C amino acid analyser. For calculation of the number of residues the molecular weight of haemoglobin and of globins (α and β) were assumed to be the same as in human and other species. The content of the stable amino acids (His, Arg, Pro, Ala, Leu, and Phe) was used to estimate the contribution in residues per mole. Extrapolation of amino acids destroyed during hydrolysis or which were liberated slowly followed the standard procedure (Hill 1965). Tryptophan was not determined quantitatively but a minimal number of residues was deduced from tryptic peptide maps after identification with Ehrlich's reagent (Muller 1961).

(*h*) N-*Terminal Amino Acid Analysis*

N-terminal analysis was carried out on the protein (0·5 μmole) by the three-cycle Edman procedure as described by Blombäck *et al.* (1966) except that coupling with phenylisothiocyanate (50 μl) was in 60% pyridine–water containing 6% *N*-ethylmorpholine. Identification of each phenylthiohydantoin amino acid (PTH) was by thin-layer chromatography on Eastman Chromagram silica gel sheets with fluorescent indicator (Jeppsson and Sjöquist 1967) and by acid hydrolysis of the PTH to regenerate the amino acid (Van Orden and Carpenter 1964) followed by determination on a Beckman Model 120C amino acid analyser. Quantitation of the PTH was by the method of Fraenkel-Conrat, Harris, and Levy (1955) from the maximum ultraviolet absorption. The ratio of the minimum at 240 nm to maximum at 267 nm varied between 0·44 and 0·65 (cf. 0·4 for pure PTH-amino acids—Sjöquist 1957).

(*i*) *Tryptic Digestion and Peptide Mapping*

SCM-globin (2 mg) was digested with TPCK-trypsin (Worthington) at 37°C for 4 hr in 1% ammonium carbonate–ammonia solution, pH 8·7, containing 0·1% phenol (Thompson and O'Donnell 1967).

The soluble peptide mixture (1–1·5 mg) was applied to Whatman 3MM paper and electrophoresis carried out in pyridine–acetic acid–water (25 : 1 : 225 v/v), pH 6·4, at 3000 V for 1 hr under Varsol in a Savant ionophoresis unit. The peptides in a 1-in. strip were sewn onto a second sheet of paper and chromatographed in n-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12 v/v —Hill, Swenson, and Schwartz 1960; Thompson, Hosken, and Air 1969). The peptides were stained with 0·2% ninhydrin in 95% ethanol.

III. Results

(*a*) *Chromatographic Separation of the Haemoglobins*

As shown in Figure 1 fowl haemoglobin can be resolved into two major components and a minor acidic fraction by ion-exchange chromatography. The relative percentage of each haemoglobin component was obtained by summation of the

extinction values at 540 nm. In six separate experiments the mean relative percentage of Hb I was 73·4 (range 66·5–78·0), of Hb II 25·7 (range 21·6–31·0), and of Hb III 0·9 (range 0·4–2·5). Thus the ratio of the haemoglobin peaks, Hb I : Hb II, is about 3 : 1.

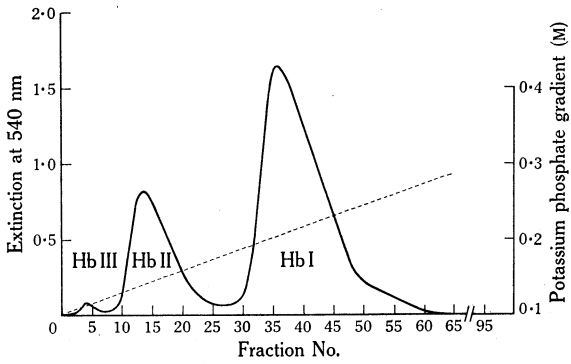


Fig. 1.—Chromatography of adult chicken carbonmonoxyhaemoglobin (125 mg) on a column of Bio-Rex 70 (1 by 35 cm) at 4°C. Buffer was potassium phosphate, pH 6·7, and a linear gradient was established between 0·1 and 0·4M at a flow rate of about 20 ml/hr. Fraction size was 4 ml. Dotted line represents the gradient.

Reproducible results were obtained with carbonmonoxyhaemoglobin provided chromatography was carried out within 1 or 2 days of preparation of the haemoglobin. Saturation of the solutions with carbon monoxide facilitated reproducibility, whereas its omission and aging of the haemoglobins yielded an additional component usually observable as a shoulder or peak after Hb I. This was probably due to methaemoglobin formation.

(*b*) *Chromatographic Separation of the Globin Chains*

As suggested by Muller (1961) fowl globin can be separated chromatographically into its two subunit classes in urea solutions at pH 1·9. In order to reduce the time necessary for the separation and to obtain more discrete peaks a slight modification of the procedure of Muller was adopted as described in the experimental procedures. Figure 2 depicts the separation of α^{I} and β^{I} chains from Hb I without prior conversion to globin or to reduced, carboxymethylated globin, whereas the α^{II} and β^{II} chains were separated in the carboxymethylated form. Generally, reduced, carboxymethylated globin was used and recovery ranged from 50–75% of the amount applied to the column.

