Biochem. J. (1975) **149**, 245–258
Printed in Great Britain

Rat Haemoglobin Heterogeneity

TWO STRUCTURALLY DISTINCT α CHAINS AND FUNCTIONAL BEHAVIOUR OF SELECTED COMPONENTS

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(*Received* 21 *June* 1974)

Six haemoglobins were separated analytically from haemolysates of adult Wistar rats (*Rattus norvegicus*) by cellulose acetate electrophoresis and preparatively by DEAE-cellulose chromatography. The globin chains were separated from unfractionated haemolysates by CM-cellulose chromatography by using a non-linear formic acid–pyridine gradient followed by CM-cellulose chromatography in 8M-urea by using a gradient of increasing Na^+ concentration in phosphate buffer, pH 6.7. Two α chains and three non-α chains were identified. Chains isolated from purified haemoglobins were correlated with chains isolated from unfractionated haemolysates by electrophoresis on urea–starch gels to make presumptive assignments of the subunit composition of the six haemoglobin tetramers. Partial amino acid sequences were determined for the major and minor α chains. The oxygen equilibria of two of the major haemoglobin components and of the unfractionated haemolysate were examined at pH 7.5 and 8.0. The two purified haemoglobins exhibited similar oxygen affinities; the haemolysate, however, had a lower oxygen affinity than either of the two purified haemoglobins. Both the haemolysate and the two haemoglobins showed an alkaline Bohr effect larger than that of human haemoglobin A.

Although the laboratory rat is commonly used in a variety of studies of erythrocytes, few studies of the structure of rat haemoglobin have been reported. Rat haemoglobin presents more technical difficulties in such studies than do the haemoglobins of many mammals, since the haemolysate contains multiple components, some of which precipitate spontaneously *in vitro* at physiological pH values.

Two observations demonstrate the desirability of knowledge about the structure of rat haemoglobin. Stein *et al.* (1971) reported that the several components of rat haemoglobin are synthesized at different times during erythrocyte maturation, and an hereditary hypochromic anaemia resembling human thalassaemia has been reported in Belgrade rats (Sladic-Simic *et al.*, 1966).

The present paper describes: (a) six rat haemoglobins; (b) their subunits (two α and three non-α polypeptide chains, which have been tentatively designated as β chains); (c) amino acid sequence data for the major ($^{I}\alpha$) and minor ($^{II}\alpha$) α chains; (d) the oxygenation properties of the haemolysate and of components containing $^{II}\beta$ chains in combination with either $^{I}\alpha$ or $^{II}\alpha$ chains (i.e. $^{I}\alpha_2\,^{II}\beta_2$ or $^{II}\alpha_2\,^{II}\beta_2$).

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Materials and Methods

Blood samples

Blood obtained by cardiac puncture from anaesthetized Carworth Farms Wistar rats (*Rattus norvegicus*) was collected in 3.8% (w/v) sodium citrate. Cells were washed four times with 0.9% NaCl–0.15M-Tris (free base), pH 9.1, at room temperature (20–23°C). Packed cells were lysed by the addition of 3vol. of 5mM-Tris–HCl (pH 8.4). Cell membranes were removed by centrifugation at 60000*g* for 1h at room temperature in a Beckman model L ultracentrifuge (Beckman Instruments, Fullerton, Calif., U.S.A.).

Separation of rat haemoglobins by DEAE-cellulose chromatography

After passage through a column (1.5cm × 60cm) of Sephadex G-25 (coarse grade; Pharmacia Fine Chemicals Inc., Piscataway, N.J., U.S.A.) equilibrated with 0.05M-Tris–HCl (pH 8.7), a portion of haemolysate containing 250mg of haemoglobin was

placed on a column (2cm×46cm) of DEAE-cellulose (Whatman DE-52; Reeve Angel, Clifton, N.J., U.S.A.) equilibrated with the same buffer. Haemoglobin components were separated by gradient elution with a 1260ml linear gradient of 0.05M-Tris-HCl, pH8.3 to 6.8. The fractions collected were 12ml. These procedures were carried out at room temperature.

Separation of rat haemoglobins by electrophoresis on cellulose acetate

Electrophoresis was carried out at 300V for 30min at 25°C in 0.04M-Tris–1mM-EDTA–0.05M-boric acid (pH8.2) (Garrick *et al.*, 1973). Cellulose acetate strips were stained with 0.2% Ponceau S in 7% (w/v) trichloroacetic acid and rinsed with 5% (v/v) acetic acid.

Globin preparation

Globin was prepared from both unfractionated haemolysate and purified haemoglobin by the method of Garrick *et al.* (1973). The sample (10–40mg/ml) was added dropwise with continuous stirring to 20vol. of 2.5% (w/v) oxalic acid in acetone at room temperature. Precipitated globin was collected by centrifugation at 18000*g* at 4°C for 10min.

Preparative purification of globin chains

Globin was fractionated by a modification of the method of Dintzis (1961). A 400mg sample, dialysed for 2h against 0.2M-formic acid–0.02M-pyridine, was placed on a column (2.5cm×18cm) of CM-cellulose (Whatman CM-32), equilibrated with the same solution. Chains were eluted with the following six-chamber gradient (160ml in each chamber): (a) 0.2M-formic acid–0.02M-pyridine; (b) 0.8M-formic acid–0.08M-pyridine; (c) 1.0M-formic acid–0.10M-pyridine; (d) 1.2M-formic acid–0.12M-pyridine; (e) 1.4M-formic acid–0.14M-pyridine; (f) 1.6M-formic acid–0.16M-pyridine. Fractions (4.5ml) were collected.

The fractions corresponding to α subunits were pooled, freeze-dried and further fractionated by the method of Clegg *et al.* (1966). The sample was dissolved in 8M-urea containing 3.9mM-Na_2HPO_4 and 1.2mM-NaH_2PO_4,H_2O (9mM-Na^+) and 0.05M-2-mercaptoethanol (pH6.7) and placed on a column (2.5cm×18cm) of CM-cellulose (Whatman CM-23) equilibrated with the same buffer. Chains were eluted with a 2200ml linear gradient of increasing Na^+ concentration (9–59mM). The second buffer was made from the first buffer by adding NaCl to give a final concentration of 0.05M. Fractions (10ml) were collected. Pooled fractions were passed through a column (3.5cm×112cm) of Sephadex G-25 (coarse grade) equilibrated with 0.5% (v/v) formic acid and freeze-dried. Fractions corresponding to β subunits were similarly separated into individual components.

Analytical separation of globin chains

Urea–starch gels were prepared by the method of Garrick *et al.* (1970), with 0.045M-Tris–1mM-EDTA–0.025M-boric acid (pH8.6) and run at 4°C for 40h at 125V. Gels were stained with Amido Black.

Amino acid analysis

Samples for analysis were dissolved in 6M-HCl containing 9mg of phenol/100ml of acid and hydrolysed *in vacuo* at 110°C for selected times. Hydrolysates were dried *in vacuo* over NaOH and analysed on a Beckman 120C amino acid analyser.

Aminoethylation of globin chains

Chains were aminoethylated by the method of Nute & Sullivan (1971). A 100mg sample was dissolved in 25ml of 0.55M-Tris–5mM-EDTA–0.28M-2-mercaptoethanol–8M-urea (pH8.6) and flushed with N_2. The mixture was stirred for 1h and then three 0.35ml portions of ethyleneimine were added at 30min intervals. At 1h after the final addition, the mixture was passed through a column (3.5cm×112cm) of Sephadex G-25 (coarse grade) previously equilibrated with 0.5% (v/v) formic acid and freeze-dried.

