

Hematologic and Biosynthetic Studies in Homozygous Hemoglobin Constant Spring

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Abstract. The elongated α -globin chains of hemoglobin Constant Spring (α^{CS} chain of HbCS) are produced in low amounts such that the α^{CS} -gene acts as a form of α -thalassemia; yet in the homozygous state the pathophysiological effects of this mutant are more severe than in the corresponding conditions that result from α -globin gene deletions. In studies designed to examine this discrepancy, we have demonstrated that a significant proportion of red cells produced in an HbCS homozygote has a much reduced red cell life span. Contrary to previous reports, we have been able to demonstrate the expected deficit in α -chain production in this condition and have shown that both the cessation of globin chain synthesis *in vitro* and the destruction of the excess β -chains occur unusually rapidly. Comparison with various deletion forms of α -thalassemia suggests that, in terms of intracellular globin chain precipitates and free β -chain pool, homozygous HbCS red cells more closely resemble those of HbH disease, with three of the four α -genes inactivated, than they do the more comparable α -thalassemia carriers with only two genes deleted.

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Introduction

Hemoglobin Constant Spring (HbCS),¹ an abnormal hemoglobin with a frequency of up to 5% of some populations in Southeast Asia, has α -chains that are 172 amino acids long, instead of the normal 141 (1, 2). The elongated α -chain is thought to result from a single base substitution in the chain termination codon of the $\alpha 2$ -gene, which results in its translation as an amino acid and allows read-through of the normally untranslated 3' flanking region of the α -globin messenger RNA (mRNA) until the next in-phase termination codon is reached. Heterozygotes for HbCS have ~1% of the variant in their red cells, instead of the 20-25% usually found in heterozygotes for α -chain hemoglobin variants. This has been shown to be due to instability of the α^{CS} -mRNA, presumably because of its unusual pattern of translation, rather than to instability of the protein (3). The net effect of the reduced synthesis of α^{CS} -chains is that the α^{CS} -gene acts as a form of α -thalassemia, and when it is inherited together with α^0 -thalassemia ($- - / \alpha^{\text{CS}} \alpha$) it produces the clinical phenotype of HbH disease (4).

When compared with other common forms of α -thalassemia in Southeast Asia, most of which are due to gene deletions, the HbCS forms show several unexplained differences. Both heterozygotes ($\alpha \alpha / \alpha^{\text{CS}} \alpha$) and homozygotes ($\alpha^{\text{CS}} \alpha / \alpha^{\text{CS}} \alpha$) have been reported to show α / β globin chain synthesis ratios > 1 , rather than the expected deficit of α -chain production (5, 6). Furthermore, since the α^{CS} -gene is associated with a very low output of α -globin chains, homozygotes should have the same clinical

1. Abbreviations used in this paper: Hb, hemoglobin; HbA and HbA₂, normal adult hemoglobins; HbF, fetal hemoglobin; HbCS, hemoglobin Constant Spring; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; PMSF, phenylmethyl sulfonyl fluoride.

phenotype as individuals homozygous for the deletion form of α^+ -thalassemia ($-\alpha/-\alpha$) or heterozygous for α^0 -thalassemia ($\alpha\alpha/-$). These individuals are mildly anemic, with microcytic, hypochromic red cells, but show no other clinical abnormalities or changes in their Hb pattern (7). HbCS homozygotes, in contrast, have a moderate hemolytic anemia with splenomegaly, relatively normal red cell indices, and elevated levels of Hb Barts (γ_4) (6, 8, 9). Furthermore, patients with HbH disease with only one α -gene ($-\alpha/-$) are less anemic and have less HbH than those who are heterozygous for both α^+ -thalassemia and the HbCS mutation ($\alpha^{CS}\alpha/-$) (10, 11).

In an attempt to clarify some of the unexplained phenotypic differences between HbCS and the deletion forms of α -thalassemia, we have carried out extensive clinical, hematological, and biochemical studies on a patient homozygous for HbCS.

Methods

Patients. P.P., a 32-yr-old Thai male, homozygous for HbCS, gave informed consent for these studies, which were carried out over a 2-wk period in Oxford. Apart from recurrent jaundice as a child, he has had no symptoms. Mild icterus and hepatosplenomegaly (4 and 3 cm, respectively) were the only abnormal physical findings; bone x-rays of chest and skull were normal.

