ABNORMAL HUMAN HAEMOGLOBINS

IV. THE CHEMICAL DIFFERENCE BETWEEN NORMAL HUMAN HAEMOGLOBIN AND HAEMOGLOBIN C

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SUMMARY

Tryptic digests of normal human haemoglobin and of haemoglobin C contain a peptide fragment (peptide 4) which apparently alone of all the peptides has a different chemical structure in the two haemoglobins. These peptides have been degraded and their amino acid sequences have been determined in the two cases. The sole alteration is the replacement of a glutamic acid residue of normal haemoglobin by lysine in haemoglobin C.

INTRODUCTION

Human haemoglobin has been shown to consist of two identical halves[1]. The globin of the human haemoglobin molecule comprises two pairs of identical polypeptide chains[2-3]. The abnormal human haemoglobin, sickle cell haemoglobin (S), was found by INGRAM[4-6] to differ from normal human haemoglobin (A) by one amino acid in each of one pair of identical polypeptide chains, that is, by one amino acid in the half molecule. We have investigated the abnormal human haemoglobin C in a similar manner, and find that it also differs from haemoglobin A by one amino acid in the half molecule. The same glutamic acid residue of haemoglobin A which is replaced by valine in haemoglobin S, has been replaced by lysine[7] in haemoglobin C. The genetic implications of this change have been dealt with previously[7].

Haemoglobin C was discovered by ITANO AND NEEL[8] together with haemoglobin S in an individual suffering from a mild form of sickle cell anaemia. The techniques employed included electrophoresis on paper and in solution where haemoglobins S and C are easily distinguishable from each other and from haemoglobin A.

Individuals have been found who are homozygous for the haemoglobin C gene and who have virtually only haemoglobin C in their erythrocytes. Family studies on other patients have indicated that they are heterozygous for the haemoglobin C

Abbreviations: DNP, dinitrophenyl; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin.

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gene and the genes for either haemoglobins A, S or the thalassaemia syndrome[9]. The haemoglobin C gene is found mostly among people of negroid descent[9], but one case of the homozygous haemoglobin C disease has been reported in a Sicilian[10]. As in the case of haemoglobin S, the mutation producing haemoglobin C is inherited as a single gene[9]. From the examination of families having the genes for both haemoglobin S and haemoglobin C, RANNEY[11] has concluded that the genes for haemoglobins S and C are allelic or closely linked; that is to say they occupy the same or closely linked loci in the chromosome (see also refs. 12, 13).

Haemoglobin C has fewer net negative charges[14] than both haemoglobins A and S. This can be demonstrated both by moving boundary and paper electrophoresis under acid (pH 6.5) and alkaline (pH 8.6) conditions. ITANO[14] calculated that at pH 8.6 the haemoglobin C molecule (mol. wt.: 66,700) has about two net negative charges fewer than haemoglobin S, and that the haemoglobin S molecule has about two net negative charges fewer than haemoglobin A. On the other hand, SCHEINBERG[15] examined the relative mobilities of haemoglobins A, S and C by paper electrophoresis over a wide pH range. He concluded that haemoglobin C has between five and eight carboxyl groups fewer per molecule than haemoglobin A. We have found[7] that there is a charge difference between haemoglobins A and C caused by the substitution of two amino groups for two carboxyl groups in the molecule. These are the only charge differences in the molecules so far detected.

ITANO[16] reported that reduced haemoglobin C has a higher solubility than reduced haemoglobin A in 2.4 *M* phosphate solutions, but HUISMAN *et al.*[17] find that carbonmonoxy-haemoglobin C is less soluble than carbonmonoxy-haemoglobin A and that the reduced haemoglobins A and C have about the same solubility in salt solutions. These findings might suggest that there is more than one type of haemoglobin C (just as there is more than one type[18] of haemoglobin D); however, we have examined three samples of haemoglobin C from unrelated negro patients and all have the same structure as far as can be determined by our methods.

Amino acid analyses of haemoglobins A and C have shown only small and statistically insignificant differences in their compositions[14], except that HUISMAN reported an increase in the lysine content of haemoglobin C, as would be expected from our findings. On the other hand, he did not observe a corresponding fall in the number of glutamic acid residues. Other differences between the two haemoglobins, reported by HUISMAN in the same paper, have not so far been substantiated by us.

The present paper deals with the comparison of the tryptic digests of haemoglobins A and C, the chymotryptic digests of their trypsin resistant "cores", and the structural analyses of the peptides in the haemoglobin A and C trypsin digests which were found to differ.

