THE AMINO-ACID COMPOSITION OF HUMAN ADULT AND FOETAL CARBONMONOXYHAEMOGLOBIN ESTIMATED BY ION EXCHANGE CHROMATOGRAPHY*

by

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It has been known for many years, that the haemoglobin of the foetus and the newborn child and the haemoglobin of adults differ in many respects. VON KÖRBER[1] and VON KRÜGER[2] in the nineteenth century demonstrated that the foetal haemoglobin (Hb-f) exhibits a much greater resistance to denaturation with alkaline reagents than does the haemoglobin (Hb-a) obtained from the blood of normal adults. Afterwards, BRINKMAN AND JONXIS[3] and SINGER *et al.*[4] used this principle for the percentual estimation of the foetal haemoglobin. Except in denaturation rate the two proteins differ in electrophoretic mobility[5], in ultra-violet spectral absorption[6], in crystal structure[7], in solubility[8,9] and in speed of spreading in monomolecular layer[10]. On the other hand, the molecular weight, the iron content and the spectral absorption at higher wavelengths were identical.

There is also evidence that the amino-acid composition of the two haemoglobins differs in some respects. The amino-acid content of human adult globin and Hb has been reported several times in the literature. The most detailed investigations were carried out in 1950 by SCHROEDER, KAY AND WELLS[11] by means of the methods of starch chromatography, which was developed by STEIN AND MOORE[12]. In this analysis only slight attention was paid to the influence of the time and method of hydrolysis of the protein. Concerning the amino-acid composition of foetal haemoglobin only partial information is available. Using microbiological methods VAN DER LINDEN[13,14] found differences in the threonine, proline, valine, methionine, *iso*leucine, tyrosine, histidine and tryptophan content of globin prepared from umbilical cord blood, which contains about 20% of adult haemoglobin next to the foetal component, and of globin from the blood of normal adults. It will be noted that PORTER AND SANGER[15] described differences in the amounts of N-terminal residues.

The object of the present paper was to compare the amino-acid composition of the haemoglobin from the blood of normal adults and of purified foetal haemoglobin, free of the adult component. The analysis was made by means of the method of ion exchange chromatography which has recently been developed by STEIN AND MOORE[16,17]. The influence of the time of hydrolysis on the recovery of the constituent amino-acids was also studied.

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EXPERIMENTAL

Preparation and purification of haemoglobins. Red blood cells which remained after removal of the plasma of the whole blood of normal adults (laboratory workers) were used as source of Hb-a. These erythrocytes were first washed three times with normal saline and three times with 1.25 % saline and finally haemolised by the addition of one volume of distilled water and 0.4 volume of toluene. This mixture was stored for 24 hours at 0° C. After centrifugation and removal of the toluene fraction the haemoglobin solution was shaken with celite 535 and slowly filtered with suction on a Buchner funnel, in order to remove the stroma protein and some other impurities. This procedure was repeated twice. The purified haemoglobin solution was adjusted to 1–2 g %, saturated with carbon monoxide, and dialysed again slowly running distilled water for two day sand against twice-distilled water for two days and against twice-distilled water for 24 hours at 4° C with four changes of the dialyzing fluid. After a final treatment with Celite 535 the solution was stored in the dark at 1° C.

Red cells from six samples of blood obtained from the foetal side of the placenta, which contain 80–90 % Hb-f next to normal haemoglobin, were first treated in the same manner as the erythrocytes of the normal adult individuals. Then a haemoglobin solution free of the adult pigment was prepared by the technique of CHERNOFF[18], which is based on the different rates of denaturation of the two proteins in alkaline media. After removing the precipitate of denatured normal haemoglobin by centrifugation the remaining solution was treated with Celite 535, saturated with carbon monoxide and dialyzed in the same manner as described for the normal haemoglobin. The final solution which contained 1–1.5 % of COHb-f was also stored in the dark at 1° C. Crystallisation of the two haemoglobins was not feasible since a sufficiently concentrated solution of foetal haemoglobin could not be obtained.

Criteria of purity. The electrophoretic homogeneity was used as an initial criterion of purity. The procedure was a slightly modified method as described by BEAVEN, HOCH AND HOLIDAY[19]. It was found that each haemoglobin moves as a single boundary, while in the combined solution the two haemoglobins move with different speed. The stock solutions were also tested for the presence of stroma protein and of the catalase. None of these impurities could be detected.