Attempts to separate the globin chains were made with:

(1) the pyridine–formic acid gradient system of Dintzis (1961);

(2) the technique of Clegg, Naughton, and Weatherall (1965), in which a CM-cellulose column was used and the developer was of 8M urea with 0·05M mercaptoethanol and phosphate buffer, pH 6·7, under the action of a linear sodium ion gradient;

(3) the method of Sasakawa (1961) and Sasakawa *et al.* (1963), in which an Amberlite CG-50 column and urea solutions 2M in formic acid at pH 2·8 were used.

These were unsuccessful. Poor resolution of the two globin chains and low recovery (less than 30%) was also obtained with the method of Matsuda, Maekawa, and

Otsubo (1965) which was a modification of Sasakawa's (1961) procedure with a convex gradient from 2M urea–3M formic acid to 10M urea–3M formic acid. However, when a formic acid gradient was included with the urea gradient (4M urea–2M formic acid to 10M urea–6M formic acid) and a smaller column (1·0 by 10 cm) and linear gradient were used a similar separation of the globin chains to that shown in Figure 2 resulted except that resolution was not as complete.

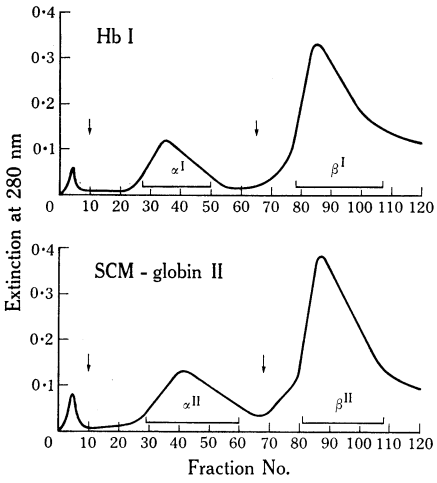


Fig. 2.—Chromatographic separation of the α- and β-chains of globin (90 mg) derived from Hb I and Hb II. The column was Bio-Rex 70 (1·5 by 10 cm) equilibrated with 10% formic acid and elution of the globin chains at room temperature was by stepwise convex gradient elution as described in the text. The arrows indicate the start and change in gradient elution respectively. The initial peaks were unretarded material. Fraction size 5 ml. Flow rate 60 ml/hr.

(c) *Gel Electrophoresis of Haemoglobins and Globins*

The results of starch-gel electrophoresis in pH 8·6 borate buffer of haemolysates obtained from fowl erythrocytes are shown in Figure 3. Two distinct components were resolved. The slower moving (cathodic) component was Hb I whereas the anodic component was Hb II. The components as isolated by chromatography were free from notable cross-contamination.

Analytical polyacrylamide-gel electrophoresis of the cyanmethaemoglobins at pH 9·0 likewise revealed that unfractionated haemoglobin was composed of two components (Fig. 4). The minor acidic component (Hb III) was not apparent probably because of its low concentration. The isolated components Hb I and Hb II migrated as single zones. Hb III gave a "step-ladder" pattern indicative of contamination with non-haem protein and heterogeneity or aggregation or both. Hb III also appeared to be contaminated with Hb II; the observed heterogeneity has been reported before (Hashimoto and Wilt 1966).

With aged preparations (5 days) of cyanmethaemoglobin both Hb I and Hb II split into two components when examined by acrylamide-gel electrophoresis. This observation may in part explain the finding of five electrophoretic cyanmethaemoglobin components by Hashimoto and Wilt (1966).

The starch-gel electrophoretic behaviour of the two globins at pH 1·9 is shown in Figure 5. The α- and β- chains of both globins showed good resolution. The two slow-moving subunits (α-chains) differed slightly in their mobility whereas the more cationic subunits (β-chains) appeared to migrate at the same rate. This latter result agrees with the finding of Hashimoto and Wilt (1966) but differs from that of Muller (1961) who found that these subunits also differed slightly in their mobility.

(*d*) *Amino Acid Compositions of SCM-Globins*

The calculated amino acid compositions of reduced, *S*-carboxymethylated globins from Hb I and HbII and of the α- and β- chains isolated from these are presented in Tables 1–3.

From a consideration of Table 1 it is evident that the present amino acid composition data for Hb I and Hb II are more compatible with those of Saha (1964) than with those of Van der Helm and Huisman (1958). The data of the latter workers indicate an overestimation of the basic amino acids with concomitant underestimation of the acidic and neutral amino acids and a complete absence of the sulphur-containing amino acids, methionine, and cysteine. Failure to detect the latter probably results from their destruction during hydrolysis in the presence of oxygen.