MATERIALS AND METHODS

Haemoglobin

The method of preparation of the haemoglobin solutions was the same as previously described by INGRAM[5], except that the haemolysates were centrifuged after dialysis. Three different samples of haemoglobin C, from persons known to be homozygous for the haemoglobin C gene, and two samples of haemoglobin A were used. One of the samples of haemoglobin C was a solution of the pure haemoglobin in

aqueous glycerine and was dialysed and centrifuged as usual. Paper electrophoresis in 0.06 *M* veronal buffer at pH 8.6 showed haemoglobin C migrating as a single band with a lower mobility than both haemoglobins A and S. No other components were visible in this test.

Digestion

Tryptic digestion of the heat denatured haemoglobin and chymotryptic digestion of the trypsin resistant core were performed in a "pH stat" as previously described[5,6].

Electrophoresis

Pyridine acetate buffers at pH 6.4 and 3.6, using AnalaR grade pyridine–glacial acetic acid–water (25 : 1 : 225) and (1 : 10 : 90), respectively, were used for one dimensional paper electrophoretic separation of the peptides as described by INGRAM[5]. "*Fingerprints*" of the resulting peptide mixtures of haemoglobins A and C were performed "in parallel" as previously described[5].

For better resolution of peptide mixtures, the tryptic and chymotryptic digests were subjected to electrophoresis on Whatman No. 3MM paper at pH 6.4 at 20 V/cm for 1.5 h. The peptide bands were located by a 0.025 % ninhydrin in water-saturated *n*-butanol spray followed by heating at 100° for about 5 min; they were then cut out and eluted with 20 % acetic acid. The bands from haemoglobin A and C digests so obtained were next run in parallel by descending chromatography[19] with *n*-butanol–acetic acid–water (4 : 1 : 5) on Whatman No. 1 paper or by paper electrophoresis at pH 3.6.

End group analysis of peptides

1-fluoro-2,4-dinitrobenzene as 5 % solution in alcohol was added to the peptide dissolved in 1 % aqueous trimethylamine, as described by SANGER AND THOMPSON[20] for the dinitrophenylation of peptides. The DNP amino acids were determined by a modification of LEVY's two dimensional paper chromatographic system[21]. The first solvent was replaced by the *tert.*-amyl alcohol solvent saturated with the pH 6 phthalate buffer of BLACKBURN AND LOWTHER[22].

EDMAN stepwise degradation was performed by coupling the peptide with phenylisothiocyanate in the triethylamine buffer described by SJÖQUIST[23]. The phenylisothiocyanate was a commercial grade, redistilled *in vacuo*; the triethylamine was redistilled from a commercial grade, but the acetone used was of AnalaR grade and was not further purified. The PTC peptide was cyclised to the PTH of the N-terminal amino acid either in acetic acid saturated with HCl, as described SJÖQUIST[23] or in 3 *N* HCl (ref. 24). The PTH amino acid was extracted with ethyl acetate from acid or neutralized solutions, as indicated (Table IIb). When acetic acid had been used for cyclisation, the reaction mixture was first diluted with water before extraction. In order to identify the PTH amino acid, the amino acid was regenerated by heating[25,26] the PTH amino acid in concentrated HI (M.A.R.) for 4 h at 140°. The amino acid was then identified by using the two dimensional chromatographic system[27] of REDFIELD. The first (methanolic) solvent had been replaced by *n*-butanol–acetic acid–water (3 : 1 : 1). This system will be referred to as the "butanol–acetic acid: REDFIELD 2" system.

Qualitative amino acid analyses of peptides eluted from paper chromatograms

were performed by hydrolysis of the peptides in hard glass capillary tubes for 14 h at 120° in constant boiling HCl which had been twice glass distilled from stannous chloride. This was followed by two dimensional chromatography in the REDFIELD system[27].

Quantitative amino acid analyses were performed in the following way. The peptides were hydrolysed as described above, and the hydrolysates chromatographed on a multisheet frame on 20 × 20 cm squares of Whatman No. 1 paper using the "butanol–acetic acid: REDFIELD 2" system. The papers were allowed to dry overnight in a fume cupboard. After development in the second dimension, the papers were dried in a Kodak film drying cabinet for 30–60 min and then steamed for 10 min to remove the excess diethylamine. The ninhydrin colour of the amino acids was developed by dipping the papers in a 5 % solution of ninhydrin in acetone which contained 5 % by volume of a 0.05 *M* phosphate solution (pH 7.2) (ref. 28) and by heating the papers at 65° for 22 min[29]. The spots were cut out with a razor blade and the ninhydrin colour eluted in 4-ml aliquots of 71 % ethanol. The O.D. of each spot so eluted was found by using a 1-cm cuvette in a Unicam SP 500 spectrophotometer at 575 mμ against a blank cut from one of the papers[29]. 10-μl aliquots of standard mixtures of amino acids of 2, 5, 8 and 10 m*M* strength were run with each determination. They contained lysine, histidine, glutamic acid, threonine, proline, valine and leucine. The amount of each amino acid in a peptide was found from the standard curves.

