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Köln Haemoglobinopathy

FURTHER DATA AND A COMPARISON WITH OTHER HEREDITARY HEINZ BODY ANAEMIAS

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SINCE 1962 there have emerged from the group of congenital non-spherocytic haemolytic anaemias several in which the anaemia is attributable to one of a number of unstable haemoglobins. A diagnostic feature of these haemoglobinopathies is the ease with which inclusion bodies can be produced in the red cells (see Table I). They may occur spontaneously in the fresh blood of splenectomized subjects or, if not present, they may be formed *in vitro* by incubation of the blood with or without the use of redox compounds. Other notable features are the heat-lability of these haemoglobins, the presence of small amounts of methaemoglobin in the fresh blood and its accumulation after incubation of the cells (Table I).

During a study of a family, five members of which had a haemolytic anaemia attributable to an unstable haemoglobin, it became apparent that they were a branch of the larger family with Haemoglobin Köln studied previously by Hutchison, Pinkerton, Waters, Douglas, Lehmann and Beale (1964). We report here findings which consolidate those reported by these workers. In addition, some further data have been obtained regarding the consequence to red cells of the presence of Haemoglobin Köln.

METHODS

Standard methods were used for haematological and biochemical investigations (Dacie and Lewis, 1963; Lehmann and Ager, 1961; Watson-Williams, Beale, Irvine and Lehmann, 1965). Starch gel electrophoresis was carried out by the method of Smithies (1959) and electrophoresis of globin in 6 M-urea by the method of Chernoff and Pettit (1964). Separation of the globin chains, cysteine aminoethylation and peptide elution from paper were carried out as described and summarized by Clegg, Naughton and Weatherall (1965).

Autohaemolysis was carried out as follows: Samples (1 ml.) of fresh, sterile, defibrinated blood were added to bijou bottles with well-fitting washers and screw-caps. Where samples were supplemented with glucose this was added as 0.1 ml. of 10 per cent (w/v) sterile solution. The bottles and contents were incubated at 37° C. for 48 hours with continuous mixing at 62 rev./min. in the vertical plane. The extent of haemolysis was determined at the end of the incubation period as described by Selwyn and Dacie (1954).

Glutathione and methaemoglobin were determined according to Grimes (1965, 1967).

CASE REPORTS

Family Tree

The family tree is shown in Fig. 1. The propositus (IV, 1) was found to have a haemolytic anaemia, as were her mother (III, 5), her half-brother (IV, 2) and her two children (V, 1 and 2).

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TABLE I

DISTINCTIVE PROPERTIES OF UNSTABLE HAEMOGLOBINS

Haemoglobin	*Abnormality of Hb molecule*	*Heat lability*	*Fresh blood inclusion bodies in splenectomized subjects*	*Inclusion body formation after* in vitro *incubation*	*Methaemoglobin in fresh blood*	*Methaemoglobin formation in blood incubated* in vitro	*Reference*
Zurich	β63 Hist→ Arg	+	Not described	With redox compounds	Nil	+	Bachmann and Marti (1962)
Ube I		Not described	+	Not described	Not described	Not described	Shibata *et al.* (1963)
Hereditary Heinz-body anaemia	–	+	+	No additives +	+	+	Grimes, Meisler and Dacie (1964); Dacie *et al.* (1964)
Köln	β98 Val → Met	+	+	No additives +	+	+	Hutchison *et al.* (1964)
Seattle	β70 Ala → Glu or β76 Ala → Glu	+	Not described	No additives +	+	+	Huehns (1965)
St. Marys	–	+	Not described	No additives +	+	+	Huehns and Shooter (1965)
Galliera Genova	–	Not described	+	With redox compounds	Not described	Not described	Sansone and Pik (1965)

An aunt (Mrs. L.S. III, 1) living in Colorado, U.S.A., has a mild haemolytic anaemia with splenomegaly and a haemoglobin of about 10–11 g. per 100 ml. An uncle (III, 4) was splenectomized in 1946 and died post-operatively, and another uncle (III, 6) was a regular soldier who in 1939 was returned to the United Kingdom for splenectomy, and also died post-operatively. Blood samples from only the first five cases quoted were examined. Blood samples from IV, 3 and V, 3 and 4 have been examined elsewhere and are said to be normal. There is no history of abnormality in III, 2 and III, 3.