Peptide 'mapping'

A 5mg sample of aminoethylated α chain was digested with trypsin (1mg/100mg of globin; Worthington TRTPCK, Worthington Biochemical Corp., Freehold, N. J., U.S.A.) for 3h at room temperature, then freeze-dried. Tryptic peptides were 'mapped' by a modification by Ranney *et al.* (1965) of the method of Clegg *et al.* (1966). After high-voltage electrophoresis and chromatography, the paper was dipped in a 0.25% (w/v) solution of ninhydrin in anhydrous acetone and heated at 60–70°C for 10min. The purple spots were cut out and eluted with 6M-HCl containing 9mg of phenol/100ml of acid. The eluates were hydrolysed for 18h and their amino acid compositions determined as described above.

Cleavage of the α chains with CNBr

About 200mg of CNBr was added to 94mg of aminoethylated α chains dissolved in 20ml of 70% (v/v) formic acid. After 20h in the dark at room temperature, the mixture was diluted to 200ml with water and freeze-dried. The sample was dissolved in 1M-acetic acid and placed on the first of four Bio-Gel P10 (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) columns (each 1.5cm×90cm) in series, previously equilibrated with 1M-acetic acid. Fractions (4.1ml) were collected.

Succinylation (3-carboxypropionylation) and tryptic digestion

A 10mg sample was dissolved in 1ml of 0.5M-$NaHCO_3$–8M-urea and succinic anhydride (10mg)

was gradually added. The pH was maintained above 8.5 by dropwise addition of 0.5M-Na_2CO_3–8M-urea. The sample was then diluted with 3ml of 0.5M-$NaHCO_3$ and digested with trypsin. After digestion, the peptides were precipitated by the addition of trichloroacetic acid to a final concentration of 10% (w/v). The precipitate was washed with 95% (v/v) ethanol–diethyl ether (1:1, v/v), then with diethyl ether and finally dried.

Sequencing of α chains

A Beckman sequencer model 890 was used for sequence studies. For intact α chains, a regular double-cleavage protein program was used (Edman & Begg, 1967). For peptides obtained by CNBr treatment of α chains, a single-cleavage Quadrol program was developed. Ethyl acetate wash-time was decreased by 30% and the thiazolinone derivatives were extracted in 5ml of butyl chloride. The first degradation cycle in certain runs was carried out without phenylisothiocyanate. This procedure gave more uniform coating and was found to be helpful in decreasing overlap of residues in later cycles. Butanedithiol (0.005%, v/v) was added to the butyl chloride to preserve serine and threonine residues (Hermodson *et al.*, 1972). The thiazolinone derivatives were converted into phenylthiohydantoin derivatives by heating at 80°C for 10min in 1M-HCl containing 0.1% (v/v) ethanethiol (Hermodson *et al.*, 1972); the products were then extracted into ethyl acetate. Extracted phenylthiohydantoin derivatives were identified with t.l.c. and a Beckman GC-65 gas chromatograph. T.l.c. was carried out on pre-coated silica-gel plates (E. Merck, Brinkmann Instruments, Westbury, N.Y., U.S.A.; type 60-F-254) with fluorescent indicator. The solvent systems used were 1,2-dichloroethane–propionic acid–heptane (25:17:58, by vol.) (Jeppsson & Sjoquist, 1967) and chloroform–methanol (9:1, v/v) (Pataki, 1966). After ethyl acetate extraction, the aqueous phases were dried and tested for arginine phenylthiohydantoin by the phenanthraquinone spot test (Yamada & Itano, 1966) and for histidine phenylthiohydantoin by the Pauly spot test (Bennett, 1967).

Oxygen equilibria

Components of rat haemoglobin isolated by DEAE-cellulose chromatography were used for the determination of oxygen equilibria. Concentration of the purified haemoglobins led to methaemoglobin formation and was therefore avoided. Samples were dialysed overnight at 4°C against 0.1M-K_2HPO_4 adjusted to pH7.5 and 8.0 with phosphoric acid. Oxygen equilibria were measured by the method of Ranney *et al.* (1965) in a tonometer equipped with a spectrophotometric cuvette. This involved deoxygenating the haemoglobin solution (60μM in haem) by alternately evacuating and flushing the tonometer with purified N_2. After deoxygenation, appropriate portions of air were added and equilibrated with the haemoglobin at 20°C. The percentage saturation was determined spectrophotometrically with a Cary 14 spectrophotometer.

Results

In this section the preparative and analytical separations of the six rat haemoglobins and the five globin chains are described. The two α and three putative β chains were correlated with the six haemoglobins observed in the haemolysate. Although not complete, amino acid-sequence data provided evidence that the two α chains are structurally distinct. The oxygen equilibria of the two components containing either $^{I}\alpha$ or $^{II}\alpha$ chains in combination with $^{II}\beta$, the major β chain, were studied.

Haemoglobin purification

Fig. 1 presents a preparative separation of rat haemolysate into nine peaks by DEAE-cellulose chromatography. These nine peaks correspond to at

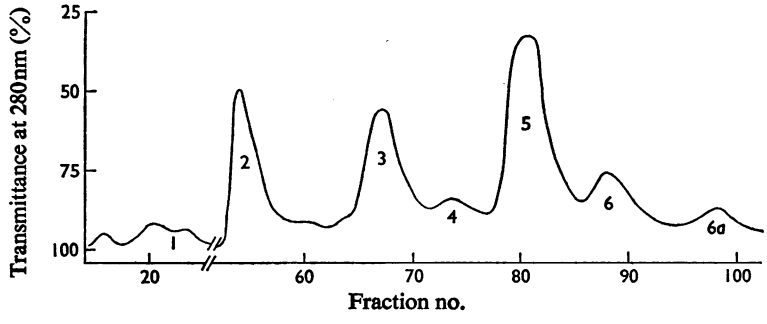


Fig. 1. *Fractionation of rat haemoglobins by DEAE-cellulose chromatography*

For details see the text.

least six haemoglobin components as described below. The analytical patterns of these components and of the haemolysate from electrophoresis on cellulose acetate are shown diagrammatically in Fig. 2. Two haemoglobins are cathodal (Hb 1 and Hb 2) and four are anodal (Hb components 3–6). Hb 1, the most unstable, had a tendency to trail during both separatory techniques. This instability probably produces the two peaks seen for Hb 1 in Fig. 1. Hb 4, present in the smallest amount, was always contaminated with Hb 5, the major component. The two bands seen for Hb 5 in Fig. 2 both contained the same globin chains, as shown below in Fig. 4(*b*) (channels 2 and 3), i.e. the slower band in Hb 5 is not Hb 4.

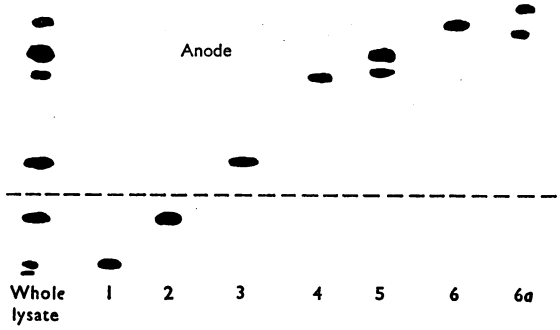


Fig. 2. *Diagrammatic representation of cellulose acetate electrophoresis of rat haemoglobins*

Fractions representing each of the haemoglobin components in Fig. 1 were concentrated by ultrafiltration. The pattern for unfractionated haemolysate is shown at the left. The anode is at the top. ----, Origin.

Table 1. *Subunits and proportions of the six rat haemoglobins*

Subunit compositions were assigned by using urea–starch-gel electrophoresis (Fig. 4). The observed percentage of the total was obtained by integrating E_{540} peaks from a DEAE-cellulose separation. The expected percentage of the total was calculated by multiplying the fractional amounts of substituent chains. These were obtained by integrating E_{280} peaks from CM-cellulose chromatography separations and are as follows: $^{I}\alpha$, 70%, and $^{II}\alpha$, 30% of total α; $^{I}\beta$, 13%, $^{II}\beta$, 62%, and $^{III}\beta$, 25% of total β.