Using the method of BRINKMAN AND JONXIS[3] we estimated the percentual amount of Hb-f in the haemoglobin solutions from cord blood before and after removing the normal pigment by alkali denaturation according to the method of CHERNOFF[18]. For this purpose 0.1 ml of the untreated solution was added to 9.9 ml 0.1 *N* NaOH (pH finally 12.7) and the extinction at 6400° A was measured in a 1 cm cuvette at different times. As the purified Hb-f solutions contained a much lower content of haemoglobin (1–1.5 g per 100 mm) it was necessary to mix 1.0 ml of these solutions with 9.0 ml 0.11 *N* NaOH (pH finally 12.7). BRINKMAN AND JONXIS have pointed out that, when the logarithms of the percentages of the unaltered haemoglobin concentrations estimated at varying intervals are plotted against time, a straight line is observed, which may be considered as "a line of disappearance of the more alkali-resistant component". By extending this straight line to zero time, the percentual quantity of this component may be estimated. It is necessary to carry out these estimations before the haemoglobin is converted into carbonmonoxyhaemoglobin as it is known that, both COHb-a and COHb-f exhibit a higher resistance to denaturation with alkali. The fractional denaturation curves given for one sample in Fig. 1 show that the concentration of Hb-f before removing the adult haemoglobin was about 78 %, while the purified Hb-f solution contains nearly 100 % Hb-f. The amounts of Hb-f in the six solutions used in our experiments and measured with this technique was 98–102 %. It will be noted that the Hb-a solutions contain no alkali-resistant component; within two minutes all the haemoglobin is denatured (Fig. 1).

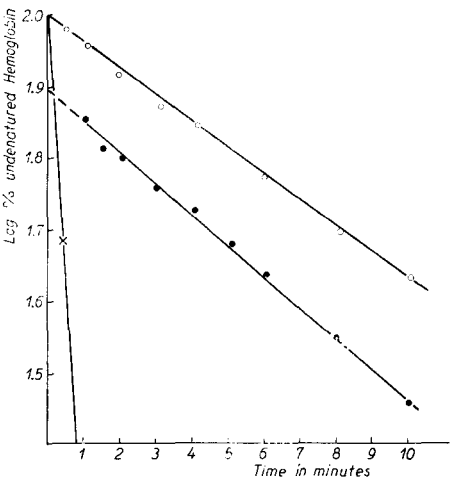

Fig. 1. Fractional denaturation of an umbilical cord blood specimen ●—●, adult haemoglobin ×—× and of a purified foetal haemoglobin solution ○—○.

Concentration of the haemoglobin solution. The concentration of the Hb solutions was based upon the spectrophotometric methods as described by DRABKIN AND AUSTIN[20]. As the tests on ammonia and sulfate in the dialysed solutions were negative, confirmation of the concentration was also obtained from the estimation of the total nitrogen, assuming the nitrogen content of both haemoglobins is 16.8 g %.

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Acidic hydrolysis of haemoglobins. Acidic hydrolysis was carried out in relatively dilute solution as suggested by SCHRAM *et al.*[21]. About 100–150 mg of COHb was added to 200 ml 6 *N* hydrochloric acid and refluxed for different periods. In order to study the effect of time of hydrolysis on the recovery of the amino-acids, the hydrolysis was carried out for 24, 48, 96 and 144 hours. During the first hour of the hydrolysis vigorous foaming occured; this could be diminished by adding one drop of octylalcohol. At the end of the period of refluxing, the excess of HCl was removed *in vacuo* at 40° C, water was added and again removed by evaporation. This process was repeated twice. The hydrolysates were taken up finally in 0.1 *M* sodium citrate-citric acid buffer (pH 2.0) and diluted till a concentration of 1 to 1.5 mg N per ml; the pH varied between 1.5 and 2.0. The solutions were kept in the dark at —10° C.

It was found that for the estimation of the amino-acid content of the haemoglobins it was not necessary to prepare first the globin; therefore only carbonmonoxyhaemoglobin was hydrolysed in all cases.

Chromatographic methods. The chromatography of the hydrolysates was performed on columns of a kation exchanger using a slightly modificated gradient elution technique as recently described by STEIN AND MOORE[16,17]. Instead of Dowex 50 (4–5 % cross linked) we used the English resin "Zeokarb 225" (particle size less than 50 μ, water regain 1.1 g per g) according to the method of CAMPBELL *et al.*[22]*. A full description of the technique used will be given elsewhere[23]. The hydrolysates of six samples of adult Hb and the same number of hydrolysates of six samples of foetal Hb have been analyzed.

RESULTS

A typical experiment showing the elution curve obtained on a column of Zeokarb 225 with a sample of COHb-a hydrolysed for 48 hours, is given in Fig. 2. With the exception of threonine and serine the separation of all amino-acids was complete.