Partial acid hydrolysis was performed by incubation of the peptide in concentrated HCl (M.A.R.) at 37°.

Peptides T-4a and T-4b were purified by paper electrophoresis of the haemoglobin C tryptic digest at pH 6.4; peptide T-4a by electrophoresis of band 2 at pH 3.6, and peptide T-4b by paper electrophoresis of band 4 at pH 3.6 (see Fig. 4).

Tests for tryptophan and cysteine were performed by using the EHRLICH[30] and the platinic iodide[31] reagents respectively.

RESULTS AND DISCUSSION

Comparison of the haemoglobin A and C tryptic digests

The fingerprints of haemoglobin A and C digests (Fig. 1) show that in the neutral band of peptides the peptide T-4 of haemoglobin A, has been replaced in haemoglobin C by the neutral peptide T-4a. The more rigorous method of paper electrophoresis at pH 6.4 followed by chromatography (Fig. 2a and 2b) showed indeed that this was the only change in the neutral band (band 2), but in addition a new peptide (T-4b) was found in the positively charged band 4. Elution of this peptide from the chromatogram showed that it was mixed with other peptides and it was finally purified as described above.

The remaining peptides of the tryptic digests of haemoglobins A and C have been examined in the following ways: (a) By fingerprinting the digests in parallel locating the peptide spots by the 0.025 % ninhydrin spray, eluting each spot from each of two fingerprints and analysing qualitatively the amino acid composition of each and every peptide. (b) By parallel electrophoresis at pH 6.4 of the digests and parallel descending chromatography of the peptide bands (Fig. 2a and 2b). The peptides were located and qualitative amino acid analyses made as before.

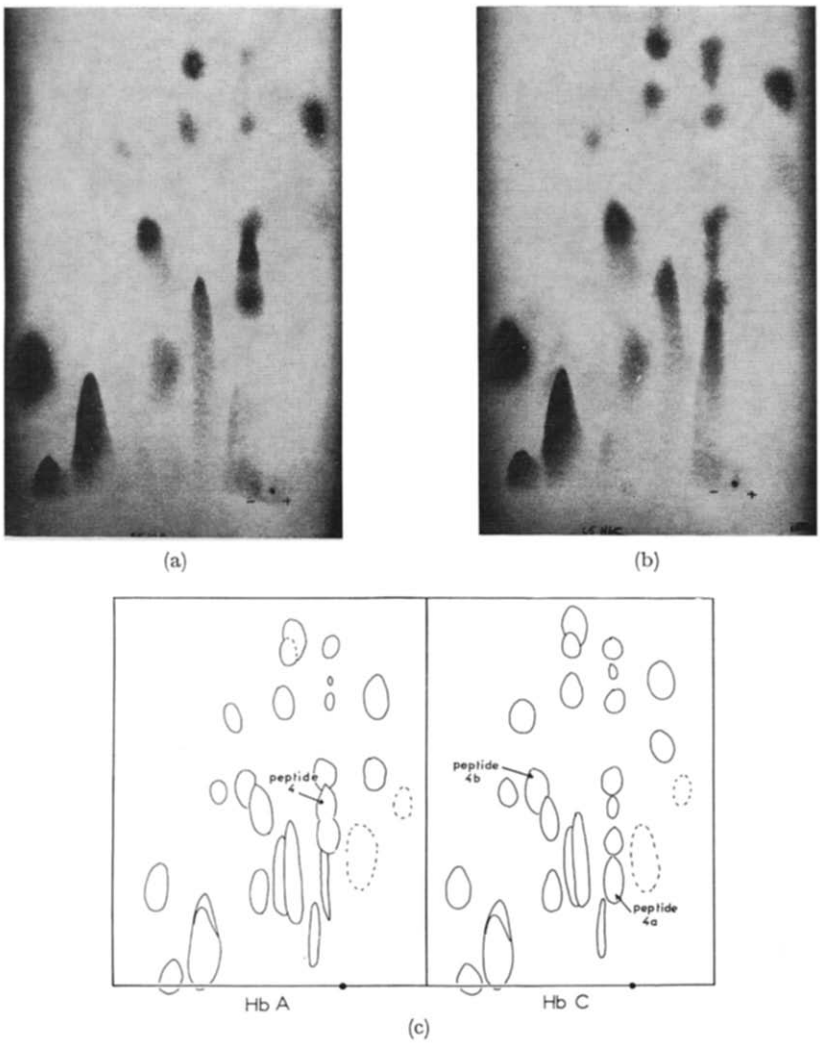

Fig. 1. Fingerprints of the trypsin digests of haemoglobins A and C. (a), Haemoglobin A; (b), Haemoglobin C; (c), Tracings showing the location of the changed peptides. The dotted lines indicate ninhydrin positive peptides which only become visible after heating.