		Proportion (% of total)	
Hb	Subunit composition	Observed	Expected
1	$^{II}\alpha_2{}^{III}\beta_2$	7	8
2	$^{I}\alpha_2{}^{III}\beta_2$	13	18
3	$^{II}\alpha_2{}^{II}\beta_2$	20	19
4	$^{II}\alpha_2{}^{I}\beta_2$	2	4
5	$^{I}\alpha_2{}^{II}\beta_2$	45	43
6	$^{I}\alpha_2{}^{I}\beta_2$	13	9

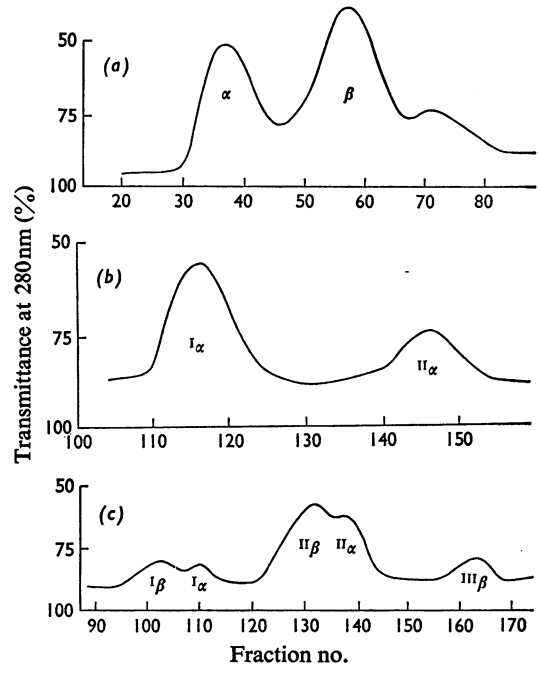


Fig. 3. *Preparative fractionations of rat globin chains*

(*a*) Separation of globin chains into α and β components by a modification of the method of Dintzis (1961). After separation the pooled fractions (30–42) and (50–62) were diluted with water to five times their original volume and freeze-dried. The peak after β is probably non-haem protein. (*b*) Separation of the two α chains by the method of Clegg *et al.* (1966). (*c*) Separation of the three β chains by the method of Clegg *et al.* (1966).

Hb 6 was often contaminated with Hb 5. Hb 6*a* contained the same globin chains as Hb 6 (Fig. 4*c*, channels 6 and 10). The peak before Hb 1 was a non-haem protein. The complex patterns in Figs. 1 and 2 probably result from the high degree of instability of rat haemoglobins. Haemolysates of all rats examined gave essentially the same pattern. The presence of six haemoglobins agrees with the findings of Stein *et al.* (1971). The amount of each component was determined as a percentage of the total by integrating the peaks at E_{540} from a DEAE-cellulose chromatography separation (Table 1).

Chain separations

Globin from the lysate was separated into α and β components by a modification of the method of Dintzis (1961). Fig. 3(*a*) shows the pattern of the effluent from the CM-cellulose column. The α and β chains were further separated by the method of Clegg *et al.* (1966). Fig. 3(*b*) presents the separation of the α chains into two components; we have designated the

major component $^{I}\alpha$ and the minor component $^{II}\alpha$ according to the convention established by Adams *et al.* (1969). Fig. 3(*c*) shows the separation of the β chains into three components: $^{I}\beta$, $^{II}\beta$ (the major component) and $^{III}\beta$. Additional peaks are due to α chain contamination, as shown in Fig. 4(*a*). This contamination can be avoided by appropriate selection of the fractions pooled from the β peak (Fig. 3*a*) without any obvious change in the relative proportions of the three types of β chains. In subsequent separations, fractions corresponding to fractions 53–62 in Fig. 3(*a*) were pooled and further separated. The percentage proportion of each chain was determined by integrating the E_{280} peaks. These proportions are as follows: $^{I}\alpha$, 70%, and $^{II}\alpha$, 30% of total α; $^{I}\beta$, 13%, $^{II}\beta$, 62%, and $^{III}\beta$, 25% of total β. The expected percentages of each haemoglobin component can be calculated by multiplying the fractional amounts of substitutent chains. These expected percentages of each type of tetramer are given in Table 1.

Electrophoresis on urea–starch gels

Fig. 4 shows schematically the electrophoretic patterns of globin chains derived from the purified haemoglobins shown in Fig. 1 and from chains purified from haemolysate shown in Fig. 3. The subunit compositions of the haemoglobins summarized in Table 1 were deduced from the patterns shown in Fig. 4. The observed charge of each of the five globin chains on urea–starch gels agreed with the migration of each of the six haemoglobins on cellulose acetate. As mentioned above, the contaminating peaks in Fig. 3(*c*) were due to $^{I}\alpha$ and $^{II}\alpha$ chains (Fig. 4*a*, channels 2 and 4). The slower band of Hb 5 (Fig. 2) apparently contained the same chains as the main band of Hb 5 (Fig. 4*b*, channels 2 and 3). The chains of Hb 4 were contaminated with those of Hb 5 (Fig. 4*c*, channel 8). Hb 6*a* was probably derived from Hb 6, since both seem to contain the same chains (Fig. 4*c*, channels 2, 6 and 10). Hb 6 was contaminated with Hb 5 (Fig. 4*c*, channel 6). These conclusions were based on comparisons of the subunits of individual haemoglobins with α and β chains purified from haemolysate. No contradictions were observed when α and β chains from an individual haemoglobin were first separated by the method of Dintzis (1961). Moreover, there is good agreement between observed and expected percentages of haemoglobin tetramers (Table 1).