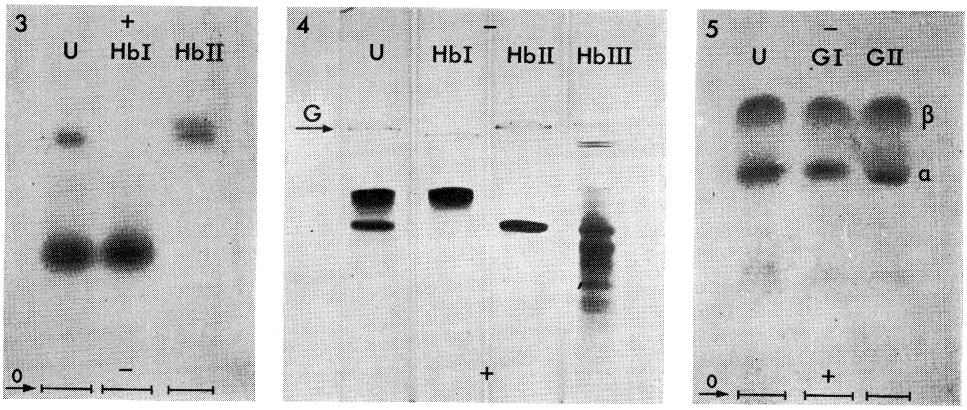


Fig. 3.—Starch-gel patterns of unfractionated carbonmonoxyhaemoglobin (U) and isolated components Hb I and Hb II. pH 8·6, discontinuous buffer system.

Fig. 4.—Polyacrylamide-gel electrophoresis pattern of unfractionated fowl haemoglobin (U) and the isolated components Hb I, Hb II, and Hb III. pH 9·0, discontinuous buffer system. Haemoglobin in the cyanmethaemoglobin form.

Fig. 5.—Starch-gel patterns of globin prepared from unfractionated fowl haemoglobin (U) and the isolated components globin I (G I) and globin II (G II). pH 1·9, 1·4M formate, ionic strength 0·02. The slow-moving subunits are the α-chains whereas the more cationic subunits are the β-chains.

In agreement with the conclusions of both Saha (1964) and Van der Helm and Huisman (1958) the present analytical data indicate that Hb II contains more of the acidic amino acids and less of the basic amino acids than Hb I.

Because of their similarity further comparisons will be between our data and that of Saha (1964) and are considered in Section IV. In all cases in our results the total number of amino acid residues exceeded the value assumed in the original calculation. This was particularly noticeable with the α^{II} chains. Only when the complete amino acid sequences have been worked out will more accurate evaluations become possible.

(*e*) *Peptide Mapping of the Isolated Globin Chains*

The isolated SCM-globin chains were digested with trypsin and then finger-printed, the results of which are presented in Figures 6–9. These tryptic peptide

TABLE 1

AMINO ACID COMPOSITION OF SCM-GLOBIN I FROM Hb I AND SCM-GLOBIN II FROM Hb II

Values are given as residues per mole of globin calculated from the μmoles recovered from the column and converted to moles per molecule of 568 residues by reference to the amino acids stable to hydrolysis

Amino Acid	Amino Acid Residues per Molecule					
	24 Hr	48 Hr	72 Hr	Mean or Extrapolated Integral Value	Saha Data*	Van der Helm and Huisman Data†
			SCM-globin I from Hb I			
Lysine	45·3	47·4	46·0	46	47	69
Histidine	33·2	36·3	34·1	35	34	53
Arginine	18·0	17·9	18·5	18	19	30
Aspartic acid	53·1	51·9	49·8	52	53	46
Threonine	35·7	34·2	32·0	(37)‡	30	26
Serine	27·9	25·6	23·6	(30)‡	24	21
Glutamic acid	46·3	45·2	43·8	45	49	32
Proline	24·1	23·6	22·0	23	25	19
Glycine	36·8	35·7	34·6	36	38	28
Alanine	71·8	71·4	70·7	71	80	70
Valine	35·4	42·0	45·5	46§	58	47
Methionine	3·8	3·6	3·8	4	7	0
Isoleucine	19·4	22·1	25·0	25§	29	26
Leucine	63·4	65·5	67·2	65	74	70
Tyrosine	12·6	12·6	12·6	13	17	12
Phenylalanine	30·1	30·7	31·0	31	33	27
SCM-cysteine	8·0	6·0	6·6	7	8	
Total				584	625	576
			SCM-globin II from Hb II			
Lysine	41·8	45·3	44·7	44	44	56
Histidine	24·7	26·6	30·3	27	26	35
Arginine	19·5	20·3	20·0	20	19	20
Aspartic acid	56·2	55·1	53·1	55	54	59
Threonine	28·6	27·5	26·9	(30)‡	28	24
Serine	30·1	28·2	26·3	(32)‡	27	34
Glutamic acid	62·0	61·2	61·8	62	61	55
Proline	22·8	21·3	23·2	22	22	18
Glycine	32·2	31·8	32·1	32	34	27
Alanine	69·7	68·6	67·6	69	70	59
Valine	39·3	43·8	45·5	46§	54	56
Methionine	8·9	8·7	6·6	9	12	0
Isoleucine	12·3	15·3	15·9	16§	18	20
Leucine	66·6	67·6	65·7	67	68	80
Tyrosine	14·7	11·5	13·8	14	17	4
Phenylalanine	31·1	31·2	30·0	31	32	29
SCM-cysteine	5·9	5·4	5·2	6	8	
Total				582	594	576

* Data taken from Saha (1964) for comparison. Based on an assumed molecular weight of 64,500.