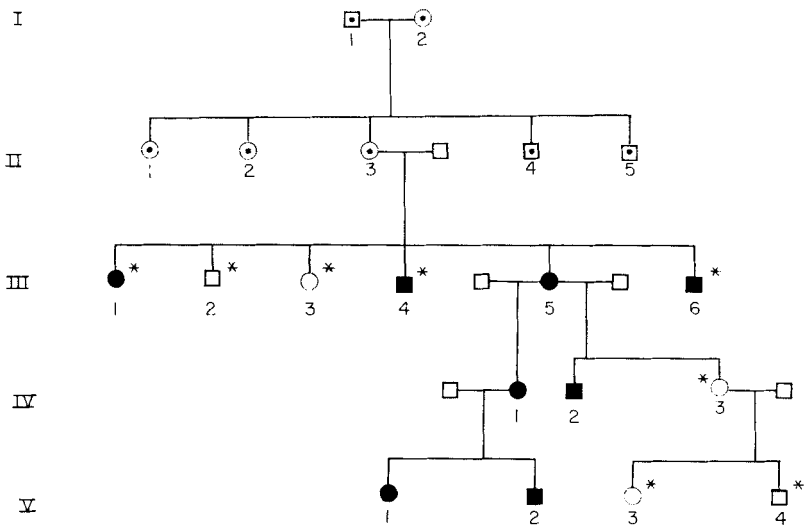

FIG. 1. Family tree. Symbols have the following meaning: □, male; ○, female. Filled symbols represent affected subjects and open symbols unaffected subjects. Asterisks indicate that the diagnosis is based on the subject's history and not on personal examination by the authors. ⊡, ⊙, no data available.

We know little about generations I and II but with the help of Dr. Hutchison of Western Infirmary, Glasgow, have established that II, 1 is the grandmother of the propositus of Hutchison *et al.* (1964) and I, 1 is the German immigrant from whom both families are derived.

Case 1 (IV, 1). The propositus, a married woman aged 28, had noticed slight jaundice and tiredness throughout her life and knew that others in the family had been affected similarly. Her urine was said to be darker than normal. Her first pregnancy was uneventful but in 1962 during the second she became increasingly tired. The spleen was not palpable, there was only slight anaemia—haemoglobin 12.2 g. per 100 ml., MCHC 27 per cent, reticulocyte count 11 per cent, serum bilirubin 1.5 mg. per 100 ml. and normal red cell osmotic fragility. In September 1965 she had a febrile illness (probably viral in origin) with muscular chest pain lasting for some 3 weeks and on September 10th appeared more anaemic—haemoglobin 9.5 g. per 100 ml., reticulocytes 12 per cent. Ten days later she had haemoglobinuria for 3 days. Analgesic tablets containing aspirin, phenacetin and codeine were taken about this time but it is not known whether they preceded the haemoglobinuria. A further blood sample showed haemoglobin 9.2 g. per 100 ml., reticulocytes 15 per cent. Ham's acid serum test for paroxysmal nocturnal haemoglobinuria was negative. After recovery her blood was studied in greater detail and on October 13th her haemoglobin was 11.6 g. per 100 ml., reticulocytes 7 per cent.

Examination of the blood film showed many hypochromic cells and target cells and some irregularly contracted cells and microspherocytes. There was an increased number of polychromatic cells. Heinz bodies were absent and a G6PD screening test was normal. Direct antiglobulin tests were negative and red cell osmotic fragility was slightly decreased with median corpuscular fragility (MCF) 0.39 per cent. The cold agglutinin titre with Group O red cells was 1 in 8 at 4° C. and 0 at room temperature. Haemoglobin electrophoresis on paper using tris buffer (pH 8.9) and starch gel (pH 8.65) showed an apparent increase in what was at first thought to be haemoglobin A_2. Alkali resistant haemoglobin was less than 1 per cent and inclusion bodies of the type demonstrable in Haemoglobin H disease ('H' bodies) could not be demonstrated. The subject was taking oral iron at the time and the serum iron was 150 μg. per 100 ml. Serum bilirubin was 0.6 mg. per 100 ml.

She was at this time considered to be a case of β-thalassaemia but further tests were undertaken because of the haemoglobinuria. Because of considerable menorrhagia she was given oral iron for some months and when tested on later occasions her haemoglobin was 12.0–12.4 g. per 100 ml., reticulocytes 8–10 per cent. Later blood films after iron therapy showed many normal cells and only occasional hypochromic cells but irregularly contracted cells, burr cells and microspherocytes were more conspicuous. Punctate basophilia was seen in 0.25–2.0 per cent of the red cells. After 2 weeks without oral iron, serum iron was 117 μg. per 100 ml.

Case 2 (V, 1). A girl aged 7 years, slightly jaundiced on occasions and said to be unduly tired at school. The spleen was palpable 2 cm. below the costal margin. Her urine was said often to be darker in colour than normal.

Case 3 (V, 2). A boy aged 4 years, slightly jaundiced on occasions and frequently tired. The spleen was moderately enlarged and palpable 5 cm. below the costal margin. His urine was said to be darker in colour than normal.

Case 4 (III, 5) is a married woman aged 55 who has continuous slight jaundice only. Her spleen was palpable 4 cm. below the costal margin and the urine was said often to be darker in colour than normal.