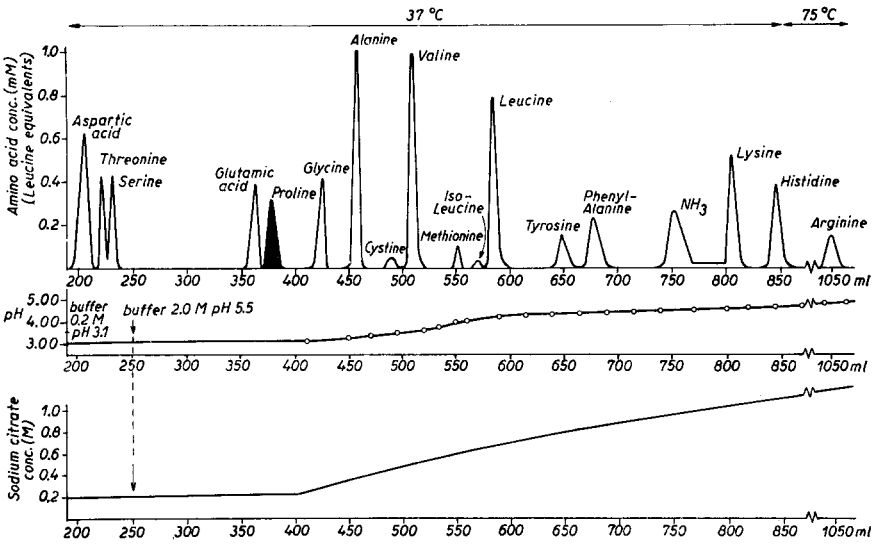

Fig. 2. Elution curve obtained by chromatography of a 48 hour hydrolysate of human adult COHb on "Zeokarb 225" by the gradient elution method of STEIN AND MOORE[16]. The values plotted on the ordinate are expressed as leucine equivalents (except for proline) and must be multiplied by four as only one fourth of each eluted fraction was analysed. In general for each run 2 ml of a hydrolysate (= 14–17 mg of protein) were used. In the lower part of the figure the gradual increase of pH (from 3.1 to 5.0) and of the sodium citrate concentration (from 0.2 *M* to 1.2 *M*) in the effluent is given. It is noteworthy that during the greater part of the elution the temperature was 37° C; only the elution of arginine needs a temperature of 75° C.

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TABLE

AMINO-ACID RECOVERIES FROM HUMAN ADULT

The data are given as g of

Amino-Acid	*Time of hydrolysis*					
	24 h		*48 h*			
Aspartic acid	10.40	10.58	10.50	10.32	10.60	10.60
Threonine	5.84	5.83	5.46	5.65	5.40	5.45
Serine	5.19	5.20	4.53	4.60	4.32	4.41
Glutamic acid	7.28	7.15	7.40	7.45	7.10	7.27
Proline	4.88	5.33	4.92	5.03	4.87	5.04
Glycine	4.66	4.52	4.62	4.47	4.48	4.56
Alanine	10.63	10.58	10.03	9.76	10.07	9.97
Cystine/2	0.99	1.16	1.01	1.00	0.99	0.97
Valine	11.39	11.32	10.60	10.77	11.19	11.13
Methionine	1.59	1.53	1.40	1.39	1.40	1.40
*Iso*leucine	0.34	0.28	0.41	0.27	0.31	0.34
Leucine	—	15.12	15.40	15.15	14.93	15.15
Tyrosine	4.38	4.15	4.08	3.93	3.65	3.74
Phenylalanine	7.80	8.22	7.83	7.80	7.76	7.92
Lysine	10.58	10.21	9.95	9.98	9.85	9.82
Histidine	8.65	8.48	8.17	8.08	8.40	8.59
Arginine	3.75	3.57	—	—	3.28	3.44
Ammonia	1.34	1.35	1.40	1.32	1.34	1.46

* These values were obtained by extrapolating to zero time of hydrolysis by the method of least squares.

Seventeen amino-acids and ammonia have been shown to be present in the two types of haemoglobin. There is no evidence of the presence of other amino-acids, while tryptophan was completely lost by the acidic hydrolysis. It will be noted that although the separation of the amino-acids is about the same, the order was different in some respects from that given by STEIN AND MOORE[16]. It was found that glutamic acid was eluted before proline, and cystine before valine. It may be that the few modifications in the technique are responsible for these alterations.