No differences in amino acid composition, apart from the ones described in this paper, could be detected between the peptides in the tryptic digests of haemoglobins A and C.

Comparison of the chymotryptic digests of the trypsin resistant "cores" of haemoglobins A and C

Examination[6] of the "core" digests of haemoglobins A and C by fingerprinting revealed no differences in the patterns of the peptides.

Examination of the amino acid compositions of each peptide obtained by method 2 again revealed no differences between the two "cores" (Fig. 3).

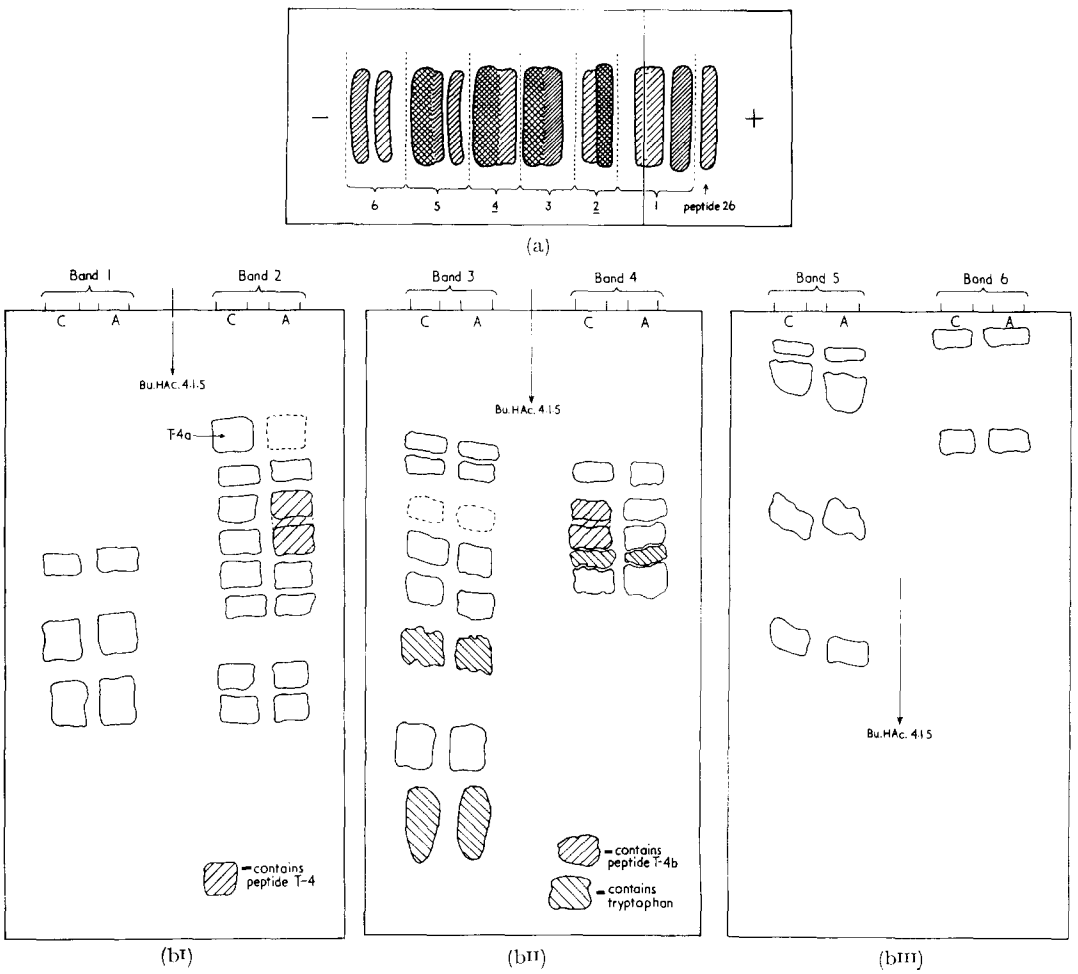

Fig. 2. (a) Tracing of the ionogram at pH 6.4 of the trypsin digest of haemoglobin A showing how the peptide bands were cut out for elution and subsequent fractionation. (Identical patterns were obtained from the trypsin digests of haemoglobin C). (b) Tracings of the peptide bands shown in Fig. 2 (a) after separation by chromatography in the *n*-butanol–acetic acid–water (4:1:5) system.