Case 5 (IV, 2) is a man aged 19. In 1959, aged 12, he became jaundiced, splenomegaly was discovered and because of the family history he was referred to Dr. R. A. Shanks at the Royal Hospital for Sick Children, Glasgow, to whom we are indebted for the following details. When seen his height was 145 cm. and weight 36.4 kg. The spleen was palpable two finger breadths below the costal margin, the liver one finger breadth. His haemoglobin varied from 9.5 to 11.0 g. per 100 ml., films showed some 30 per cent of spherocytes and some isolated crenated cells. Reticulocytes were 4.8 per cent, serum bilirubin 4.5 mg. per 100 ml. and osmotic fragility was not increased. Splenectomy was performed in January 1960, and recovery was uneventful. Four months later his haemoglobin was 10.9 g. per 100 ml. and serum bilirubin 3 mg. per 100 ml. The family moved from Glasgow and was lost to follow-up.

In April 1966 he was symptomless and his mother considered that his health had been much improved by splenectomy in that jaundice was no longer obvious and minor febrile infections were no longer disabling. His haemoglobin was 14.3 g. per 100 ml., MCHC 29 per cent, reticulocytes 9 per cent. His urine was said often to be darker than normal.

RESULTS

Some of the haematological data are summarized in Table II.

Red Cell Morphology

Case 1 (IV, 1). Most red cells were normocytes, occasional hypochromic cells were present as were a few irregularly contracted cells, burr cells and microspherocytes. Punctate basophilia was present in 0.25–2.0 per cent of the red cells on different occasions. On two occasions methyl violet staining revealed small Heinz bodies in up to 2 per cent of the red cells.

TABLE II

HAEMATOLOGICAL DATA IN FIVE CASES OF KÖLN HAEMOGLOBINOPATHY

	Propositus (*IV*, 1)	*V*, 1	*V*, 2	*III*, 5	*IV*, 2
Sex and age	F, 28	F, 7	M, 4	F, 55	M, 19
Haemoglobin (g./100 ml.)	9.2–12.4	10.9	9.9–10.3	12.2	14.3
Reticulocytes (%)	7–15	4–12	11–12	12	9
MCHC (%)	25–30	28	27.5–28	28	29
MCV (cu.μ)	108	—	97	112	117
Platelets (No./cu.mm.)	130,000	—	200,000	160,000	870,000*
Heinz bodies (% of red cells)	0–2	Nil	Nil	Nil	80
Serum bilirubin (mg./100 ml.)	1.1	—	—	1.3	1.1
Alkali-resistant haemoglobin (%)	1.0	2.1	3.7	1.0	1.0
Splenectomy	No	No	No	No	Yes

* Whole blood count was inaccurate owing to presence of Heinz bodies released by lysis of red cells.

Cases 2 *and* 3 (V, 1) *and* (V, 2). Many red cells were hypochromic, target cells were present and also occasional irregularly contracted cells and microspherocytes. Punctate basophilia was present in 0.5–1.0 per cent of the red cells. Heinz bodies could not be demonstrated.

Case 4 (III, 5). Similar to IV, 1 but punctate basophilia was seen less frequently.

Case 5 (IV, 2). The film showed a majority of normocytes with some irregularly contracted cells and target cells. There were also cells containing Howell-Jolly bodies and Pappenheimer bodies. Approximately 2 per cent of the red cells showed punctate basophilia. Heinz bodies, mainly single and often large, were seen in approximately 80 per cent of the red cells after staining with methyl violet. These bodies failed to stain with Feulgen and iron stains but after supravital staining with 0.01 per cent aqueous acridine-orange, yellow fluorescence was obtained implying the presence of DNA.

Osmotic Fragility

Results are shown below:

Subject	*Fresh blood MCF (NaCl per cent)*	*Incubated blood MCF (NaCl per cent)*
IV, 1	0.41	0.315
V, 1	0.39	—
V, 2	0.375	0.36
III, 5	0.415	0.405
IV, 2	0.44	0.52
Normal	0.4–0.445	0.465–0.590

Osmotic fragility was obviously reduced only with V, 1 and V, 2 in whom iron deficiency could not be excluded, but in all except the splenectomized patient IV, 2 there were tails of resistant cells. However, after incubation for 24 hours the osmotic fragility was markedly decreased with all subjects tested except IV, 2 in whom it was normal apart from a few abnormally fragile cells which haemolysed in 0.75 and 0.85 per cent NaCl solution.

Electrophoresis

On paper electrophoresis in tris buffer (pH 8.9) and starch gel by discontinuous tris–citrate-borate (pH 8.65), a haemoglobin was found moving slightly faster than Haemoglobin S on paper and like Haemoglobin S on starch. Its proportion was about 15 per cent of the total. It resembled in its electrophoretic behaviour Haemoglobin Köln as described by Pribilla (1962) and Hutchison *et al.* (1964).

Structure of the Globin

The following studies were carried out on globin prepared from the purified slow fraction of the haemoglobin of the propositus (IV, 1).

Electrophoresis of this globin in 6 M-urea at pH 8.6 gave bands running identically with the α and β bands of Globin A. Hybridization of the haemoglobin gave a normal $\alpha_2^{\text{human}}\beta_2^{\text{canine}}$ hybrid but the $\alpha_2^{\text{canine}}\beta_2^{\text{human}}$ hybrid was missing.