Table I lists the results of the analyses for the various amino-acids in samples of adult carbonmonoxyhaemoglobin, which were hydrolysed for different periods. The data show that the recovery of the greater number of amino-acids is independent of the time of hydrolysis; only for aspartic acid, threonine, serine, methionine, tyrosine and lysine were marked losses found after longer hydrolysis. As it is known that peptide bonds involving serine, threonine and aspartic acid are among those which are readily hydrolysed, it may be expected that these amino-acids are completely liberated after hydrolysis for 24 hours. Therefore the recoveries of these amino-acids and also of methionine, tyrosine and lysine are plotted against time. The results given in Fig. 3 show a linear decrease with time of hydrolysis for all these amino acids. By extrapolation to zero time the original amount of each amino-acid can be easily established. Our results found for haemoglobin are in this respect in good agreement with those given by SMITH AND STOCKEL[24] for crystalline carboxypeptidase and by SMITH, STOCKELL AND KIMWELL[25] for crystalline papaine. It will be noted that the recovery of ammonia increased with longer time of hydrolysis.

As shown in Table I, the yields of the other amino-acids are essentially constant

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I

CARBONMONOXYHAEMOGLOBIN HYDROLYSATES

amino-acid per 100 g protein.

Time of hydrolysis				Average or extrapolated value	Schroeder[11] et al.	Van der Linden[13] globin
96 h		144 h				
10.00	10.32	9.80	10.64	10.60* ± 0.10**	11.02	10.7
5.00	5.08	4.56	4.90	6.10* ± 0.04[5]	5.85	5.6
3.81	3.84	3.13	3.26	5.50* ± 0.03	5.05	—
7.14	7.20	6.88	7.18	7.20 ± 0.05	7.17	6.5
4.96	4.96	4.68	5.30	5.00 ± 0.05[5]	4.39	4.8
4.63	4.53	4.36	4.40	4.52 ± 0.03	4.85	—
10.30	10.30	10.15	10.19	10.20 ± 0.06	10.19	—
0.96	1.12	—	1.05	1.03 ± 0.02[5]	—	—
11.35	11.50	—	11.48	11.09 ± 0.11	10.65	10.3
1.24	1.29	1.10	1.16	1.60* ± 0.01	1.38	1.39
0.34	0.30	0.32	0.30	0.32 ± 0.01[5]	0.21	0.4
14.88	16.02	14.60	15.72	15.22 ± 0.14[5]	15.06	14.3
3.78	3.81	3.54	3.62	4.40* ± 0.04	2.99	3.1
7.83	8.30	7.75	8.15	7.93 ± 0.06[5]	7.66	7.15
9.92	9.82	9.52	9.20	10.60* ± 0.05[5]	9.64	10.0
8.71	8.60	8.60	8.42	8.47 ± 0.07	8.44	7.8
3.26	3.44	3.15	3.54	3.43 ± 0.04[5]	3.28	3.5
1.50	1.75	1.76	1.90	1.10* ± 0.03		

** Standard deviations of the means and for the extrapolations standard errors of estimate (see W. J. Dixon and F. J. Massey, *Introduction to statistical analysis*, 1951, p. 153).

after hydrolysis for different periods. This is of special interest, since it was to be expected from earlier work on the hydrolysis of proteins, notably that of Harfenist and Craig[26], that the recovery of *iso*leucine and valine was incomplete on short hydrolysis and increased with longer times, when *iso*leucine and valine are coupled together in peptide linkage. This was also found for carboxypeptidase[21] and for papaine[25]. In spite of the relatively low amount of *iso*leucine in COHb-a it will be clear, that this coupling is not

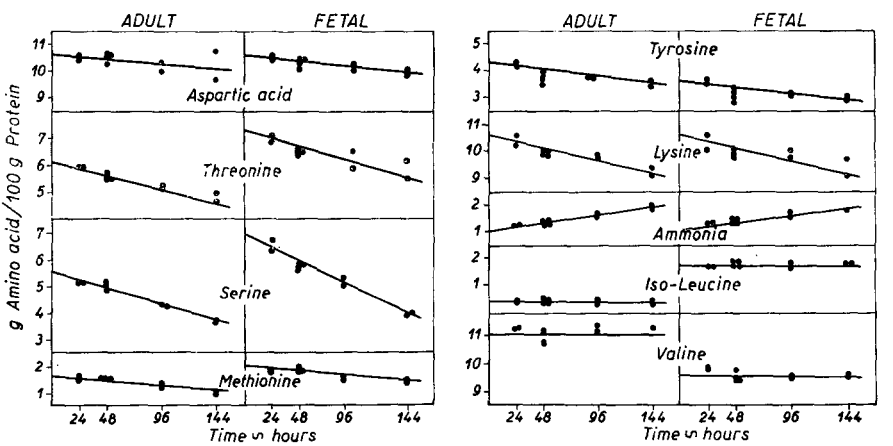

Fig. 3. Recoveries as a function of time of hydrolysis of various amino-acids and of ammonia.