† Data taken from Van der Helm and Huisman (1958) but converted to same basis as used here for comparison.

‡ Obtained by linear extrapolation to zero time. § 72-hr values only.

TABLE 2

AMINO ACID COMPOSITION OF α- AND β-CHAINS OF SCM-GLOBIN I FROM Hb I

Values are given as residues per mole of globin calculated from the μmoles recovered from the column and converted to moles per molecule of 140 residues (α-chain) or 144 residues (β-chain) by reference to the amino acids stable to hydrolysis

Amino Acid	Amino Acid Residues per Molecule					
	24 Hr	48 Hr	48 Hr*	72 Hr	Mean or Extrapolated Integral Value	Saha Data†
			α-Chains			
Lysine	12·4	12·2	11·5	12·0	12	12
Histidine	9·7	9·8	9·6	9·5	10	8·5
Arginine	3·4	3·3	2·9	3·3	3	4·5
Aspartic acid	12·1	11·9	11·8	12·6	12	13·5
Threonine	8·8	8·3	8·2	8·0	(9)‡	7·5
Serine	6·0	4·9	4·6	4·5	(7)‡	5·5
Glutamic acid	9·4	9·5	9·8	9·9	10	11·5
Proline	5·4	5·6	6·2	6·0	6	6·5
Glycine	8·9	9·2	9·5	9·6	9	9·5
Alanine	17·7	18·0	18·7	18·0	18	20
Valine	11·2	11·9	12·1	12·5	13§	14·5
Methionine	0·7	0·7	1·1	0·2	1	2
Isoleucine	6·3	6·7	7·1	7·0	7	7
Leucine	14·7	15·2	15·2	15·6	16§	18·5
Tyrosine	3·3	3·6	4·3	3·8	4	4·5
Phenylalanine	7·2	7·2	7·3	7·1	7	8
SCM-cysteine	1·0	0·6	1·1	0·6♯	1	2
Tryptophan					0‖	
Total					145	155·5
			β-Chains			
Lysine	10·3	10·4	10·0	11·9	11	11·5
Histidine	7·4	7·9	7·1	8·0	8	8
Arginine	5·3	5·6	4·7	5·6	5	5
Aspartic acid	13·4	13·6	13·6	13·4	14	13
Threonine	6·9	6·7	6·4	6·5	(7)‡	7
Serine	5·6	5·0	4·7	5·0	(6)‡	6
Glutamic acid	12·4	12·7	13·9	12·8	13	12·5
Proline	5·3	5·4	5·6	5·6	5	6·5
Glycine	8·4	8·6	8·9	8·9	9	9·5
Alanine	17·1	17·3	17·2	17·8	17	20
Valine	12·1	12·6	12·4	12·6	13	14
Methionine	0·6	0·8	1·3	0·8	1	1·5
Isoleucine	6·3	6·9	6·6	6·7	7	7·5
Leucine	18·0	18·5	18·2	18·1	18	19
Tyrosine	2·3	2·4	2·4	2·5	3	3·5
Phenylalanine	8·0	8·2	8·3	8·0	8	8·5
SCM-cysteine	1·3♯	1·2	2·4	0·9	2	2
Tryptophan					2‖	
Total					149	155

* Preparation from a different White Leghorn fowl. Preparation and analyses 6 months later than the other 48-hr sample.

† Data taken from Saha (1964) for comparison. Based on an assumed molecular weight of 31,000 for α_2 (or β_2 for β-chain analysis) and now divided by 2 to correspond to α_1 (or β_1).

‡ Obtained by linear extrapolation to zero time. § From 72-hr value only.

‖ Deduced from tryptic peptide map. ♯ Cysteic acid included.

TABLE 3

AMINO ACID COMPOSITION OF α- AND β-CHAINS OF SCM-GLOBIN II FROM Hb II

Values are given in residues per mole of globin calculated from the μmoles recovered from the column and converted to moles per molecule of 140 residues (α-chain) or 144 residues (β-chain) by reference to the amino acids stable to hydrolysis