The globin chains were separated on a carboxymethylcellulose column in 8 M-urea using an ionic gradient. The α and β chains of the abnormal globins were found to elute at the same ionic concentrations as the α and β chains of haemoglobin A. Aminoethylation of the β chain followed by tryptic digestion and finger printing gave a peptide pattern (Fig. 2) identical with that of haemoglobin A. This finding along with the behaviour of the globin in strong urea solution suggested that there was no difference in charged residues between this globin and Globin A. Specific colour tests carried out on fingerprints, however, showed one difference from the β chain of Globin A, namely, a positive divalent sulphur test for the peptide βTpXI of the abnormal globin. This peptide comprises residues 96–104 of the 146 residues of the β chain. Amino acid analysis of this peptide gave the result shown in Table III. This confirms the presence of methionine which is seen to have replaced a corresponding amount of valine, giving the composition of βTpXI as shown below.

Residues	96	97	98	99	100	101	102	103	104
β^{A}TpXI	Leu	His	*Val*	Asp	Pro	Glu	Asn	Phe	Arg
$\beta^{\text{Köln}}$TpXI	Leu	His	*Met*	Asp	Pro	Glu	Asn	Phe	Arg

Thus, the haemoglobin was concluded to be identical with that of the Haemoglobin Köln from Glasgow described by Carrell *et al.* (1966), i.e. α_2 β_2 98 Val.→Met.

TABLE III

AMINO-ACID ANALYSIS OF βTpXI FROM THE ABNORMAL HAEMOGLOBIN OF THE PROPOSITUS AND FROM HAEMOGLOBIN A

	Abnormal haemoglobin		*Haemoglobin A*	
	μmoles	*Residues per peptide*	*μmoles*	*Residues per peptide*
Aspartic acid	0.096	1.9	0.081	2.1
Glutamic acid	0.053	1.0	0.037	1.0
Proline	0.047	0.9	0.030	0.8
Valine*	0.017	0.3	0.042	1.1
Methionine†	0.030	0.6	Nil	Nil
Leucine	0.053	1.0	0.036	0.9
Phenylalanine	0.053	1.0	0.043	1.1
Histidine‡	+	1	+	1
Arginine‡	+	1	+	1

* There is no difference in charged residues between the abnormal haemoglobin and Haemoglobin A. It is virtually impossible, therefore, to separate them completely by electrophoretic methods and traces of the β^{A} peptide containing 98 Valine contaminate the β^{Koln} peptide.

† Methionine usually gives low values on amino acid analysis because the acid hydrolysis used here destroys about one-fifth of this amino acid.

‡ A positive specific colour test was considered to represent one residue.

Urine

Specimens of urine were examined from all five patients—those of IV, 1, V, 2 and IV, 2 had an abnormal golden-brown colour. Those from V, 1 and III, 5 were normal. No bile or excess urobilinogen was present in the three abnormally-coloured urines. No further studies were undertaken.

Phenacetin Study

Red cells (10 ml.) from IV, 1 were labelled with ^{51}Cr as sodium chromate and injected into a compatible normal recipient. The time for half-clearance ($T_{\frac{1}{2}}$) was 7 days. At this stage, phenacetin was taken by mouth, 2.7 g. daily for 3 days without measurable effect on the slope of red cell elimination.

Autohaemolysis

The results of autohaemolysis are shown in Table IV. In the case of V, 2 the test was carried out also on an aliquot of the blood using the original method of Selwyn and Dacie (1954). The results show that in the four subjects studied there was abnormal autohaemolysis in the absence of additives. In all subjects this was reduced by added glucose, which is characteristic of 'Type I' abnormality. However, only in case IV, 2 was there a marked response to added glucose.