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TABLE

AMINO-ACID RECOVERIES FROM HUMAN FOETAL

The data are given as g of

Amino-acid	*Time of hydrolysis*					
	24 h			*48 h*		
Aspartic acid	10.53	10.48	10.08	10.19	10.37	10.41
Threonine	7.12	6.90	6.50	6.66	6.46	6.42
Serine	6.74	6.37	5.93	5.76	5.95	5.67
Glutamic acid	7.36	7.44	7.50	7.68	7.82	7.60
Proline	4.43	4.36	—	—	4.24	4.20
Glycine	3.94	4.60	4.70	4.71	4.57	4.42
Alanine	9.40	9.46	9.57	9.80	9.54	9.80
Cystine/2	0.97	0.95	0.98	0.98	0.95	0.94
Valine	9.83	9.90	9.47	9.38	9.81	9.38
Methionine	1.81	1.86	1.82	1.79	1.76	1.97
*Iso*leucine	1.80	1.74	1.91	1.79	1.79	1.88
Leucine	14.70	15.30	15.69	15.50	14.99	14.98
Tyrosine	3.59	3.55	3.37	3.20	3.09	3.02
Phenylalanine	8.35	7.78	7.75	7.98	7.83	7.97
Lysine	10.00	10.62	9.68	9.86	9.77	10.11
Histidine	7.45	7.60	7.33	7.20	7.38	7.61
Arginine	3.34	3.50	-	—	3.37	3.22
Ammonia	1.31	1.37	1.36	1.48	1.34	1.43

* and **, see corresponding footnotes to Table I.

present. As shown in Fig. 3 these two amino-acids are completely liberated after hydrolysis for 24 hours.

In Table I our final results are also compared with the quantities which have been reported by SCHROEDER *et al.*[11] for adult carbonmonoxyhaemoglobin and by VAN DER LINDEN[13] for human globin. In general they are in good agreement. Apart from aspartic acid however, the values of the five amino acids, which were calculated by extrapolation to zero time, and of proline and valine were found to be higher, while the amounts of aspartic acid and of glycine were lower.

Table II summarizes the results of the analysis of purified foetal haemoglobin, while the recoveries as a function of time of hydrolysis of aspartic acid, threonine, serine, methionine, tyrosine, lysine, ammonia, *iso*leucine and valine are given in Fig. 3. These data show that apparent destruction occurs in COHb-f of the same amino acids as found in COHb-a, while also the yields of *iso*leucine and valine did not increase with longer time of hydrolysis. Comparison of our results with the data of VAN DER LINDEN[13] is not completely appropriate, since this author studied globin prepared from umbilical cord blood, which contained about 20% of the normal adult component.

Table III presents a comparison between the amino-acid composition of the two haemoglobins. The nitrogen recoveries, which are for COHb-a about 103.5% and for COHb-f about 102.8%, indicate that the composition of these proteins has been satisfactorily estimated. As no tryptophan analysis was carried out, the values for this amino-acid given by VAN DER LINDEN[13] were used in calculation. In general the number of individual amino-acids present in these haemoglobins and calculated per molecule of 68,000 molecular weight is reasonably satisfactory too, although the values for some

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II

CARBONMONOXYHAEMOGLOBIN HYDROLYSATES

amino acid per 100 g protein.

Time of hydrolysis				Average or extrapolated value	VAN DER LINDEN[13] globin***
96 h		141 h			
10.20	10.10	10.00	9.97	10.60* ± 0.03**	10.6
6.52	5.78	6.22	5.64	7.30* ± 0.05	6.4
5.31	5.03	4.18	4.13	6.90* ± 0.05	—
7.68	7.60	7.33	7.59	7.56 ± 0.05	6.6
4.23	4.22	4.34	4.27	4.29 ± 0.03	4.1
4.52	4.40	4.50	4.46	4.48 ± 0.07	—
9.25	9.88	9.45	9.57	9.64 ± 0.07	—
1.07	1.05	0.92	1.00	0.98 ± 0.02	—
9.48	9.62	9.60	9.55	9.60 ± 0.06	9.1
1.40	1.58	1.29	1.39	2.05* ± 0.02[5]	1.76
1.83	1.89	1.85	1.86	1.83 ± 0.02	1.6
14.93	15.80	14.35	15.90	15.20 ± 0.12[5]	14.1
3.05	3.18	2.90	2.95	3.60* ± 0.02[5]	2.8
8.30	7.96	8.23	7.80	7.99 ± 0.07	7.1
9.75	10.15	9.09	9.82	10.60* ± 0.07	9.9
7.71	7.25	7.65	7.35	7.45 ± 0.05[5]	7.3
3.32	3.40	2.93	3.46	3.31 ± 0.05	3.4
1.72	1.47	—	1.66	1.10* ± 0.04[5]	