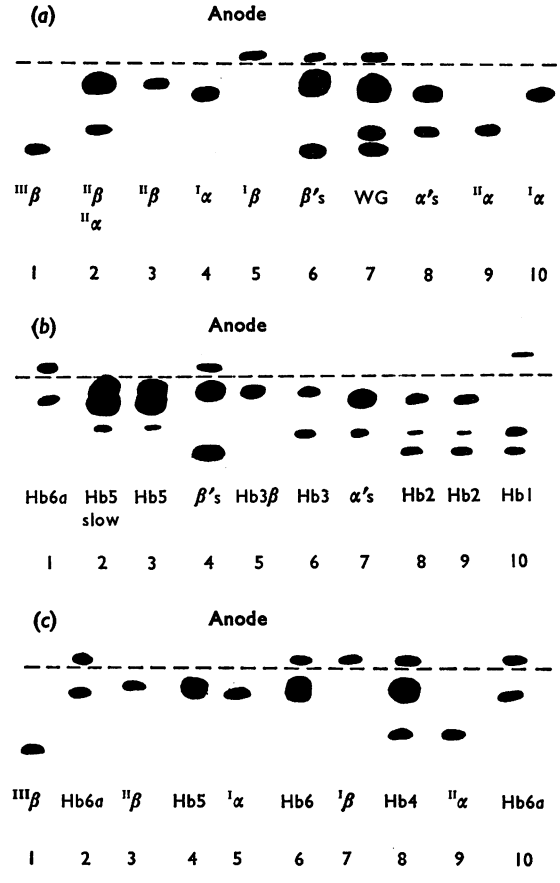
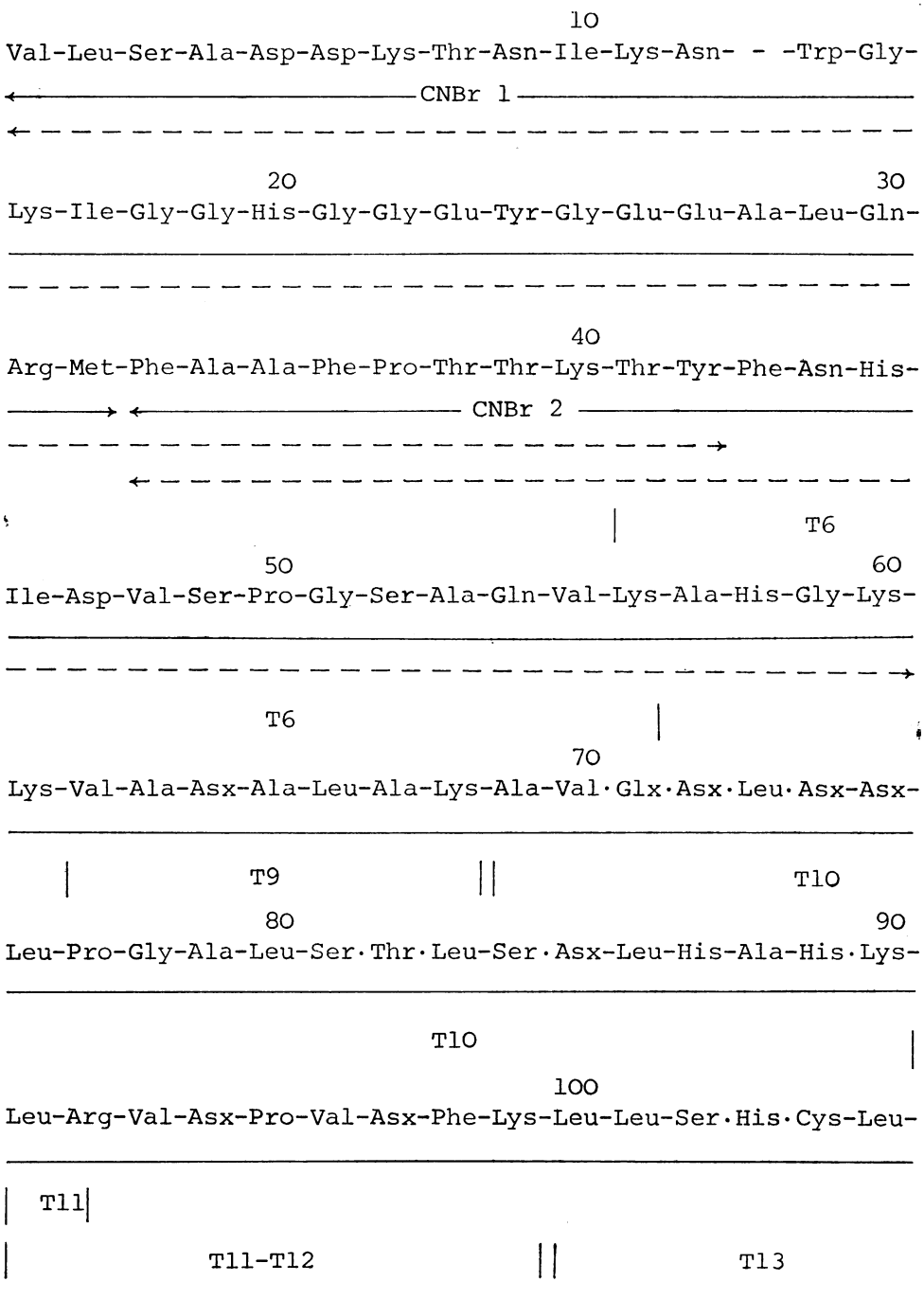


Fig. 4. *Diagrammatic representations of urea–starch-gel patterns of rat globin chains and purified haemoglobins*

(*a*) WG, Whole globin made from unfractionated haemolysate. α, α peak in Fig. 3(*a*); β, β peak corresponding to fractions 53–62 in Fig. 3(*a*). $^{I}\alpha$ in channel 10 is fraction 117 and $^{II}\alpha$ in channel 9 is fractions 143–145 in Fig. 3(*b*). The following samples are from Fig. 3(*c*): $^{I}\beta$ in channel 5 is fractions 100–101, $^{I}\alpha$ in channel 4 is fractions 111–113, $^{II}\beta$ in channel 3 is fraction 128, $^{II}\alpha+^{II}\beta$ in channel 2 is fraction 139, and $^{III}\beta$ in channel 1 is fractions 162–163. (*b*) Globin from the more cathodal band of Hb 5 (Fig. 2) is shown as Hb 5 slow. The anode is at the top. ----, Origin. (*c*)

Serine, threonine and proline contents of the five chains

In the amino acid compositions of mouse and rabbit haemoglobin chains (Dayhoff, 1972) the values for serine, threonine and proline are higher in α than in β chains. This distribution of these particular amino acids is also found in rat α and β chains (Table 2). Thus $^{I}\alpha$ and $^{II}\alpha$ chains appear to be authentic α chains and, on the basis of low serine, threonine and proline content (Table 2), the others are probably β chains ($^{I}\beta$, $^{II}\beta$ and $^{III}\beta$). The sequence data below confirm this conclusion for the two α chains.


10
Val-Leu-Ser-Ala-Asp-Asp-Lys-Thr-Asn-Ile-Lys-Asn- - -Trp-Gly-
CNBr 1
20
30
Lys-Ile-Gly-Gly-His-Gly-Gly-Glu-Tyr-Gly-Glu-Glu-Ala-Leu-Gln-
40
Arg-Met-Phe-Ala-Ala-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Asn-His-
CNBr 2
T6
50
60
Ile-Asp-Val-Ser-Pro-Gly-Ser-Ala-Gln-Val-Lys-Ala-His-Gly-Lys-
T6
70
Lys-Val-Ala-Asx-Ala-Leu-Ala-Lys-Ala-Val·Glx·Asx·Leu·Asx-Asx-
T9
T10
80
90
Leu-Pro-Gly-Ala-Leu-Ser·Thr·Leu-Ser·Asx-Leu-His-Ala-His·Lys-
T10
100
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T11
T11-T12
T13

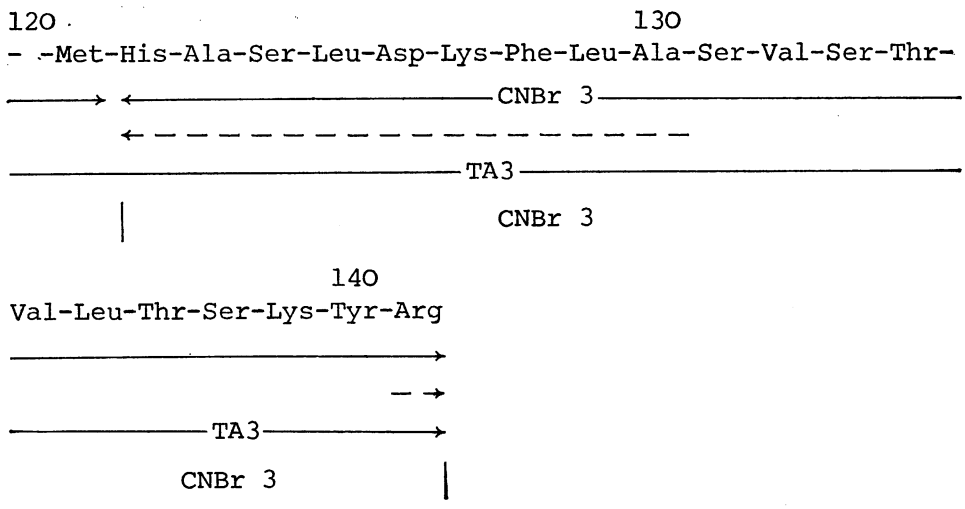


Fig. 5. *Partial amino acid sequence of rat $^{I}\alpha$ chain*

The CNBr fragments are shown by solid lines. ----, Residues identified by sequencer analysis. Residues placed by homology to mouse and rabbit α chains (Dayhoff, 1972) are shown by bracketing the tryptic or CNBr peptide with vertical bars. Commas between amino acids indicate that the amino acid to the left could not be positioned with confidence by homology. Median points between amino acids indicate that the amino acid to the left has been placed with a high degree of confidence by homology. This punctuation is taken from Dayhoff (1972). The blank at position 13 indicates a residue which could not be identified.