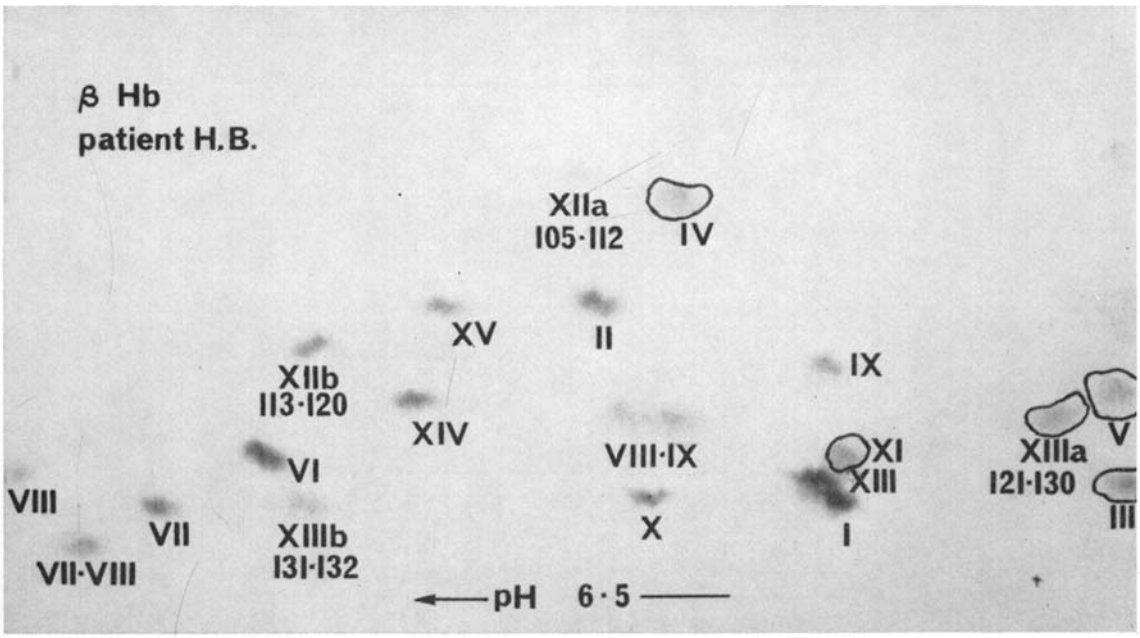

FIG. 2. Peptide chromatogram ('fingerprint') of the aminoethylated β chain of the abnormal haemoglobin of the propositus. Because of the aminoethylation all of the 15 tryptic peptides of the β chain are represented (including the two parts of the SH-containing peptide βTpXII). The picture is much the same as that obtained with the β chain of Haemoglobin A, but βTpXI differs in its amino acid composition. It stains positive for methionine and on analysis methionine is found to replace valine.

(*Facing p.* 400)

Methaemoglobin

All the four subjects examined had small amounts of methaemoglobin in their fresh blood. In all, incubation of the blood for 48 hours at 37° C. with continuous mixing resulted in

TABLE IV

RESULTS OF AUTOHAEMOLYSIS TESTS

Subject	*Haemolysis (per cent)*	
	No additives	*Added glucose*
Normal range (30 subjects)	0–1.7	0–1.1
III, 5	1.8	1.1
IV, 2	2.8	0.7
IV, 1*	2.7	1.0
	3.1	1.5
	2.2	1.0
V, 2	4.7	2.5
Method of Selwyn and Dacie (1954) Normal range (20 subjects)	0.5–3.6	0–1.3
V, 2	6.6	3.6

* Three replicates were obtained on different occasions.

TABLE V

METHAEMOGLOBIN IN FRESH AND INCUBATED WHOLE BLOOD

Subject	*Methaemoglobin (per cent total haemoglobin)*		
	Fresh blood	*After 48 hours incubation*	
		No additives	*Added glucose*
Normal range* (12 subjects)	Assumed nil	0–10	0–6
IV, 1†	3.5	22	11
	2.9	18	11
	3.4	15	11
III, 5	3.0	17	11.5
IV, 2	3.1	12	4
V, 2	4.6	18	12
Autohaemolysis method of Selwyn and Dacie (1954) Normal range (three subjects)	Assumed nil	5–7	2–5
V, 2	4.6	33	24

* Blood was incubated under the condition described for the autohaemolysis test in 'Methods'.

† Three replicates were obtained on different occasions.

abnormally high amounts of methaemoglobin (Table V). Added glucose diminished significantly the amount of methaemoglobin formed in its absence. Where no glucose is added

to the blood cellular metabolism ceases after consumption of endogenous glucose. This occurs after several hours and thereafter methaemoglobin accumulates through cessation of methaemoglobin reductase activity which is linked to glycolysis.

Glutathione (GSH)

Fresh blood values are shown below:

Normal range (μmoles-SH/ml. RBC)	*IV*, 1	*V*, 2	*III*, 5	*IV*, 2
2.40–3.80	2.56 2.02	1.83 2.05	2.31	1.20 1.42