For comparison, studies were also carried out on several patients with various forms of α -thalassemia that were shown by restriction enzyme mapping to be due to gene deletions. The two patients with HbH disease were of Filipino and Chinese origin; they had the typical features of this condition, with HbH levels of 11 and 8%, respectively, and reticulocyte counts of 7 and 5%. Two patients of Sudanese and Nigerian origins, homozygous for α^+ -thalassemia ($\alpha-\alpha-$), also had typical hematological findings but were clinically unaffected, as was the α^0 -thalassemia heterozygote ($\alpha\alpha/-$) (previously reported as III in family L of reference 12).

Hematologic studies and hemoglobin analysis. Hematologic studies on blood and bone marrow were performed by standard procedures. Methods for hemoglobin analysis by starch gel electrophoresis, quantitative analysis on cellulose acetate, and measurement of fetal Hb (HbF) by alkaline denaturation have been described previously (13).

For electron microscopy, aspirated bone marrow was taken into heparinized Hanks' solution, fixed in glutaraldehyde (2.5% in 0.1 M phosphate buffer, pH 7.3), and processed for transmission electron microscopy (14). At least 600 consecutive erythroid cell profiles were assessed for the presence of precipitated globin chains.

Red cell fractionation. Age stratification of red cells was achieved by centrifugation of a column of 14 ml packed red cells at 200,000 g for 60 min at 4°C.

Bone marrow erythroid cells were fractionated according to their degree of maturity on bovine serum albumin gradients (15).

Ferroketic studies. Ferroketic studies were carried out after intravenous injection of 9 ml of the patient's plasma that had been labeled with 6 μ Ci ^{59}Fe ferric citrate (specific activity 10 $\mu\text{Ci}/\mu\text{g}$ iron; Radiochemical Centre, Amersham Corp., Amersham, England). Subsequent determination of plasma and erythroid iron turnover was as described (16). Plasma iron and total iron binding capacity were determined by standard methods (17, 18).

Red cell survival studies were carried out using the patient's cells

labeled with ^{51}Cr by the sodium chromate/acid-citrate-dextrose method. Subsequent peripheral red cell ^{51}Cr counts were corrected for elution (19).

Surface ^{59}Fe and ^{51}Cr counts over heart, sacrum, liver, and spleen were measured with a collimated sodium iodide scintillation probe. The injection of ^{51}Cr -labeled red cells was delayed until 30 min after that of ^{59}Fe -labeled plasma to allow determination at each site of the proportion of ^{59}Fe which would cross-count with ^{51}Cr . All later ^{51}Cr counts were then corrected for ^{59}Fe cross-counts and analyzed for any excess accumulation of ^{51}Cr in liver and spleen (20).

Globin chain synthesis studies. Peripheral blood and bone marrow samples were incubated with [^3H]leucine for increasing periods of time (21). For peripheral blood incubations, reticulocyte enrichment was unnecessary in the HbCS homozygote and the patients with HbH disease but was carried out in the other α -thalassemia cases. White cells were removed by cellulose columns (22) in all cases. For determination of the total globin chain synthesis pattern, aliquots were removed from the incubation mixture at various times and added directly to 2% HCl in acetone at -20°C , without prior washing.

In pulse-chase experiments, incubated samples were washed three times in reticulocyte saline (NaCl, 0.13 M; KCl, 0.005 M; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0024 M). Fresh incubation medium that contained 10 mM nonradioactive leucine was added to half of the sample and the incubation was continued for 2 h more, while the other half was immediately converted to globin by acid-acetone precipitation. The reincubated aliquot was similarly converted to globin, without further processing.

For gel filtration, incubated samples were washed, freed of membranes by centrifugation at 100,000 g 30 min, and loaded immediately onto Sephadex G-75 columns in 0.05 Tris-HCl, pH 7.4, at 4°C (15). After the radioactive profile of the eluate had been obtained, appropriate fractions were pooled, nonradioactive autologous hemolyzate (50 mg) was added as carrier, and globin was precipitated with acid acetone for chain separating.