Amino Acid	Amino Acid Residues per Molecule					
	24 Hr	48 Hr	48 Hr*	72 Hr	Mean or Extrapolated Integral Value	Saha Data†
			α-Chains			
Lysine	10·7	11·9	10·9	10·6	11	11·5
Histidine	5·9	6·5	6·7	6·6	7	7
Arginine	3·8	4·1	4·3	4·1	4	4·5
Aspartic acid	14·6	14·3	13·2	13·5	14	13·5
Threonine	7·0	6·5	5·8	5·9	(8)‡	7
Serine	7·8	6·6	5·2	5·8	(9)‡	7
Glutamic acid	17·8	18·0	17·8	17·8	18	16·5
Proline	5·7	5·0	5·2	5·3	5	5·5
Glycine	7·9	8·1	7·4	7·9	8	8
Alanine	19·1	18·0	17·2	18·1	18	18
Valine	11·0	11·8	11·9	12·3	12	14·5
Methionine	0§	trace§	4·3	3·6	4	3·5
Isoleucine	2·2	2·1	2·5	2·2	2	3·5
Leucine	13·0	14·8	14·8	15·0	15	17
Tyrosine	4·9	4·4	4·8	4·9	5	4
Phenylalanine	7·4	7·5	6·9	7·2	7	7·5
SCM-cysteine	0	0·7	1·1	0·3	1‖	1·5
Tryptophan					1#	
Total					149	150
			β-Chains			
Lysine	9·8	11·0	10·4	11·3	11	10·5
Histidine	6·9	7·1	7·2	6·9	7	5·5
Arginine	5·8	5·5	5·9	5·8	6	5
Aspartic acid	14·1	13·7	14·3	13·9	14	13
Threonine	6·6	6·1	6·3	6·2	(7)‡	6·5
Serine	6·1	5·5	4·9	5·2	(7)‡	6·5
Glutamic acid	14·5	14·2	15·1	14·5	15	13·5
Proline	5·3	5·3	5·8	5·2	5	5·5
Glycine	8·6	8·6	8·8	8·6	9	8·5
Alanine	17·3	16·9	17·0	17·3	17	16
Valine	12·2	12·1	12·2	12·4	12	12·5
Methionine	1·1	0·9	1·6	1·4	1	2·5
Isoleucine	5·6	5·7	5·9	5·7	6	5
Leucine	17·8	17·1	17·9	17·8	18	17
Tyrosine	2·5	2·7	2·4	2·4	3	4·5
Phenylalanine	8·0	8·1	8·3	7·8	8	8·5
SCM-cysteine	1·2	0·9	2·5	1·7	2	2·5
Tryptophan					2#	
Total					150	143

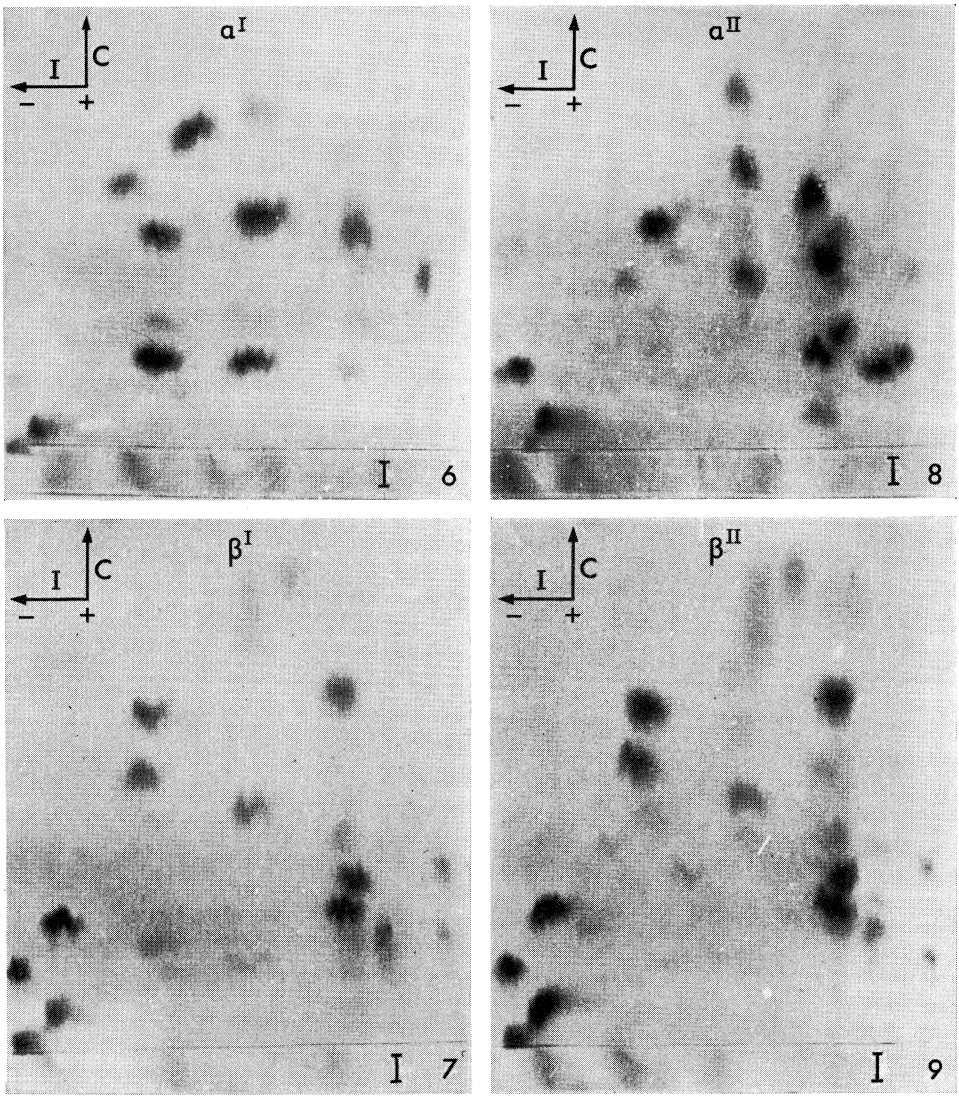
* Preparation from a different White Leghorn fowl. Preparation and analyses 6 months later than the other 48-hr sample.