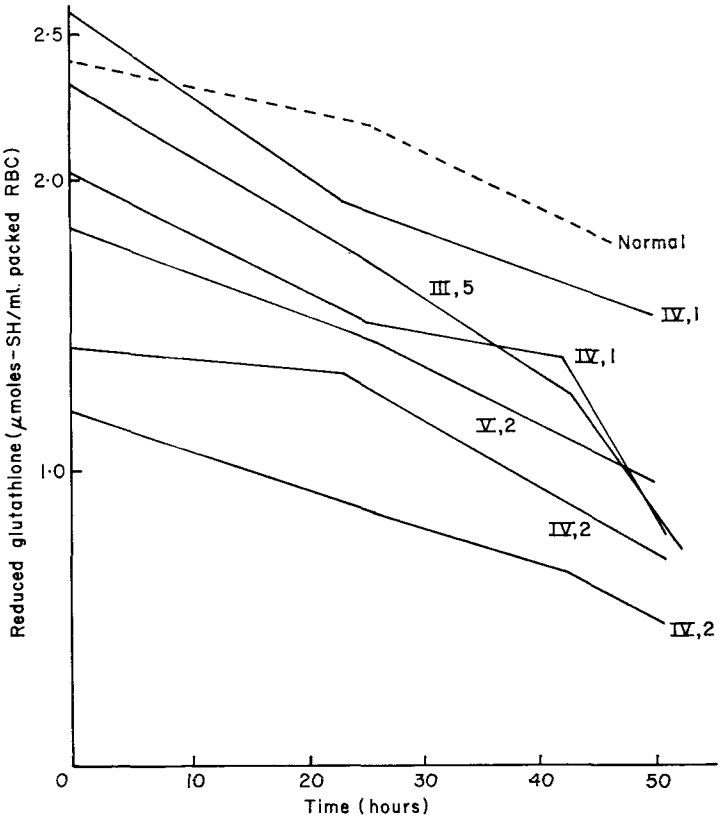

FIG. 3. Decline in GSH activity of red cells incubated with adequate glucose for up to 50 hours. Experimental conditions were similar to those for autohaemolysis described in text. Broken line shows the values obtained in one of 12 normal controls. This particular control sample is illustrated since it showed the most rapid decline in GSH and, coincidentally, gave the lowest zero-time value for GSH.

Three of the subjects had values somewhat lower than the normal range. The splenectomized subject (IV, 2) demonstrated markedly low values on two occasions. Determinations were made also upon blood incubated with added glucose for 48 hours under the conditions used for autohaemolysis and the results are shown in Fig. 3. It is clear that the GSH levels in cells from IV, 1 fell, on two occasions, somewhat more rapidly than in those from normal subjects.

This was true also for III, 5. Subjects V, 2 and IV, 2 yielded values not different from the most rapid drop in GSH shown by normal blood.

Heat-stability of Haemolysates

The blood of all four subjects tested gave an insoluble precipitate when buffered haemolysates were heated at 50° C. as described earlier (Dacie, Grimes, Meister, Steingold, Hemsted, Beavan and White, 1964). In the case of IV, 1 the insoluble precipitate obtained after 4.5 hours heating amounted to 8 per cent of the total globin.

Presence of Inclusion Bodies in Red Cells

Abundant Heinz bodies were present in the fresh red cells of the splenectomized subject IV, 2 and were found in occasional cells of IV, 1. Subjects V, 2 and III, 5 had no inclusion bodies in their fresh red cells. When blood was incubated for 48 hours under the conditions of the autohaemolysis test of Selwyn and Dacie (1954), inclusions staining with methyl violet were present in most cells of all four subjects.

DISCUSSION

The subjects of the present study are related to the family with Haemoglobin Köln reported earlier by Hutchison *et al.* (1964). Some of the data confirmed those obtained in that study. It is appropriate to consider the findings of the current study in relation to those in other congenital non-spherocytic haemolytic anaemias resulting from unstable haemoglobins.

Diagnostic Features

Heat lability of the abnormal haemoglobin. The four subjects of the present report yielded a heat labile fraction when buffered fresh haemolysates were incubated at 50° C., in confirmation of the heat lability of Haemoglobin Köln reported earlier (Hutchison *et al.*, 1964). This property is now considered typical for the abnormal haemoglobins of hereditary Heinz-body anaemia (Grimes and Meisler, 1962; Grimes, Meisler and Dacie, 1964; Dacie *et al.*, 1964), Haemoglobins Seattle and St. Marys (Huehns, 1965; Huehns and Shooter, 1965) and Haemoglobin H (Grimes, 1967).

Inclusion bodies in red cells of splenectomized subjects. The splenectomized patient of the present study had inclusion bodies in the fresh red cells. This was a feature of the other splenectomized patient of this family reported by Hutchison *et al.* (1964) and inclusion bodies were also found in the splenectomized subjects studied by Cathie (1952), Schmid, Williams and Clemens (1958), Schmid, Brecher and Clemens (1959), Scott, Haut, Cartwright and Wintrobe (1960), Worms, Bernard, Bessis and Malassenet (1961), Grimes *et al.* (1964), Dacie *et al.* (1964), Shibata, Iuchi, Miyaji, Ueda and Takeda (1963), André, Dreyfus and Le Boloc'h Combrisson (1964), Goudemand, Biserte, Habay and Voisin (1964) and Sansone and Pik (1965). The case of Lange and Akeroyd (1958) as later described by Gregoratos, Vennes and Moser (1964) is omitted since the latter authors expressed doubts about the diagnosis of hereditary Heinz-body anaemia. Also omitted is that of Lelong, Fleury, Alagille, Malassenet, Lortholary and Para (1961) because only 2–3 per cent of the red cells contained Heinz-bodies after splenectomy.

In some of these cases, abnormal haemoglobins have been found: Ube I (Shibata *et al.*,

1963); Köln (Pribilla, 1962; Hutchison *et al.*, 1964) and Galliera Genova (Sansone and Pik, 1965). Others, which have not been named. were observed by Scott *et al.* (1960), Dacie *et al.* (1964) and Goudemand *et al.* (1934).