For Amberlite CG-50 (British Drug Houses Ltd., Poole, England) chromatography, stroma-free samples were dialyzed overnight against three changes of developer 2 (23) and loaded onto the column in the same buffer at 4°C. When the fast-moving hemoglobins (HbH and Hb Barts) had been eluted, the column was equilibrated at 25°C for elution of the remaining hemoglobins (normal adult Hb [HbA, HbA₂]) (24). Pooled fractions were converted to globin for chain separation as described above.

Separation of globin chains from all the above samples was carried out by carboxymethyl-cellulose chromatography (21, 25) in 8 M urea-2-mercaptoethanol, using a gradient of 0.005–0.033 M Na_2HPO_4 , pH 6.8. Specific activities of the peak tubes were measured after dialysis against 0.5% formic acid and expressed as counts per minute per milligram, using appropriate extinction coefficients (21).

Results

Hematologic studies

At the time of study the hematologic findings in P.P. were as follows: white blood cells, $9.0 \times 10^9/\text{liter}$; erythrocytes, $4.38 \times 10^{12}/\text{liter}$; Hb, 11.5 g/dl; hematocrit, 0.38; mean corpuscular volume (MCV), 87.0 fl; mean corpuscular Hb (MCH), 26.3 pg; mean corpuscular Hb concentration, 30.2 g/dl; and reticulocytes, 11.5%. The peripheral blood film showed polychromasia and anisocytosis, with marked basophilic stippling. The serum bil-

irubin level was raised to 74 $\mu\text{mol/liter}$, but no bilirubin or urobilinogen was detectable in the urine. Other biochemical measurements were normal, as were serum folate and B₁₂ levels. Serum haptoglobin was undetectable and serum ferritin was 363 $\mu\text{g/liter}$.

The osmotic fragility curve on fresh blood was within the normal range (50% lysis at 0.435% NaCl, control 0.45%) with no evidence of subpopulations more or less sensitive to lysis. However, after 24 h of storage the red cells showed a markedly increased resistance (50% lysis at 0.26% NaCl vs 0.545% in the control). Autohemolysis showed 1.40 and 3.35% lysis after 48 h with and without glucose, respectively. The whole-blood oxygen dissociation curve was shifted left slightly, with an oxygen pressure at 50% Hb saturation of 24.5 mmHg (control 29.0 mmHg) and a 2-3-diphosphoglycerate level of 6.55 mmol/liter.

Red cell enzyme measurements showed normal levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconate, adenosine deaminase, glucosephosphate isomerase, phosphofructokinase, lactic acid dehydrogenase, glyceraldehyde phosphate dehydrogenase, and aldolase, with increased levels of pyruvate kinase, hexokinase, glutathione peroxidase, and erythrocyte glutathione reductase, consistent with the high reticulocyte count. Parantrophenol and pyridine 5'-nucleotide levels were also slightly increased.

A bone marrow aspirate showed hypercellularity that was mainly due to erythroid cells (myeloid/erythroid, 1:3.1) with marked basophilic stippling of the late normoblasts. Macrophage iron was moderately increased and siderotic granules were seen in many of the erythroid precursors. Under the electron microscope, inclusion bodies were observed in the cytoplasm of the erythroblasts (Fig. 1). Approximately 4% of the cells con-

tained branching inclusions similar in appearance to those seen in HbH disease, while in 0.6% of the erythroblasts, multiple rounded inclusion bodies, similar in appearance to those found in β -thalassemia, were observed. The corresponding figures for marrow reticulocytes were 10.8 and 1.0%, respectively. These findings are compared with results from other forms of α -thalassemia in Table I.

Ferrokinetic and red cell survival studies

The results of the ferrokinetic measurements are shown in Table II. The erythroid iron turnover was approximately five times normal, in keeping with the reversed myeloid/erythroid ratio in the marrow. The reticulocyte production index was increased comparably to almost six times normal (11.6% reticulocytes with a hematocrit of 36%). This suggests that erythropoiesis is largely effective in delivering red cells to the circulation and that the erythroid expansion is a response to an increased rate of peripheral red cell destruction.

The ⁵¹Cr red cell studies confirmed a shortened red cell survival, $t_{1/2}$ of ⁵¹Cr = 14 d. An arithmetic plot (Fig. 2) suggests that there may be two components to the survival curve, with 15% of the labeled cells having a mean lifespan of 5 d and the rest a life span of 47 d. This possibility is supported by two other findings. The red cell ⁵⁹Fe utilization was lower than normal (Fig. 3), which, in the absence of significant intramedullary red cell destruction, is consistent with the early removal of young cells from the circulation. Furthermore, surface counting demonstrated an unexpected accumulation of ⁵⁹Fe counts over the spleen, reaching a plateau by 4 d and over the liver, which showed a more gradual rise over the whole 10-d period (Fig.