*** This globin was prepared from blood of newborn infants without removing the adult haemoglobin.

amino acids, for instance glutamic acid and leucine, must be treated with some reserve. Comparison of the number of residues in COHb-a and COHb-f shows that in general the same values were found for aspartic acid, glycine, half cystine, leucine, phenylalanine, lysine and arginine. It is not really clear if the content of glutamic acid in the two haemoglobins is the same. It may be that the quantity of this amino-acid in the foetal haemoglobin is slightly greater than in the adult component ($P < 0.02$). The amounts of the other amino-acids differ to a greater or lesser extent. They may be divided into two categories as follows:

(a) Amino-acids, which were found in a greater amount in the foetal carbonmonoxyhaemoglobin, *i.e.* threonine, serine, methionine, *iso*leucine and tryptophan (the last of these amino-acids according to VAN DER LINDEN[13]). For all these amino-acids $P < 0.001$.

(b) Amino-acids, who are present in a smaller amount in COHb-f, *i.e.* proline. alanine, valine, tyrosine and histidine (always $P < 0.001$). These differences agree well with the results of VAN DER LINDEN[13], which are also summarized in Tables I and II, The much higher content of *iso*leucine in human foetal haemoglobin is one of the most striking features of this protein.

DISCUSSION

It is obvious that the differences in amino-acid composition in our analyses of purified foetal haemoglobin and of adult haemoglobin are somewhat greater than those described by VAN DER LINDEN[13], as in his investigations cord blood was used, which

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TABLE III

COMPARISON OF THE AMINO-ACID COMPOSITION OF HUMAN ADULT AND FOETAL CARBONMONOXYHAEMOGLOBIN

Amino-Acid	*Adult Carbonmonoxyhaemoglobin*				*Foetal Carbonmonoxyhaemoglobin*				*Differences between number of residues*
	g of amino-acid per 100 g COHb	*N as % of total N*	*Calculated number of residues for mol. wt of 68,000*	*Assumed no. of residues*	*g of amino-acid per 100 g COHb*	*N as % of total N*	*Calculated number of residues for mol. wt of 68,000*	*Assumed number of residues*	
Aspartic acid	10.60	6.60	54.2	54	10.60	6.60	54.2	54	
Threonine	6.10	4.25	34.8	35	7.30	5.07	41.6	42	+ 7
Serine	5.50	4.35	35.6	36	6.90	5.45	44.7	45	+ 9
Glutamic acid	7.20	4.07	33.3	33	7.56	4.26	34.9	35	+ 2?
Proline	5.00	3.60	29.5	29	4.29	3.08	25.3	25	− 4
Glycine	4.52	5.00	40.9	41	4.48	4.97	40.7	41	
Alanine	10.20	9.52	78.0	78	9.64	8.98	73.6	74	− 4
Cystine/2	1.03	0.71	5.9	6	0.98	0.67	5.6	6	
Valine	11.09	7.90	64.2	64	9.60	6.83	55.4	55	− 9
Methionine	1.60	0.89	7.3	7	2.05	1.14	9.3	9	+ 2
*Iso*leucine	0.32	0.20	1.7	2	1.83	1.20	9.7	10	+ 8
Leucine	15.22	9.65	78.9	79	15.20	9.62	78.6	79	
Tyrosine	4.40	2.02	16.5	16	3.60	1.64	13.4	13	− 3
Phenylalanine	7.93	3.98	32.5	33	7.99	4.01	32.7	33	
Lysine	10.60	12.05	49.3	49	10.60	12.05	49.3	49	
Histidine	8.47	13.60	37.1	37	7.45	12.06	32.7	33	− 4
Arginine	3.43	6.55	13.4	13	3.31	6.32	12.9	13	
Ammonia	1.10*	5.38	44.1*	44*	1.10*	5.38	44.1*	44*	
Total	113.21	100.32	613.1	612	113.38	99.33	614.6	616	+ 4
Tryptophan[13]	1.20	0.96	4.0	4	1.57**	1.25	5.2	5	+ 1
Heme calculated	3.80	2.26			3.80	2.26			
Final total		103.54	617.1	616		102.84	619.8	621	+ 5

* This value is omitted from the total.

** Calculated from the value given by V. D. LINDEN[13] by multiplying it by 5/4.

contained a possible amount of 20% of the normal adult component. The information in Table I and II makes it clear that the differences in amounts of threonine, proline, valine, methionine, *iso*leucine, tyrosine and histidine are slightly greater than those described by VAN DER LINDEN. This investigator did not carry out serine and alanine analyses.