† Data taken from Saha (1964) for comparison. Based on an assumed molecular weight of 31,000 for α_2 (or β_2 for β-chain analysis) and now divided by 2 to correspond to α_1 (or β_1).

‡ Obtained by linear extrapolation to zero time. § Not included in averaging.

‖ Only results from 48 hr* considered. # Deduced from tryptic peptide map.

maps or fingerprints clearly show that the α-chains differ in structure from each other and from the β-chains. However, as suggested from their similar amino acid compositions and electrophoretic and chromatographic rates, the β-chains of Hb I and Hb II have almost identical fingerprints, differing at most by two peptides. It can



Figs. 6–9.—Peptide maps obtained by ionophoresis at pH 6·4 (*I*) and chromatography (*C*) in butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12 v/v) of tryptic digests of the isolated *S*-carboxymethylated globin chains α^{I}, β^{I}, α^{II}, β^{II} of fowl haemoglobin.

be inferred from this that the β-chains are probably structurally similar and a close resemblance in their amino acid sequence exists, although admittedly more definitive identities of the two globin chains must await complete amino acid sequence

determination. Some peptides in the fingerprint of α^{II} globin are similar in ionophoretic and chromatographic rates to those in the β-chains.

As indicated by Kabat (1968) an advantage of using SCM-globin for peptide mapping is that only about 5% insoluble "core" material remains following trypsin treatment. The present work supports this finding.

If it is assumed that Hb I and Hb II have the structure $\alpha_2\ \beta_2$, the number of tryptic peptide spots expected from α- and β-globins (calculating from the lysine plus arginine contents of 15–17 and 16–18, respectively, in the isolated chains) agrees favourably with the number obtained in the peptide maps.

(*f*) N-*Terminal Amino Acid Analyses*

The *N*-terminal sequences of the isolated α- and β-globin chains were deduced from the data presented in Table 4 and gave the following results:

α^{I}: Val-Leu-Ser-	β^{I}: Val-His-Gly-
α^{II}: Met-Leu-Thr-	β^{II}: Val-His-Gly-

Thus it can be concluded that Hb I consists of four chains, all of which have free *N*-terminal valine. Hb II also consists of four chains, the two α-chains of which have free *N*-terminal methionine, and both β-chains have free *N*-terminal valine. The α^{II} sequence Met-Leu-Thr has not been detected previously by other workers.

IV. Discussion

It is clear from the literature that many contradictions exist concerning the number of adult chicken haemoglobins and the number of different globin subunits. The number of haemoglobins that have been reported varies from two to five. Most evidence favours two haemoglobin components which occur in a ratio of about 3 : 1 (see Introduction).

As far as haemoglobins are concerned it is important to distinguish between real heterogeneity and heterogeneity due to changes subsequent to haemolysis (Manwell *et al.* 1963). Kabat (1968) and Washburn (1968*a*) also draw attention to the fact that apparent heterogeneity can result from repeated freezing and thawing, or aging of fowl haemoglobin solutions. Thus the Hb I and Hb II components characteristic of fresh haemolysates each gave rise to two or more electrophoretic bands when subjected to these conditions. Intra- or interpolypeptide chain disulphide bonding has been used as a plausible explanation for apparent heterogeneity in turtle and frog haemoglobins (Riggs, Sullivan, and Agee 1964; Trader and Frieden 1966; Sullivan and Riggs 1967) and mouse haemoglobins (Riggs 1965), and may "account for the above-mentioned heterogeneity of chicken haemoglobins reported in the literature" (Kabat 1968).

Methaemoglobin formation involving a change in the oxidation state of haem iron may also produce apparent heterogeneity (Hashimoto and Wilt 1966). The accepted practice therefore is to employ the usually more stable cyanmethaemoglobin derivative in the presence of 0·01% cyanide during fractionations. However, a finding of the present studies was that although fresh cyanmethaemoglobin solutions gave the Hb I, Hb II pattern, aged solutions resulted in the splitting of both

haemoglobins into two components. The carbonmonoxyhaemoglobin derivatives were found to give the most consistent results with both fresh and aged preparations. This observation contrasts with that of Hashimoto and Wilt (1966) who found reproducibility only with cyanmethaemoglobin.