Table 2. *Serine, threonine and proline contents of α and β chains in rat haemoglobin*

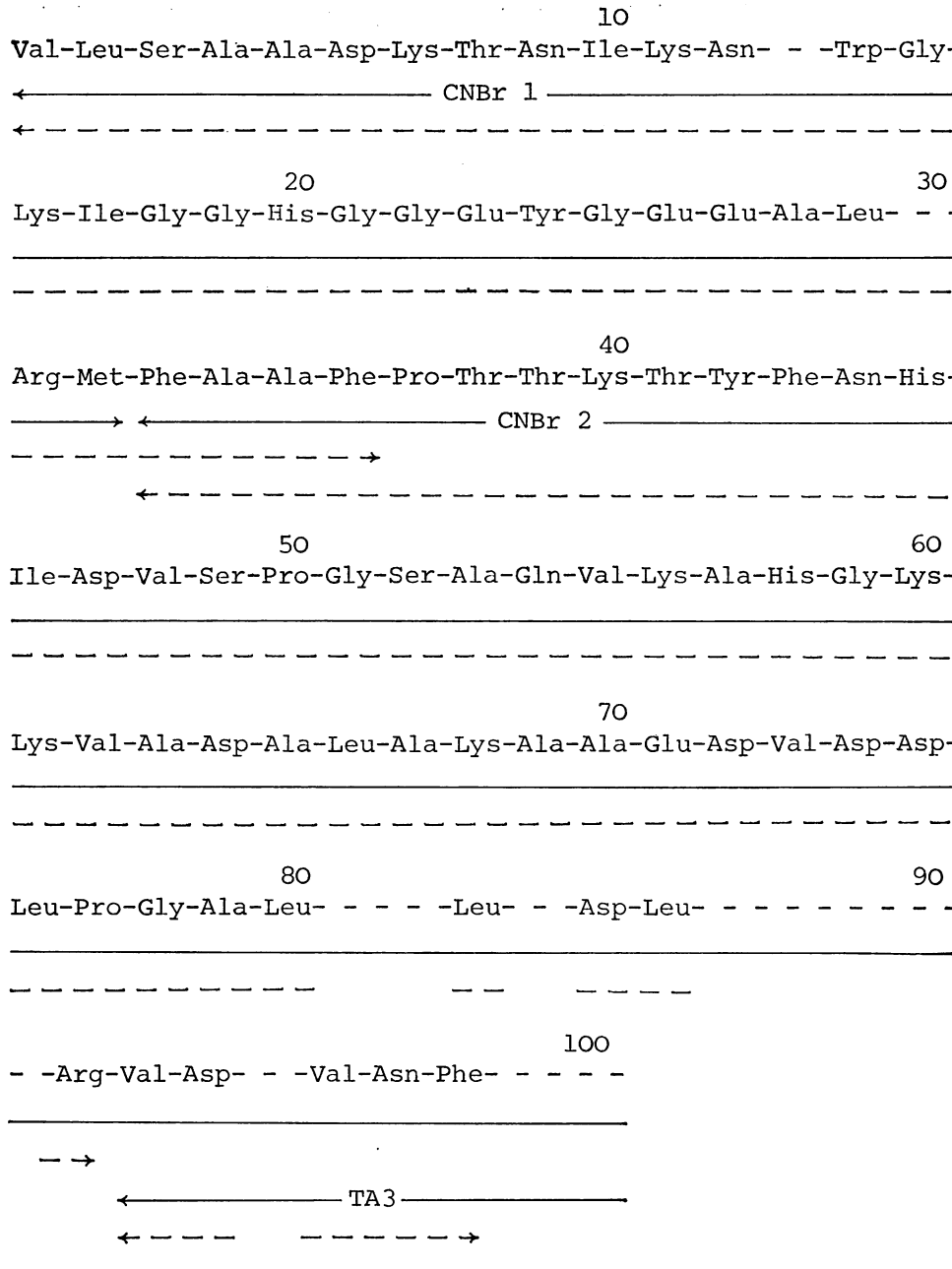
For $^{I}\alpha$ and $^{II}\alpha$ chains the values were based on a total of 141 amino acid residues/chain. For the three types of β chain the values were based on a total of 146 amino acid residues/chain. Values were calculated from 24h hydrolysates.

Amino acid	Chain ...	No. of residues				
		$^{I}\alpha$	$^{II}\alpha$	$^{I}\beta$	$^{II}\beta$	$^{III}\beta$
Serine		10.6	9.8	5.2	5.3	5.0
Threonine		8.8	8.6	2.7	2.5	2.2
Proline		6.5	6.1	3.9	4.2	3.2

Sequences of $^{I}\alpha$ and $^{II}\alpha$ chains

The partial sequences of $^{I}\alpha$ and $^{II}\alpha$ chains are presented in Figs. 5 and 6 respectively. These sequences were obtained by using a combination of (a) sequencer analysis on intact chains and fragments produced by CNBr cleavage and (b) amino acid analysis of tryptic peptides and CNBr-cleaved fragments. Since there were two methionine residues in each α chain, according to amino acid composition, each chain was cleaved into smaller fragments by CNBr after aminoethylation. The first methionine was at residue 32; the second, at residue 121. Therefore CNBr cleavage produced three pieces: CB1 (residues 1–32); CB2 (residues 33–121) and CB3 (residues 122–141). Fig. 7 shows the separation of these peptides by gel filtration. The leading edge of peptide CB2 is contaminated by products of incomplete cleavage. No contamination was observed, however, when the trailing edge of peptide CB2 was sequenced. The amino acid composition of peptide CB3 (Table 3) indicates that it is probably slightly contaminated with peptide CB1.

Automated sequencing was used to determine the sequences of the following: intact $^{I}\alpha$ chain, residues 1–42; intact $^{II}\alpha$ chain, residues 1–36; $^{I}\alpha$ CB2 peptide, residues 33–60; $^{II}\alpha$ CB2 peptide, residues 33–92; $^{I}\alpha$ CB3 peptide, residues 122–128; $^{II}\alpha$ CB3 peptide, residues 122–130 and residue 141. Arginine and histidine residues were identified by spot tests (Yamada & Itano, 1966; Bennett, 1967). All other residues were identified by at least two of the following criteria: g.l.c. of phenylthiohydantoin-amino acids and/or of trimethylsilyl derivatives and t.l.c. in two solvent systems (see the Materials and Methods section). Ambiguities caused by contaminating residues and out-of-phase cleavage were minimal, so that all identifications shown were made with confidence. Because the *N*-terminal end of peptide CB2 overlapped the sequence determined for the intact chain, the identities


10
Val-Leu-Ser-Ala-Ala-Asp-Lys-Thr-Asn-Ile-Lys-Asn- - -Trp-Gly-
CNBr 1
20
30
Lys-Ile-Gly-Gly-His-Gly-Gly-Glu-Tyr-Gly-Glu-Glu-Ala-Leu- - -
40
Arg-Met-Phe-Ala-Ala-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Asn-His-
CNBr 2
50
60
Ile-Asp-Val-Ser-Pro-Gly-Ser-Ala-Gln-Val-Lys-Ala-His-Gly-Lys-
70
Lys-Val-Ala-Asp-Ala-Leu-Ala-Lys-Ala-Ala-Glu-Asp-Val-Asp-Asp-
80
90
Leu-Pro-Gly-Ala-Leu- - - - -Leu- - -Asp-Leu- - - - - - - - - -
100
- -Arg-Val-Asp- - -Val-Asn-Phe- - - - -
TA3