Formation of red-cell inclusions following incubation in vitro. The family with hereditary Heinz-body anaemia studied by Dacie *et al.* (1964) included one splenectomized member; the remaining members had a haemolytic anaemia but had not been splenectomized and their fresh red cells contained no inclusion bodies. However, when the whole blood of these subjects was incubated inclusions appeared in the red cells in greater profusion than in normal red cells similarly treated. Subsequently Haemoglobins Seattle and St. Marys were shown to display this behaviour (Huehns, 1965; Huehns and Shooter, 1965). The members of the Haemoglobin Köln family of Hutchison *et al.* (1964) also showed this effect and data upon the three unsplenectomized subjects of the present report confirm their earlier finding.

Mean corpuscular haemoglobin concentration. It is of interest that this has been low, varying in the range 24–30, in most of the cases cited above except that of Goudemand *et al.* (1964) where it was 31 and of Pribilla (1962) where the red cells were described only as hypochromic. One reason for this might be that some of the unstable haemoglobin is removed from solution by intracellular precipitation and is then either removed from the red cells by the spleen (Crosby, 1959) or remains *in situ* as an inclusion body, which is not accounted for in haemoglobin measurement. In the present study the amount of inclusion body material in an aliquot of fresh red cells from the splenectomized subject was isolated and dried. The weight of material so obtained was corrected for the stromal content by comparison with a normal control. It amounted only to 0.6 per cent by weight of the total globin and could not therefore account for the low MCHC in this subject's cells. Whether insoluble material is formed and repeatedly removed by the reticulo-endothelial system is not known.

Punctate basophilia has been described in a number of cases: it was present before splenectomy in 30 per cent of the red cells of the cases of Cathie (1952) and in 10 per cent in one case (L Pr) of Dacie *et al.* (1964). After splenectomy in the cases of Schmid *et al.* (1959), Scott *et al.* (1960) and Worms *et al.* (1961) a marked punctate basophilia was present. In a number of other cases a few red cells showed punctate basophilia before splenectomy (Grimes *et al.*, 1964; Dacie *et al.*, 1964; Mozziconacci, Attal, Pham-Hu-Trung, Malassenet and Bessis, 1961; Goudemand *et al.*, 1964; André *et al.*, 1964), or after splenectomy (Sansone and Pik, 1965). Although Pribilla and ourselves noted punctate basophilia in the red cells of patients with Haemoglobin Köln before splenectomy, Hutchison *et al.* (1964) did not find it to be present before or after splenectomy, nor was this feature present after splenectomy in the case of Shibata *et al.* (1963) with Haemoglobin Ube I. It would therefore appear that punctuate basophilia is certainly not invariable, even after splenectomy, and when present it may be quite inconspicuous.

Methaemoglobin in fresh and in incubated blood. A feature of the subject with hereditary Heinz-body anaemia studied earlier (Grimes and Meisler, 1962) was the presence of methaemoglobin in the fresh blood which increased to large amounts on incubation *in vitro*. Formation of methaemoglobin on incubation of cells *in vitro* is a feature of Haemoblogins St. Marys and Seattle (Huehns, 1965; Huehns and Shooter, 1965), of Köln (Hutchison *et al.*, 1964) and of Haemoglobin H (Grimes, 1967).

Recent X-ray crystallographic studies of myoglobin by Nobbs, Watson and Kendrew

(1966) have helped to provide an explanation of these observations. The oxygenation of myoglobin involves a combination of oxygen with the haem iron which is converted into the ferric state according to Weiss (1964). As the folding of the polypeptide chain around the haem is very tight, and the amino acids lining the pocket in which the haem lies are hydrophobic, water cannot interfere when the oxygen is removed on deoxygenation. Thus on deoxygenation the iron atom returns again to the ferrous state. If the folding is less close, the iron atom is more easily accessible to water which can take up the position vacated by the oxygen to give the more permanent ferric form, metmyoglobin. Assuming that these considerations apply to haemoglobin, it would be expected that the introduction of a physically larger amino acid in the region of the haem could in some way widen the pocket and permit the entry of water thus facilitating methaemoglobin formation. Denaturation of protein also involves unfolding of polypeptide chains. A molecule such as Haemoglobin Köln with its change in the folding near the haem is in a state nearer towards unfolding, methaemoglobin formation and denaturation with precipitation, than is Haemoglobin A. For this reason the same degree of mild heating which does not affect Haemoglobin A will cause denaturation of Haemoglobin Köln.

GSH values in fresh and incubated blood. The four subjects of this report had low or low-normal GSH levels in their fresh red cells. The splenectomized patient had a markedly low value. The severely affected patient with hereditary Heinz-body anaemia reported earlier (Grimes and Meisler, 1962) had a low-normal level, and low values have been found in four subjects with Haemoglobin H disease (Grimes, 1967). The reason for this is not known but it is suggested that the presence of an unstable haemoglobin in red cells causes some utilization of the GSH which becomes bound to the haemoglobin during its precipitation. Studies *in vitro* showed that during 48 hours incubation of sterile whole blood from four subjects of the present study there was a tendency for the GSH to disappear more rapidly than normally despite the presence of adequate glucose (Fig. 3). This was certainly so with IV, 1 and III, 5 and the remaining two subjects showed a fall in GSH that was comparable to the most rapid fall obtained with 14 different normal blood samples.