TABLE 4

STEPWISE PHENYLISOTHIOCYANATE DEGRADATION OF GLOBIN FROM Hb I AND Hb II

The phenylthiohydantoin (PTH) amino acids were identified by thin-layer chromatography according to Jeppsson and Sjöquist (1967). Wherever possible, identification was confirmed by regeneration to the amino acid (Van Orden and Carpenter 1964) and determination on a Beckman model 120C amino acid analyser. Serine and threonine could not be determined because of destruction during regeneration

Step No.	Whole Globin		α-Chain		β-Chain	
	Amino Acid	Yield*	Amino Acid	Yield†	Amino Acid	Yield†
			Hb I			
1‡	Valine	3·8(2·9)	Valine	0·87(0·65)	Valine	0·85(0·66)
2§	Leucine and histidine		Leucine		Histidine	
3§	Serine and glycine		Serine		Glycine	
			Hb II			
1‡	Valine	(1·4)	Methionine	0·92(0·60)	Valine	0·87(0·64)
		3·6				
	Methionine	(1·2)				
2§	Leucine and histidine		Leucine		Histidine	
3§	Threonine and glycine		Threonine		Glycine	

* Yield in moles/64,000 molecular weight.

† Yield in moles/16,000 molecular weight.

‡ Calculated from ultraviolet absorption using the average molar extinction coefficient of 16,000 (Fraenkel-Conrat, Harris, and Levy 1955). Values in parenthesis are yields calculated from the amount of amino acid regenerated, corrected only for hydrolytic losses using the factors of Van Orden and Carpenter (1964).

§ In the second and third steps the phenylisothiocyanate-globins were insoluble and the yields of PTH-amino acids were always lower in these degradation steps, ranging from 50–90% of the first step. Quantitation is also difficult in the case of PTH-histidine because it does not extract completely from acid solution, or when serine and threonine are *N*-terminal because of the formation of more than one PTH-derivative.

In the present work two main haemoglobins, Hb I and Hb II, in a ratio of about 3 : 1 were consistently found in haemolysates of adult fowl erythrocytes. As well as these haemoglobins, a third minor acidic component, Hb III, was also found. Similar chromatographic data have been obtained by other workers using slightly different conditions. Matsuda and Takei (1963) and Hashimoto and Wilt (1966) employed carboxymethylcellulose and fractionated the carbonmonoxyhaemoglobin and cyanmethaemoglobin derivatives respectively. Van der Helm and Huisman (1958),

Kabat (1968), and Saha (1964) used Amberlite IRC-50 and stepwise gradient elution with sodium citrate buffers.

The relative percentages of Hb I and Hb II obtained here (73·4 and 25·7% respectively) are in agreement with those reported by Huisman and Schillhorn Van Veen (1964) (71·7 and 28·3% respectively) in which fractionation of the haemoglobins was by DEAE-cellulose chromatography and elution with sodium phosphate buffers of different pH. Fraser (1961) obtained essentially the same results (72 and 28%) with carboxymethylcellulose chromatography and stepwise gradient elution with sodium phthalate buffers at pH 5·9.

The appearance of Hb III in the void volume of the column represents a situation not unique to fowl haemoglobins. Muller (1961) found similar fractions in all samples of haemoglobins examined and cites the evidence of Huisman and Meyering (1960) that these fractions contained methaemoglobin reductase and non-haemoglobin proteins, in addition to some haemoglobin. Since these fractions were minor, heterogeneous, and in part non-haemoglobin in character, and probably represented to some extent column artefacts, they were discounted by Muller (1961). Because of its low concentration (about 1% of the total) and contamination with other proteins, characterization of so-called Hb III is incomplete. Thus, it is not known whether it results from secondary modification of either Hb I or Hb II or is a separate, distinct haemoglobin. Heterogeneity of human haemoglobins by such secondary modifications as blocking of amino-terminal groups (Holmquist and Schroeder 1966; Bookchin and Gallop 1968) and binding of glutathione (Muller 1961) is known to occur. Muller presented evidence that during chromatography of fowl haemoglobin on carboxymethylcellulose, Hb I was fractionated into two components, of which the minor one differed only in that it contained glutathione.

The recent findings of Washburn (1968*a*, 1968*b*) may shed some additional light on the problem of heterogeneity of fowl haemoglobins. He found by electrophoretic analysis on cellulose acetate strips that, besides the usual Hb I, Hb II pattern, a second minor haemoglobin component, migrating faster than Hb II, could also occur. Hb I was found to be present in all the adult chickens studied but the fast and slow Hb II components appeared either separately or together, depending on the particular fowl. It was demonstrated that the appearance of either or both of these Hb II components was under genetic control and were due to allelic codominant genes. Two haemoglobin components were produced in the homozygote and three in the heterozygote.