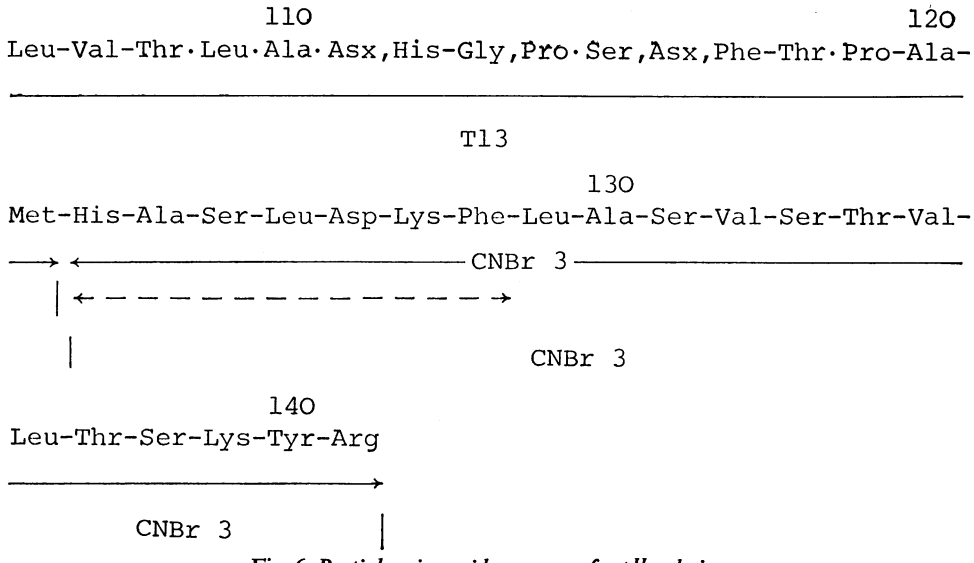


Fig. 6. *Partial amino acid sequence of rat $^{II}\alpha$ chain*

The CNBr fragments are shown by solid lines, as is also TA3, the third fragment produced by tryptic cleavage at arginine residues (actually obtained from succinylated peptide CNBr 2). Residues placed by homology to mouse and rabbit α chains (Dayhoff, 1972) are shown by bracketing the CNBr peptide with vertical bars. Blanks in the sequence indicate residues which were not identified.

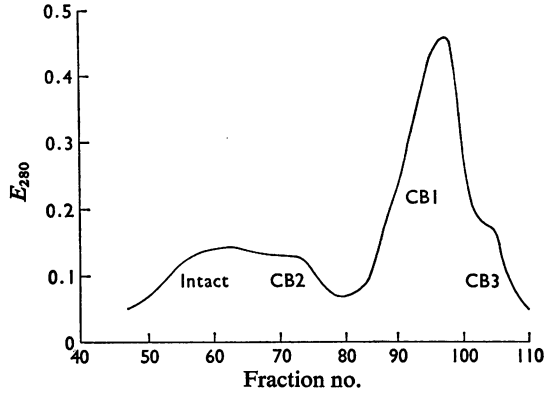


Fig. 7. *Gel filtration of fragments obtained by CNBr treatment of component $^{II}\alpha$*

Pooled fractions (CB1, CB2 and CB3) were diluted with water and freeze-dried. The only tryptophan in component $^{II}\alpha$ is at residue 14, hence the absorbance of peptide CB1 is greater than that of the other peptides.

and order of peptides CB1, CB2 and CB3 were unambiguously established.

According to amino acid composition there were three arginine residues in $^{II}\alpha$. These were located at positions 31, 92 and 141 by automated sequencing. Succinylation of $^{II}\alpha$ CB2 peptide, followed by digestion with trypsin, resulted in cleavage after the arginine residue at position 92. Since succinylation occurs at both α- and ε-amino groups, the original *N*-terminus (residue 33) was blocked. Therefore it was not necessary to separate the two resulting fragments (residues 33–92 and 93–121) before automated sequencing. On sequencing only one identifiable residue was obtained at each stage; however, the yield of the desired peptide was low and only residues 93–98 could be identified.

The problems commonly encountered in automatic peptide sequencing have been discussed by Hermodson *et al.* (1972). Thus often in a particular cycle it was not possible to identify any residue. Such blanks appear to arise mainly with labile residues such as serine and threonine. The sequenator products of aspartic acid, glutamic acid, asparagine and glutamine residues are not extracted well from the cup and/or chromatograph poorly on a SP.400 g.l.c. column. As a result, the yields of these residues are poor and in later degradation cycles may become insufficient for a definite identification of a residue. The blanks left in the sequences shown in Figs. 5 and 6 arise mainly for the reasons cited above. As mentioned in the introduction, the sequence of the α chains is not complete; the main purpose for obtaining these data was to establish the existence of two different types of α chains in rat haemoglobin.

For remaining residues, we digested $^{I}\alpha$ CB2 peptide with trypsin, and separated the peptides by high-

Table 3. *Amino acid compositions of $^{I}\alpha$ CB2 tryptic peptides and peptides $^{I}\alpha$ CB3 and $^{II}\alpha$ CB3*

Amino acid	T6 (CB2 41–56)	T9 (CB2 62–68)	T10 (CB2 69–90)	T11 (CB2 91–92)	T11-12 (CB2 91–99)	T13 (CB2 100–121)	$^{I}\alpha$ CB3	$^{II}\alpha$ CB3
Lysine	1.0	1.5	1.0		1.0		2.0	2.0
Histidine	0.9		2.3			2.0	0.9	0.9
Arginine				1.0	0.6		0.9	0.8
Aspartic acid	2.0	1.1	4.4		2.0	1.5	1.0	1.4
Threonine	0.8		1.2			2.1	2.0	2.2
Serine	2.9		2.0			1.4	3.2	3.8
Glutamic acid	1.1		1.0			0.3	0.4†	0.2†
Proline	1.2		1.2		1.0	2.4		
Glycine	1.1		1.0			1.3	0.4†	0.5†
Alanine	1.0	3.0	2.7			2.3	2.0	2.3
Half-cystine						0.5*		
Valine	2.1	0.9	0.8		1.9	1.0	2.2	2.4
Methionine								
Isoleucine	1.1							
Leucine		1.0	5.0	0.9	0.7	4.3	2.9	3.1
Tyrosine	0.8						0.9	1.0
Phenylalanine	0.6				1.0	1.3	1.0	1.0

* A low value is expected, since no measures were taken to prevent oxidation. This sample had not been treated with ethyleneimine.

† Peptide CB3 is probably contaminated with a small fraction of CB1 (Fig. 7); the values for glutamic acid and glycine may be attributed to this contamination.

voltage electrophoresis and chromatography. Spots eluted from the peptide maps were hydrolysed for the determination of their amino acid compositions (Table 3). The compositions were fitted into the sequence by homology to mouse and rabbit α chains. The amino acid compositions of $^{I}\alpha$ CB3 and $^{II}\alpha$ CB3 peptides were similarly matched by homology to provide the sequences of residues 122–141 (Table 3).

Oxygen equilibria

Fig. 8 gives the Hill plots for Hb 3 and Hb 5 in 0.1 M-potassium phosphate at pH7.6 and 8.0 and at 20°C. p_{50} is the partial pressure of O_2 at which the haemoglobin solution is half saturated. The log p_{50} and h values of these two haemoglobins calculated from Fig. 8 are given in Table 4. These values are similar for the two haemoglobins at a given pH value; thus the structurally distinct α chains contribute similarly to the functional properties of the haemoglobin when combined with the same β chain ($^{II}\beta$).