Comparing the results of the amino-acid analyses of the two haemoglobins the question arises how far the possibility will be present that the solutions of purified foetal haemoglobin contained small quantities of the adult compound. Using the alkali denaturation method of BRINKMAN AND JONXIS[3] the amounts of Hb-f in these solutions were found to be 100 ± 2%, while electrophoretically this compound moves as a single boundary. Moreover CHERNOFF[18] demonstrated by immunological experiments that foetal haemoglobin prepared in the same manner was free of the adult pigment. Therefore it may concluded that the possible amounts of normal haemoglobin in our preparations of purified foetal haemoglobin are negligible and do not disturb our values for the amino-acid composition of this protein.

Also it may be possible that small amounts of foetal haemoglobin will be present in the solutions of normal adult haemoglobin. Both SINGER *et al.*[4] and ROCHE AND DERRIEN[9]

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reported, that small quantities of the foetal compound are always present in the blood of adult individuals. According to SINGER *et al.*[4] these quantities range from 0.5 to 1.7% of the total amount of haemoglobin present. Recent investigations in our laboratory (to be published) furnished evidence that an alkali-resistant haemoglobin fraction is regularly encountered in normal human blood, which also shows the specific differences in amino-acid composition as given for the foetal haemoglobin. The quantities found however, were much lower than those given by SINGER *et al.*[4] and do not succeed 0.5% of the total haemoglobin. Therefore it will be clear, that our amino-acid analyses of normal Hb are not influenced by notable amounts of Hb-f.

The question arises whether the dissimilarity in the electrophoretic mobility of COHb-a and COHb-f—according to ZINSSER[27] the isolectric points vary from 6.91 to 6.94 for COHb-a and from 6.97 to 7.03 for COHb-f in 0.2 *M* phosphate buffer pH 7.1—can be explained in terms of the differences in amino-acid content of the two haemoglobins. A lesser content of 4 histidine residues and a possible greater content of glutamic acid and of one residue tryptophan in the foetal haemoglobin would cause a decrease in isoelectric point, which is contrary to the results of ZINSSER[27]. On the other hand, since the hydroxyl groups of serine and threonine are able to form good hydrogen bonds the possibility exists that the much greater amounts of these two amino-acids in the foetal haemoglobin would bring a decrease in the polar side chains of these protein by folding or coiling the polypeptide chains in Hb-f in a different manner than in Hb-a. It is surprising that the differences in the isoelectric points of Hb of sickle-cell anaemics (Hb-b) and of normal Hb is much greater than that of the foetal and adult haemoglobin, while the amino-acid compositions of Hb-b and Hb-a are nearly the same[11, 31]. The present study gives no evidence of differences in the content of ammonia (or amides), but our investigations on this point are not sufficiently complete to allow any conclusions in this respect.

There can be little doubt that the yields of valine and *iso*leucine were complete after hydrolysis for 24 hours and showed no increase with longer times as found for insulin[26] and some crystalline enzymes[24, 25]. Therefore it is unlikely that a coupling of these two amino-acids in adult and in foetal carbonmonoxyhaemoglobin is present.

In these two types of haemoglobin, four heme residues are present in one molecule (molecular weight 68,000). If the globin molecule is built up with four similar polypeptide chains, it would be necessary that the total amounts of residues for each amino-acid should be divided by four. The data from Table III shows that for some amino-acids this is impossible, while the limits for the method—the results generally fall within the range of 100 $\pm$ 3%—cannot explain these differences. Several different explanations are possible. First it may be that the globin molecule consists of one long polypeptide chain (molecular weight about 66,000) with four heme residues or two similar chains of a molecular weight of about 33,000, each with two heme residues. On the other hand, both COHb-a and COHb-f may be composed of haemoglobin fractions with a different amino-acid composition, while each fraction may be built up of similar peptide chains. Which of these possibilities is valid cannot be decided at the present time. However BRINKMAN *et al.* with alkali denaturation methods[29], ROCHE AND DERRIEN[9] with a salting-out technique and VAN FOSSAN[29] by column chromatography, have demonstrated the existence of different types of human adult Hb and human foetal Hb. It may be that a quantitative study of the amino-acid composition of these fractions will bring about elucidation of these problems.

As the differences in amino-acid composition of COHb-f for some of the constituent

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acids (serine, valine, *iso*leucine) are relatively rather great, this method promises to become an aid in differentiating the foetal haemoglobin from the normal component. In particular the estimation of the amino-acid composition will be of great help in deciding if an alkali-resistant pigment in the blood is identical with the foetal haemoglobin[30]. Using this technique, we consider it probable[31] that the alkali-resistant fraction which sometimes occurs in the blood of patients with sickle-cell anaemia up to 20%, is not foetal haemoglobin.