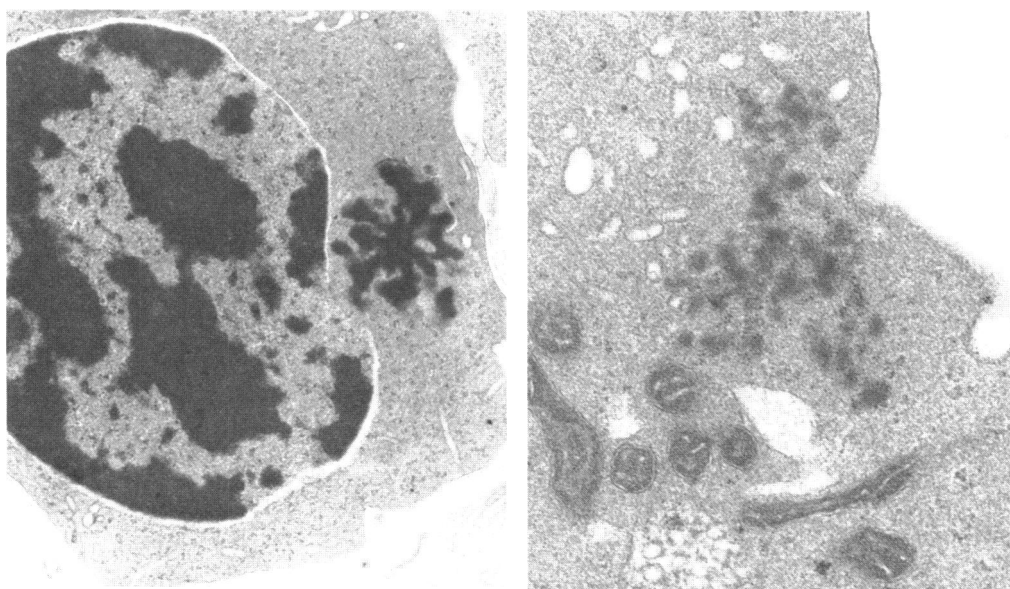


Figure 1. Electron micrographs of bone marrow cells from the HbCS homozygote showing an erythroblast with an intracytoplasmic branching inclusion (left, $\times 17,050$) and a reticulocyte containing multiple rounded inclusions (right, $\times 45,425$).

Table I. Prevalence of Globin Chain Precipitates within the Erythropoietic Cells in Homozygous HbCS Compared with Other Forms of α -Thalassemia

Condition (No. of cases studied)	% Erythroblasts with		% Nonnucleated* cells with	
	Branching inclusions	Multiple rounded inclusions	Branching inclusions	Multiple rounded inclusions
HbCS homozygote (P.P.)‡	3.9	0.6	10.8	1.1
α^+ -thal heterozygote (1)§	0	0.14	0.16	0
α^0 -thal heterozygote (2)§	0.6, 1.4	0, 0.11	0.8, 1.0	0, 0.12
HbH disease (4)§	1.1–5.1	0–0.22	2.9–12.0	0

* Containing ribosomes and mitochondria but not including part of a nucleus (i.e., profiles of marrow reticulocytes and some late erythroblasts).
 ‡ 332 consecutive erythroblast profiles and 268 consecutive nonnucleated profiles were assessed. § Data from reference 34. Inclusion bodies are not observed in erythroblasts of normal individuals.
 Thal, thalassemia.

4). In contrast, there were no excess ^{51}Cr counts over these organs during the whole 10-d period. Since ^{59}Fe specifically labels young red cells, while ^{51}Cr labels cells regardless of age, the surface-counting data support the idea of the removal of many young cells soon after release from the marrow. Those cells that survive this early cull seem to have a much longer lifespan of ~ 50 d.

Hemoglobin analysis

Quantitation of the various hemoglobin components gave: HbCS, 6.8%; HbA₂, 1.4%; HbF, 1.3%; and Hb Barts, 1.5%, the remainder being HbA.