Also included in Fig. 8 are the Hill plots for rat haemolysate; the log p_{50} and h values are given in Table 4. These p_{50} values are different from those of the purified haemoglobins.

Table 4 also contains the log p_{50} values of normal human Hb A under similar conditions (Antonini, 1965). The log p_{50} value for rat haemolysate at pH7.6 (0.770) is similar to that of Hb A (0.780). At pH8.0, however, the value for rat haemolysate (0.388) is smaller than that of Hb A (0.550). Thus the difference in log p_{50} values between pH8.0 and 7.6 is larger for rat haemolysate than for Hb A. This larger difference is also seen in the two purified haemoglobins. Rat haemoglobins therefore appear to have a larger alkaline Bohr effect than human Hb A at these pH values. Unfortunately, extensive methaemoglobin formation occurred in rat haemolysate at pH7.0 or lower, preventing a more thorough examination of the Bohr effect.

Discussion

Rat haemoglobins and their respective chains

The six haemoglobin components isolated in the present studies from rat erythrocytes resembled those identified by Stein *et al.* (1971). From a comparison of their charge and relative quantities, our haemoglobins appeared to correspond to those of Stein *et al.* (1971) as follows: our Hb 1 to their fraction I; Hb 2 to fraction II; Hb 3 to fraction III; Hb 5 to fraction IV; and Hb 6 to fraction V. Their Hb fraction VI probably corresponds to our Hb 6*a*. Our Hb 4 was probably not well resolved in the isoelectric-focusing technique of Stein *et al.* (1971). We would expect it to be found between their Hb components III and IV.

As would be expected from the presence of six haemoglobins, five globin chains were found. From their amino acid sequences, two of these chains were α chains. The remaining three chains were probably β chains, since they combine with α chains to form haemoglobin tetramers (Table 1). The relative amounts of serine, threonine and proline in the five chains supported their designations as α and β (Table

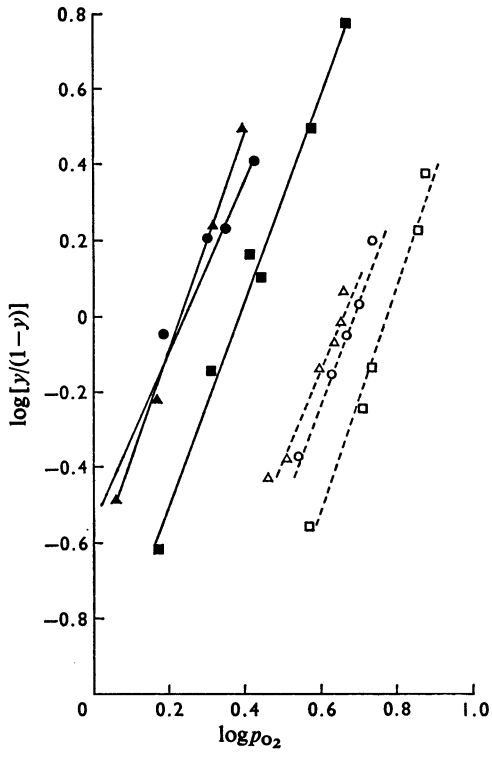


Fig. 8. *Oxygen equilibrium studies of Hb*3, *Hb*5 *and unfractionated haemolysate in* 0.1 M-*potassium phosphate at* 20°C

In this Hill plot $\log[y/(1-y)]$ (where $y = \%$ saturation with O_2) is graphed against $\log p_{O_2}$. The slope of such a plot is equal to h in the Hill equation. All the lines were fitted by least-squares analysis. Hb 3: ●, pH 8.0; ○, pH 7.6. Hb 5: ▲, pH 8.0; △, pH 7.5. Haemolysate: ■, pH 8.0; □, pH 7.6.

Table 4. *Oxygen equilibria of rat haemoglobin compared with human*

The values for Hb 3, Hb 5 and haemolysate were calculated from the data in Fig. 8. The values for Hb A were taken from Antonini (1965).

	$\log p_{50}$		h	
	pH 7.6	pH 8.0	pH 7.6	pH 8.0
Hb 3	0.682	0.240	2.7	2.4
Hb 5	0.652	0.233	2.4	3.0
Haemolysate	0.770	0.388	3.0	2.8
Human Hb A	0.780	0.550	2.7	2.7

2), but the sequences of the three β chains have not yet been studied.

The fraction of each haemoglobin component and of each globin chain was determined respectively from DEAE-cellulose and CM-cellulose chromatography separations. The expected proportions of the various haemoglobins can be calculated by multiplying the fractional amounts of their substituent chains given in Table 1. There is excellent agreement between expected and observed values for Hb components 1, 3 and 5. The discrepancy between prediction and observation for Hb 4 is probably due to difficulties in estimating the amount for such a minor component. There is more Hb 6 found than predicted. Since some Hb 5 often contaminates this region, this finding is not unexpected. No explanation is readily available for the less-than-predicted amount of Hb 2. With the possible exception of Hb 2, it therefore is likely that there is no preferential association of any particular α and β chains.

Sequences of the two α chains

The sequence of chain $^{I}\alpha$ is very similar to that of chain $^{II}\alpha$. Three differences were observed in the 108 residues placed in the $^{II}\alpha$ sequence. The only charge difference was found at residue 5, which is aspartic acid in chain $^{I}\alpha$ and alanine in $^{II}\alpha$. This agrees with peptide 'maps' of the $^{I}\alpha$ and $^{II}\alpha$ chains, which showed a difference in charge for only tryptic peptide 1. Two other differences were also found: residue 70 in $^{II}\alpha$ chain was alanine and in $^{I}\alpha$ it was most probably valine; residue 73 in chain $^{II}\alpha$ was valine and in $^{I}\alpha$ it was most probably leucine. All these interchanges can result from a single base-substitution in the particular codon. The sequence of chain $^{I}\alpha$ in this region is derived from the amino acid composition of tryptic peptide 10 and placed by homology with mouse and rabbit α chains. Therefore it is also possible that residue 70 is leucine in chain $^{I}\alpha$ and 73 is valine in both $^{I}\alpha$ and $^{II}\alpha$ chains. This latter set of assignments, however, would require two base changes in comparing residue 70 for rats and mice or for $^{I}\alpha$ and $^{II}\alpha$ chains.

The presence of two α chains in the rat probably reflects a gene duplication. Over 200 rats were examined in our laboratory and all had six haemoglobins. Stein *et al.* (1971) had also examined over 100 rats of various strains and always found the same six components. In addition, the rats examined were inbred and it is unlikely that all would be heterozygous for two alleles at one locus. Finally, the sequences for $^{I}\alpha$ and $^{II}\alpha$ chains reveal amino acid interchanges at a minimum of three positions so that the difference between them is truly structural and is not due to post-synthesis modification. Since $^{I}\alpha$ and $^{II}\alpha$ chains are so structurally similar, the duplication is probably recent and occurred around 20 million years ago. This tentative calculation is based on the acceptance in haemoglobin evolution of 14 point mutations/100 amino acid residues per 100 million years (Dayhoff, 1972) and on an observed three differences in the 108 residues that can at present be compared for $^{I}\alpha$ and $^{II}\alpha$ chains. Since all rats examined carried the duplication, it must have occurred before