SUMMARY

1. Haemoglobin from the blood of normal adult individuals and purified foetal haemoglobin have been analysed for seventeen amino-acids and ammonia using, with slight modifications, the gradient elution chromatographic method as recently developed by STEIN AND MOORE. It was found that purified foetal carbomonoxyhaemoglobin contains more threonine, serine, methionine and *iso*leucine and less proline, alanine, valine, tyrosine and lysine. The most striking differences between the two haemoglobins are the much higher content of serine and *iso*leucine and the lower amount of valine in the foetal haemoglobin.

2. As the yields of valine and *iso*leucine were complete after a short time of hydrolysis and showed no increase with longer times as found for other proteins, it is unlikely that these two amino-acids are combined in peptide linkage in these two haemoglobins.

3. Since for some amino-acids the number of residues could not be divided by four, it is unlikely that both adult and foetal globin consist of four identical polypeptide chains. Some possibilities concerning the structure of the protein molecule are discussed.

4. It is pointed out that the differences in amino-acid composition of COHb-a and COHb-f will be of great help for the detection of the presence of the foetal compound in the blood in different diseases.

RÉSUMÉ

1. Les teneurs de l'hémoglobine du sang d'individus adultes normaux et de l'hémoglobine foetale purifiée en dix-sept amino acides et en ammoniaque ont été déterminées à l'aide de la technique chromatographique d'élution par gradient, mise au point par STEIN ET MOORE et légèrement modifiée. La carboxyhémoglobine foetale purifiée contient plus de thréonine, de sérine, de méthionine et d'*iso*leucine et moins de proline, d'alanine, de valine, de tyrosine et de lysine. Les différences les plus frappantes entre les deux hémoglobines sont les teneurs beaucoup plus élevées en sérine et *iso*leucine et la teneur plus faible en valine de l'hémoglobine foetale.

2. Les rendements en valine et *iso*leucine étant quantitatifs après un temps d'hydrolyse court et ne présentant pas d'augmentation avec des temps plus longs, contrairement à ce que l'on trouve avec d'autres protéines, il est peu vraisemblable que ces deux amino acides soient liés par une liaison peptide dans ces deux hémoglobines.

3. Le nombre de résidus de quelques amino acides n'étant pas divisible par quatre, il est peu vraisemblable que les deux globines foetale et adulte, soient constituées par quatre chaînes polypeptidiques identiques. Quelques hypothèses sur la structure de la molécule protéique sont discutées.

4. Les auteurs insistent sur le fait que les différences de composition en amino acides de COHb-a et de COHb-f seront d'un grand secours pour la détection de la présence du composé foetal dans le sang au cours de différentes maladies.

ZUSAMMENFASSUNG

1. Hämoglobin aus dem Blute normaler Erwachsener und gereinigtes Foetushämoglobin wurden mit Hilfe der jüngstens von STEIN UND MOORE entwickelten, und von uns etwas abgeänderten, Stufenchromatographie auf 17 Aminosäuren und Ammoniak untersucht. Es wurde festgestellt, dass gereinigtes Foetuscarbomonoxyhämoglobin mehr Threonin, Serin, Methionin und *Iso*leucin, aber weniger Prolin, Alanin, Valin, Tyrosin und Lysin enthält. Am stärksten unterschieden sich beide Hämoglobine durch den viel höheren Gehalt an Serin und *Iso*leucin und den niedrigeren Gehalt an Valin des foetalen Hämoglobins.

2. Da die Ausbeuten von Valin und *Iso*leucin bereits nach kurzer Hydrolysenzeit vollkommen waren, und nicht, wie bei anderen Proteinen, durch längere Hydrolysenzeit gesteigert werden

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konnten, scheint es uns unwahrscheinlich, dass diese beiden Aminosäuren in den beiden Hämoglobinen durch Peptidbindungen verknüpft sind.

3. Da die Zahl einiger Aminosäurenreste nicht durch vier dividierbar ist, scheint es unwahrscheinlich, dass das Globin Erwachsener oder das Foetusglobin aus vier identischen Polypeptidketten besteht. Einige Hypothesen über die Struktur des Proteinmoleküls werden erörtert.

4. Es wird betont, dass die Unterschiede zwischen COHb-a und COHb-f, hinsichtlich ihrer Aminosäurenzusammensetzung, von grosser Bedeutung für die Bestimmung der foetalen Komponente im Blute pathologischer Fälle sein könnte.

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