The amino acid sequence of haemoglobin A peptide T-4

The original sequence found by INGRAM[4,7] was shown to be incorrect by HUNT AND INGRAM[32], and HILL AND SCHWARTZ[33] and the corrected sequence is now:

val·his·leu·thr·pro·glu·glu·lys

Val, valine; his, histidine; leu, leucine; thr, threonine; pro, proline; glu, glutamic acid; lys, lysine. The γ-carboxyl groups of both glutamic acid residues are free and carry negative charges at neutral pH.

The amino acid sequence of haemoglobin C peptide T-4a (Fig. 1)

Equal amounts of glutamic acid and lysine were found by qualitative amino acid analysis of the peptide in the REDFIELD system as judged by eye. Tryptophan

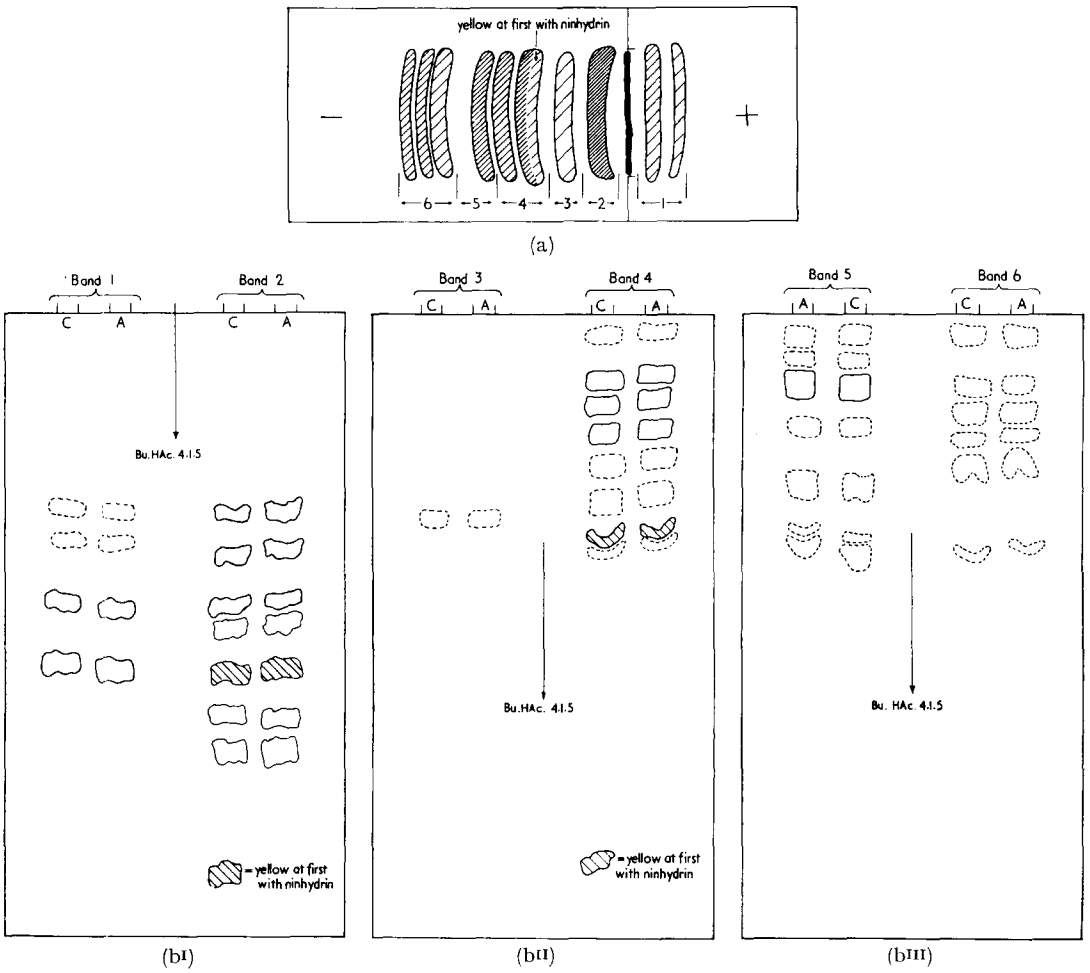

Fig. 3. (a) Tracing of the ionogram at pH 6.4 of the chymotrypsin digest of the trypsin-resistant "core" of haemoglobin (see Fig. 2(a)). (b) Tracings of the peptide bands shown in Fig. 3(a) separated by chromatography in the *n*-butanol–acetic acid–water (4 : 1 : 5) system.