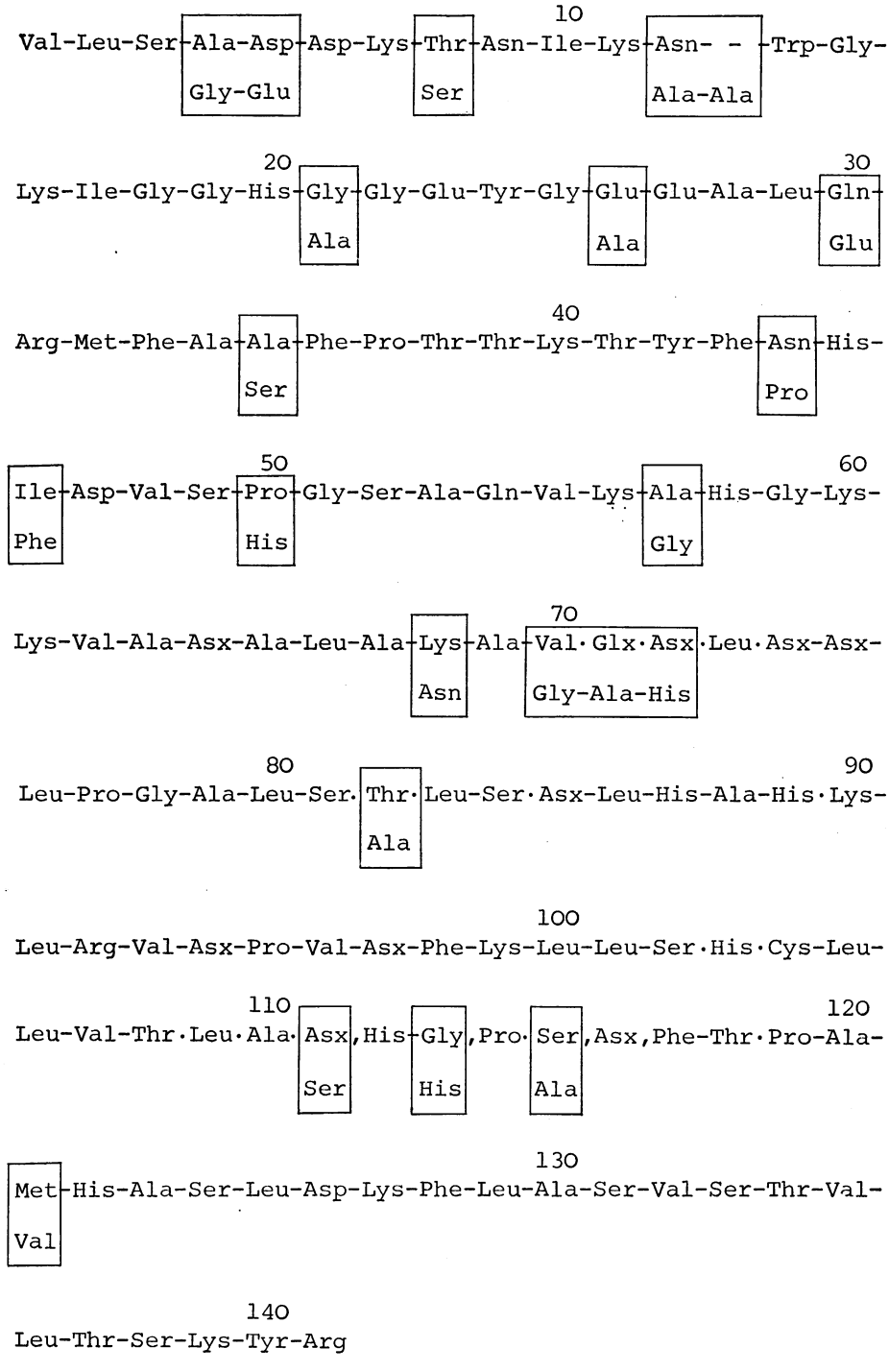


Fig. 9. *Comparison of the amino acid sequence of rat $^{1}\alpha$ and mouse α chain*

The sequences are identical except as indicated in the boxed positions below the continuous line of the rat $^{1}\alpha$ sequence. The sequence for mouse α chain (strain C57BL) is taken from Dayhoff (1972); in this reference the regions placed by homology are delineated.

the species *Rattus norvegicus* diverged. It would be of interest to examine the haemoglobins of other rat species.

Rat $^{I}\alpha$ chain differs from mouse α chain in at least 22 positions (Fig. 9). All these interchanges can occur by a single base-change in the particular codon, except for the following, which require two base-substitutions: positions 12, 44 and 113.

Most of the differences between rat and mouse α chains are not found in residues important for oxygenation function. Of particular interest is the region containing residues 44–50. In this region, which is distal to the haem group, rat α chain, like carp α chain (Dayhoff, 1972), differs from most α chains. Instead of the usual proline at position 44, rat α chain contains asparagine and carp α chain has alanine. Residue 46, which is in the haem-contact region, is usually phenylalanine. In the rat, however, it is isoleucine and in carp, tryptophan. Histidine usually occupies position 50, but in both rat and carp, proline is found instead. Whether this has any effect on the functional characteristics of the molecule is not yet clear.

Several other normally invariant residues of the α chain are altered in the rat, namely: residue 26, glutamic acid instead of alanine; residue 72, aspartic acid instead of histidine; and residue 121, methionine instead of valine. Residue 107, which is in the $\alpha^{1}\beta^{1}$-contact region (Perutz *et al.*, 1968), is probably valine in the rat, by homology with other species. Since we inferred the sequence of this region from amino acid composition, however, it is also possible that residue 107 is glycine and residue 113 is valine.

Oxygen equilibrium studies

Logp_{50} values at pH 7.6 and 8.0 are similar for Hb 3 and Hb 5 (Table 4). Thus the two structurally distinct α chains, in combination with the major β chain, contribute similarly to the oxygen-binding properties of the molecule. This similarity is not surprising, since only a minimum of three amino acid interchanges occurs between the two α chains: residue 5, aspartic acid or alanine; residue 70, valine or alanine; and residue 73, leucine or valine. Apparently none of these interchanges involves residues critical to the molecule's function.

The haemoglobins in the haemolysate have a lower oxygen affinity than the two purified haemoglobins. The higher affinity of the purified components may be an artifact, however, caused by some denaturation during purification of the haemoglobins. The h values for both the purified haemoglobins and the haemolysate were similar to that of human Hb A (around 2.7). Thus rat haemoglobins exhibit normal co-operativity. Two of the h values for the purified haemoglobins were somewhat low, however (2.4 compared with 2.7–3.0), perhaps because of changes induced during purification.

Rat haemolysate, Hb 3 and Hb 5 all show a larger Bohr effect than human Hb A between pH 7.6 and 8.0. This effect could be intrinsic to the haemoglobin molecule, i.e. involve substitutions at amino acids critical for the Bohr effect, or extrinsic, i.e. due to the presence of different effectors.

Note Added in Proof (Received 7 April 1975)

We appreciate having been sent the results of Chua *et al.* (1975) before publication and are pleased by the extensive agreement between our work and theirs. We agree fully with the 'Note Added in Proof' to their paper resolving the discrepancies between the two sequences for the major α chain. The reasons for the consensus are (1) that direct sequencing is obviously far superior to placement by homology and (2) further results for the $^{I}\alpha$ chain obtained in unpublished work by L. M. Garrick, R. L. Sloan, T. W. Ryan & M. D. Garrick. We can now state that the three chains previously postulated to be β chains are indeed β chains. About 70% of the residues for $^{II}\beta$ chain and about 30% of those for $^{I}\beta$ and $^{III}\beta$ chains have now been placed in sequence. Four substitutions between $^{II}\beta$ and $^{III}\beta$ chains have been found.

These studies were supported in part by Grant AM-15234 from the National Institutes of Health and by the Richard E. Wahle Fund. The additional work mentioned in the 'Note Added in Proof' was supported by General Research Grant RRO 5400 from the General Research Support Branch, Division of Research Resources, National Institutes of Health.

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