The heterogeneity of the haemoglobins Hb I and Hb II isolated in the present work results from the presence of four analytically distinct globin chains. The α- and β-globin chains from each haemoglobin were isolated chromatographically and the α^{I}-, β^{I}-chains and α^{II}-, β^{II}-chains were different in electrophoretic mobility, peptide map analysis of the tryptic digests, amino acid composition, and N-terminal end-group analysis. They must, therefore, have differences in amino acid sequence (see also Muller 1961). Our amino acid composition data for Hb II (Table 1), with the exception of serine and valine and possibly methionine and tyrosine, show very close resemblance to the data of Saha (1964). Conversely, although the Hb I data (Table 1) have obvious affinities the resemblance is not as great; the major differences being between the threonine, serine, alanine, valine, methionine, and leucine residues. The

reasons for these differences are not clear. As far as can be ascertained the values obtained by Saha for serine and threonine have been corrected for hydrolytic losses (Saha and Ghosh 1965). Summation of the residues of α_2- and β_2-chains from Hb I and Hb II agrees well with the values we have obtained from analyses of the whole globins, which was also the case with the data of Saha (1964).

When the analyses of the isolated α- and β-chains are compared (Tables 2 and 3), general agreement with the data of Saha (1964) becomes evident. The slight deviations that do exist, however, are sufficient to create differences in interpretation. For example, in comparing β_2^{I} and β_2^{II} Saha found 310 and 286 amino acid residues, respectively, whereas our results suggest 298 and 300 residues respectively. Furthermore, a comparison by Saha of the number of amino acid changes between the α_2- and β_2-chains implied that the α_2^{I}- and β_2^{I}-chains (15 changes) showed most resemblance. Little similarity was envisaged between β_2^{I}- and β_2^{II}-chains (40 changes). In contrast our data suggests little similarity between α_2^{I}- and β_2^{I}-chains (44 changes) but a closer relationship between β_2^{I}- and β_2^{II}-chains (14 changes); the greatest difference was between the α_2^{I}- and α_2^{II}-chains (64 changes). Although it may reasonably be concluded that Hb I and Hb II do not contain a common globin chain, the analytical data suggests a closer relationship between the β-chains than previously reported by Saha (1964). The β^{I}- and β^{II}-chains have virtually identical chromatographic and electrophoretic rates and very similar but non-identical amino acid compositions and tryptic peptide maps. Both have the *N*-terminal amino acid sequence Val-His-Gly. The "many common amino acid sequences" shared by Hb I and Hb II as reported by Kabat (1968) and based on the criteria of immunodiffusion analysis and tryptic peptide mapping, probably were derived from these closely related β-chains. In agreement with Hashimoto and Wilt (1966) we have found the most extensive differences between the two haemoglobins in the α-chains.

The Edman phenylisothiocyanate analysis of whole globin from Hb I and Hb II and of their isolated α- and β-chains is consistent with the view that no *N*-terminal masking groups are present. This interpretation conflicts with the data of Satake, Sasakawa, and Maruyama (1963), in which, for unfractionated haemoglobin, the α-chains had *N*-terminal Val-Leu- as in mammalian haemoglobin, and the β-chains were found to have an *N*-terminal acetyl-Val-Thr-Leu- sequence; no Val-His- sequence was detected. Matsuda and co-workers (1964, 1965) found that both β^{I} and β^{II} had the *N*-terminal sequence Val-His-, and the $^{+}$-chain of Hb I terminated with Val-Leu-, but no *N*-terminal end group was detected in α^{II}. This latter result was attributed to masking by an acetyl group. Our finding of the *N*-terminal sequences Val-Leu-Ser- in α^{I} and Val-His-Gly- in both β^{I} and β^{II} supports the data of Matsuda and co-workers (1964, 1965). Our finding of *N*-terminal Met-Leu-Thr- in α^{II} was unexpected in view of the masked end-group analysis data of Matsuda and co-workers (1964, 1965). It was also unexpected because the particular sequence Met-Leu-Thr- is generally associated with unusual vertebrate β-chains rather than α-chains (e.g. β-chains of sheep and cattle —Eck and Dayhoff 1966).

The conflicting data reported here on (1) amino acid compositions and the similarity of some globin chains; (2) *N*-terminal end-groups; and (3) *N*-terminal acetylation is not confined to fowl haemoglobins. Moss and Ingram (1968) document an analogous situation with the major components of tadpole and adult bullfrog

(*Rana catesbeiana*) haemoglobin. It is difficult to believe that the reported contradictions are due to the use of different species or subspecies of the animal under investigation, but at present no feasible alternative explanation can be offered.

The reported uptake of acetyl groups by fowl haemoglobin (Marchis-Mouren and Lipmann 1965) is considered in another paper (Moss and Thompson 1969).*

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VI. References

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* *Note added in proof.*—We have recently come across a publication "International Symposium on Comparative Haemoglobin Structure", Thessaloniki, April 11–13, 1966, containing a paper "Comparaison des hémoglobines chez la poule et chez divers oiseaux" by A. G. Schnek, C. L. Paul, and J. Léonis. Communication with Professor Léonis has revealed the extensive studies made in his laboratory on the amino acid composition and sequence of avian haemoglobins. Their amino acid compositions of the α- and β-chains of the adult fowl haemoglobin are in good agreement with those reported in this paper.

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