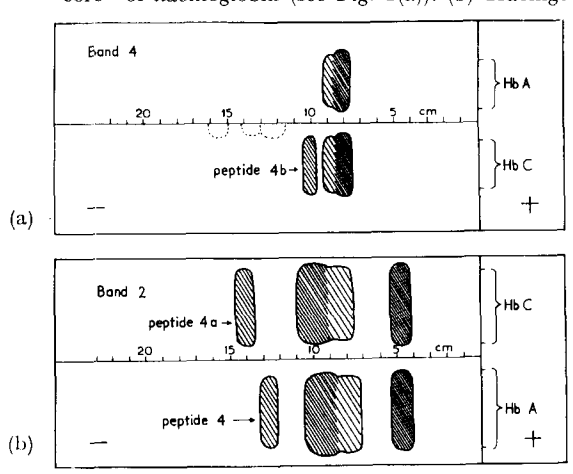

Fig. 4. (a) Tracing of the ionogram at pH 3.6 of the peptide band 2 from Fig. 2(a) of haemoglobins A and C run side by side to show the absence of the peptide A T-4 and the presence of the peptide C T-4a in haemoglobin C. (b) Tracing of the ionogramat pH 3.6 of the peptide band 4 from figure 2(1) of haemoglobins A and C run side by side to show the presence of the peptide C T-4b in haemoglobin C.

and cysteine could not be detected by the specific staining reactions for these two amino acids. No other amino acids were present.

End group analysis by the 1-fluoro-2,4-dinitrobenzene method yielded DNP-glutamic acid, identified by chromatography in the *tert.*-amyl alcohol–pH 6 phthalate system. It was the only ether soluble product after hydrolysis of the DNP-peptide T-4a. The acid soluble portion of the DNP-peptide contained only ε-DNP-lysine as determined by chromatography in the REDFIELD system[27]. Using the EDMAN stepwise degradation method[23], glutamic acid was again found to be N-terminal by hydrolysis of the PTH-amino acid in concentrated HI. The residue of the peptide after hydrolysis contained only lysine.

Thus the amino acid sequence of peptide T-4a must be glutamyl-lysine. This is in agreement with the known specificity of trypsin.

The amino acid sequence of haemoglobin C peptide T-4b

Qualitative amino acid analysis of peptide T-4b showed that the peptide contained histidine, leucine, lysine, proline, threonine and valine, while the specific staining reactions for tryptophan and cysteine were negative. Quantitative amino acid analysis using the "butanol–acetic acid, REDFIELD 2" system showed that the amino acids were present in equimolar quantities (Table I).

TABLE I

AMINO ACID ANALYSIS OF HAEMOGLOBIN C PEPTIDE T-4b

	Time of hydrolysis			
	18 h		*14 h*	
	μmoles found	*μmoles/μmoles Peptide**	*μmoles found*	*μmoles/μmoles Peptide**
Lys	0.024	1.0	0.057	1.0
His	0.020	0.9	0.061	1.1
Thr	0.027	1.2	0.045	0.8
Pro**	(0.02)	(1)	(0.05)	(1)
Val	0.020	0.9	0.054	1.0
Leu	0.023	1.1	0.065	1.2
Mean	0.023		0.056	

* Based on the mean value of μmoles of amino acid obtained in each experiment (omitting proline).

** Since the O.D. of proline is not great enough under these conditions to give accurate values the amount of proline in each hydrolysate was estimated by eye in comparison with the known standards.

End group analysis by the 1-fluoro-2,4-dinitrobenzene method failed to give any ether soluble DNP-amino acid after hydrolysis of the DNP-peptide while the residue of the peptide contained no histidine, either as the free amino acid or as the imidazole-DNP derivative, and valine was reduced in amount (Table IIa). Because of the absence of histidine in the hydrolysate, histidine was originally suspected[7] as the N-terminal amino acid in the peptides T-4 and T-4b. However, by EDMAN stepwise degradation of the peptide it was found that valine, and not histidine, was N-terminal. No amino acid could be identified after a second step of degradation, which should

TABLE II

END GROUP ANALYSIS OF PEPTIDE T-4b

a By dinitrophenylation

	Lys	*His*	*Thr*	*Pro*	*Val*	*Leu*
Before dinitrophenylation	+++	++	++	++	+++	++++
After dinitrophenylation*	++	—	+++	++	++	++++

Lysine was determined as its ε-DNP derivative*

b By EDMAN *stepwise degradation*

	Lys	*His*	*Thr*	*Pro*	*Val*	*Leu*	*α-amino butyric acid*
Before degradation	+	+	+	+	+	+±	—
1st step extract from an acid solution	±	—	—	—	++	—	—
2nd step extract from an acid solution	—	—	—	—	±	—	—
2nd step extract from neutral solution**	—	—	—	—	—	—	—
3rd step extract from acid solution	±	—	—	—	—	++	±
4th step extract from acid solution	—	—	—	—	—	+	+

* α-Amino butyric acid is probably formed by the reduction of PTH-threonine during hydrolysis with hydriodic acid.