Other Features

Thrombocytopenia. We have failed to find in our cases the moderate thrombocytopenia noted by Hutchison *et al.* (1964) in two of their subjects. Normal platelet counts before splenectomy were reported by Cathie (1952), Sansone and Pik (1965), Mozziconacci *et al.* (1961) and by Shibata *et al.* (1963), but most authors do not mention the platelet counts before splenectomy.

Splenectomy. The effect of splenectomy has often been disappointing and available data are shown in Table VI. Improvement was striking in the cases of Scott *et al.* (1960) and Shibata *et al.* (1963) and the operation was considered beneficial by Goudemand *et al.* (1964) and Hutchison *et al.* (1964). The only severely affected patients to derive much benefit seem to have been those of Scott *et al.* (1960) and Shibata *et al.* (1963) whereas those of Cathie (1952), two cases of Schmid *et al.* (1958) and that of Mozziconacci *et al.* (1961) derived no benefit from splenectomy. Although the spleen is not the only site of haemolysis it appears that splenectomy has a definite place in the therapy of hereditary anaemia with unstable haemoglobins, including Haemoglobin Köln disease.

TABLE VI

EFFECT OF SPLENECTOMY

Author	*Age at splenectomy*	*Before splenectomy*				*After splenectomy*				*Clinical benefits*
		Hb (g./100 ml.)	*Retics (%)*	*$T_{\frac{1}{2}}{}^{51}Cr$*	*Bilirubin (mg./100 ml.)*	*Hb (g./100 ml.)*	*Retics (%)*	*$T'_{\frac{1}{2}}{}^{51}Cr$*	*Bilirubin (mg./100 ml.)*	
KÖLN										
1. Hutchison *et al.*	24	13.4	7	9.5	1.5	—	—	14	—	Definite improvement
2. Present	12	9.5–11.0	4.8	—	4.5	14.3	9	—	1.1	Definite improvement
UBE I										
Shibata *et al.*	7	6.4	—	—	—	10.8–14.0	2.4–7.2	—	1.3	Considerable improvement
GALLIERA GENOVA										
Sansone and Pik	16	—	—	—	—	11.4	3.6	—	1.0	No improvement
OTHERS										
Cathie	16/12	7	37–70	—	—	7.4–8.7	23–88	—	—	No improvement
Schmid *et al.*										
1	13	—	—	—	—	8.2–8.6	40–70	4	—	Transient improvement
2	20/12	8.2	13	—	—	8.4–10	20–40	6	—	No improvement
Scott *et al.*	32/12	Anaemia	Periodic transfusions			13.7	11–15	8	1.2–1.5	No further transfusions required
Worms *et al.*	20	10.2	5	—	—	9–12	1.7–3.0	—	—	Transient improvement
Mozziconacci *et al.*	11	5–8.9	17–24	10	3.3–6.4	—	—	—	—	No improvement
Dacie *et al.*	11	10.8–14.6	12	—	3.0	12.1–13.6	3.4–10	—	2.3–3.5	No improvement
Goudemand *et al.*	30	13.6	20	5	1.5	16.5	20	25*	1.9	Subjective improvement

* 1 month after splenectomy. Other results 6 months after.

Frequency of occurrence. The cases of Köln haemoglobinopathy described by Hutchison *et al.* (1964) and ourselves now number 16. In addition we know of a further relative in Scotland of this family together with another patient, both of whom have a haemolytic anaemia and an unstable haemoglobin resembling Köln. To this list may possibly be added the patient with Haemoglobin Ube-I (Shibata *et al.*, 1963) in whom the clinical, haematological and chemical findings resemble the observations on Köln haemoglobinopathy. Thus at present Köln haemoglobinopathy is the commonest cause of hereditary Heinz-body anaemia.

SUMMARY

Five patients with haemolytic anaemia from one family were studied; three had splenomegaly and one who had been splenectomized had numerous inclusions in his fresh red cells. An abnormal haemoglobin was found by electrophoresis. This was isolated and identified as $\alpha_2\ \beta_2$ 98 Valine→Methionine, i.e. Haemoglobin Köln.

Abnormal red cell GSH and methaemoglobin values were found in fresh and incubated blood. The presence of a heat-labile haemoglobin fraction and the formation of inclusion bodies in red cells incubated *in vitro* was confirmed. Red-cell morphology and the benefit of splenectomy are discussed.

The findings in patients with Haemoglobin Köln are compared with those reported in other hereditary Heinz-body anaemias. It appears that at present Köln haemoglobinopathy is the commonest member of this group of disorders.

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