** Attempt to find PTH-histidine (see text).

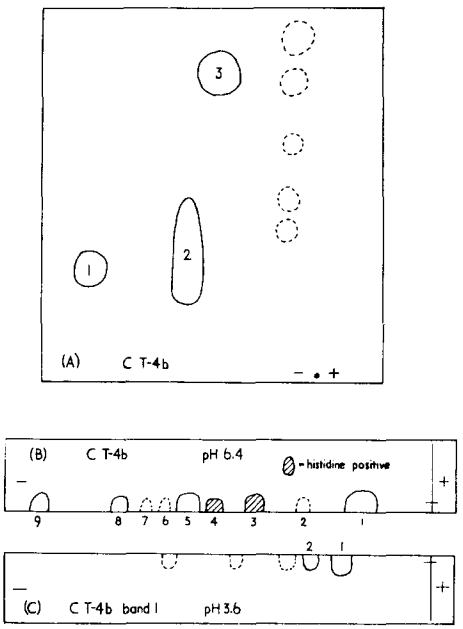

Fig. 5. (a) Tracing of the fingerprint of the partial acid hydrolysate of the haemoglobin C T-4b peptide. (b) Tracing of the ionogram at pH 6.4 of the partial acid hydrolysate of the haemoglobin C T-4b peptide. (c) Tracing of the ionogram at pH 3.6 of band 1 from Fig. 5(b).

TABLE III

PARTIAL ACID HYDROLYSIS OF PEPTIDE T-4b

a 40 h partial acid hydrolysate separated by Fingerprinting (Fig. 5a)

Peptide	*Lys*	*His*	*Thr*	*Pro*	*Val*	*Leu*
A-1	?	—	—	—	—	—
A-2	+++	—	+++	+++	—	—
A-3	—	++	—	—	++	+++

b 90 h partial acid hydrolysate separated by paper electrophoresis at pH 6.4 (Fig. 5b)

Peptide	*Lys*	*His*	*Thr*	*Pro*	*Val*	*Leu*
B-3	—	+++	—	—	+++	++++
B-4	±	++	±	±	++	—
B-5	+++	—	+++	+++	—	—
B-8	+	—	—	+	—	—
B-9	++	—	—	—	—	—

c Band B-1 separated by paper electrophoresis at pH 3.6 (Fig. 5c)

Peptide	*Lys*	*His*	*Thr*	*Pro*	*Val*	*Leu*	*Remarks*
C-1	—	—	++	±	++	+++	Probably free amino acids
C-2	—	—	++	++	—	—	

TABLE IV

END GROUP ANALYSIS OF THE FRAGMENTS FROM PEPTIDE T-4b

a By dinitrophenylation

Peptide B-3	*His*	*Val*	*Leu*	*DPN amino acid*
Residue after dinitrophenylation	—	+	++	None

Peptide B-5	*Lys*	*Thr*	*Pro*	*DPN amino acid*
Residue after dinitrophenylation Lysine was determined as the ε-DNP derivative	++	±	++	Threonine

b By EDMAN *stepwise degradation*

Peptide B-3	*His*	*Val*	*Leu*
Before degradation	++	++	+++
1st step			
extract from acid solution	±	++	±
extract from neutral solution	—	—	—
2nd step			
extract from acid solution	—	±	±
extract from neutral solution	—	—	—
3rd step			
extract from acid solution	—	—	+
Unhydrolysed residue	—	—	—

have given histidine, but leucine was found on the third step, as shown in Table IIb. The reason for this behaviour is not yet clear.

Partial acid hydrolysis of the peptide T-4b, derived from about 40 mg of haemoglobin, in concentrated HCl at 37° for 40 h, followed by separation of the peptide fragments obtained by fingerprinting, gave two main peptides, A-2 and A-3 (Fig. 5a). The qualitative amino acid analyses of these fragments are shown in Table IIIa. Hydrolysis of the peptide under the same conditions but for 90 h gave more fragments which were readily separated by paper electrophoresis at pH 6.4 and 3.6 (Fig. 5b and 5c). The qualitative amino acid analyses of some of these peptide fragments is shown in Table IIIb. Both peptides B-3 and B-5 were dinitrophenylated; B-3 gave no DNP amino acid and the residue contained no histidine and less valine than leucine as is shown in Table IVa. B-5 gave DNP-threonine in the ethereal extract and the residue of the hydrolysed DNP-peptide contained proline and lysine as shown in Table IVa. Three steps of the EDMAN degradation were performed on the peptide B-3 yielding valine for the first step, nothing for the second step and leucine for the third step, while the unhydrolysed residue contained no amino acids (Table IVb).

From these results the sequence of peptide T-4b can be constructed in the following way:

Composition	$val_1 his_1 leu_1 thr_1 pro_1 lys_1$
EDMAN degradation	val– ? –leu–(thr, pro, lys)
Partial acid hydrolysis	
A-2	(thr, pro, lys)
A-3	(val, his, leu)
B-3	val–his–leu.
B-4	(val, his)
B-5	thr–(pro, lys)
B-8	(pro, lys)
C-2	(thr, pro)
Sequence	val·his·leu·thr·pro·lys

Assuming that the T-4a peptide is joined to the C-terminal end of peptide T-4b then the sequence of the peptide in haemoglobin C equivalent to T-4 is:

val·his·leu·thr·pro·lys·glu·lys,

compared with peptide T-4 in haemoglobin A:

val·his·leu·thr·pro·glu·glu·lys

and peptide T-4 in haemoglobin S:

val·his·leu·thr·pro·val·glu·lys

It can be seen that there is a single amino acid change in haemoglobin C of a glutamic acid to a lysine in exactly the same place as the glutamic acid to valine change in haemoglobin S. These are the only differences so far detected by us in the haemoglobin half-molecule, and so are likely to represent the whole of the charge difference between the haemoglobins. Thus haemoglobin C has two less carboxyl groups and two additional amino groups per molecule, a total of four less negative charges per molecule of mol. wt. 66,700 than in haemoglobin A. SCHEINBERG[15] had estimated that the charge difference was due to five or six fewer carboxyl groups. He came to this conclusion because he was unable to detect a difference in electrophoretic mobility at low pH between molecules of haemoglobin A and C with positive

charges of about 110 and 112. It is unlikely that his method would be sensitive enough.

One of the most striking differences in the properties of haemoglobins A, S and C are their solubilities in the deoxygenated form. Since the only detectable differences in the molecules so far found are the amino acid substitutions, it is quite likely that these cause the difference in the solubilities. Conceivably the charge differences affect the pattern of charges on the surface of the molecules, so as to alter the aggregation of the molecules under conditions of high salt concentrations, either facilitating or hindering precipitation. This suggested mechanism will have to serve until the detailed three dimensional structure of haemoglobin is known and understood. The fact that haemoglobin C is able to replace haemoglobin S up to 50 % without inhibiting the gelling of the reduced haemoglobin S solution, while haemoglobin A will only replace[34] up to 25 % of haemoglobin S, is still to be explained.

Both haemoglobins S and C are found close together in Africa, and it has been suggested by MOURANT[35] that the haemoglobin C mutation has arisen from the haemoglobin S mutation, although there is no evidence to support this theory. Genetically haemoglobins A, S and C are of great interest because they illustrate biochemically the effects of allelism. These results should also be viewed in conjunction with haemoglobin G (Neel). This haemoglobin has been found by HILL AND SCHWARTZ[33] to change in the same peptide T-4, but in this case it is the second glutamic acid residue which changes to a glycine.

The present experiments show not only that a gene mutation can affect just a single amino acid residue, but that individual mutations can alter the same amino acid in polypeptide chain in different ways.

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DEOXYPENTOSE NUCLEIC ACIDS

XI. THE DENATURATION OF DEOXYRIBONUCLEIC ACID IN AQUEOUS SOLUTION: CONDUCTIVITY AND MOBILITY MEASUREMENTS

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SUMMARY

Various electrical properties have been derived from conductivity and electrical transport experiments for aqueous solutions of DNA. The specific conductivity shows a discontinuity when plotted against DNA concentration. Below this critical concentration the material transport number falls rapidly with decreasing concentration. The critical concentration corresponds to a similar discontinuity observed in the variation of O.D. with DNA concentration, measured at 2590 Å. A possible explanation of this phenomenon involves the denaturation of DNA at concentrations below the critical concentration. Above this concentration zone native DNA predominates with a charge fraction of 0.4 while below this concentration denatured DNA exists with a very high charge, but lowered mobility.

Abbreviation: DNA, deoxyribonucleic acid.

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