In order to examine the *in vivo* stability of HbCS, 14 ml of packed red cells from which the buffy coat had been removed were stratified according to age by centrifugation. Starch gel electrophoresis and quantitation of HbCS after cellulose acetate electrophoresis showed no difference in the proportion of HbCS between the youngest cells (62% reticulocytes) and the oldest

cells from the bottom of the column (0.4% reticulocytes), as shown in Fig. 5.

Upon storage, HbCS tends to be degraded into the components with fewer amino acids in the α^{CS} -chain ($\alpha^{\text{CS}2}$ -169 and $\alpha^{\text{CS}3}$ -154 residues), finally ending up as a component designated $\alpha^{\text{CS}5}$, which has not been characterized structurally but which migrates between HbF and HbA (2, 26). No difference in the pattern of these components was observed between the old and young red cells on the day they were taken. During storage at 4°C, these lysates began to accumulate significant amounts of HbCS2 and HbCS3 after 5 d and by 2 wk had been almost entirely converted to HbCS5. This degradation pattern did not change consistently between young or old cell lysates but, as reported previously (26), could be almost entirely prevented by

Table II. Ferrokinetic Studies on the HbCS Homozygote

	P.P.	Normal
Plasma iron ($\mu\text{g}/\text{dl}$)	98	60–175
Plasma total iron binding capacity ($\mu\text{g}/\text{dl}$)	179	250–400
Hematocrit (%)	36	47 \pm 7
Plasma radioiron clearance, $t_{1/2}$ (min)	23	64–101
Plasma iron turnover (mg/dl whole blood per d)	2.88	0.58–0.88
Erythroid iron turnover (mg/dl whole blood per d)	2.66	0.50–0.66
Plasma volume* (ml/kg)	45	45 \pm 5
Red cell volume (ml/kg)	23	30 \pm 5

* Determined from the extrapolated zero time ^{59}Fe counts per milliliter plasma.

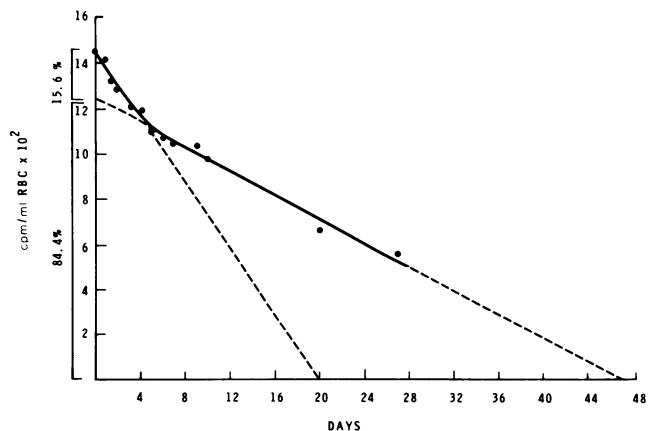


Figure 2. Arithmetic plot of ^{51}Cr autologous red cell survival, analyzed as a double population of cells (19) in the HbCS homozygote. The overall mean cell lifespan (20 d) arises from the combination of a population of longer lived cells (84.4% of total) having a mean cell lifespan of 47 d and a population (15.6%) with a mean cell lifespan of 5 d. RBC, erythrocytes.

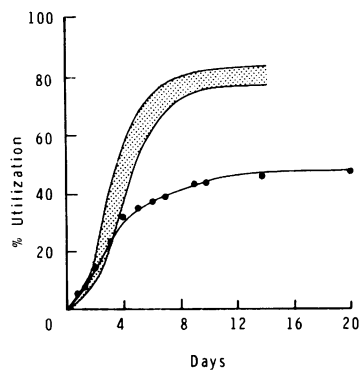


Figure 3. Utilization of ^{59}Fe -labeled plasma in the HbCS homozygote compared with normal individuals (shaded curve).

the addition of phenylmethyl sulfonylfluoride (PMSF). The slower changes observed in this study, compared with the previous one (26), may stem from a more rigorous removal of white cells before lysis, as these would be a potent source of proteolytic enzymes.

Globin chain synthesis

Peripheral blood. The pattern of globin chain synthesis in the peripheral blood reticulocytes of P.P. is shown in Fig. 6 and the results are summarized in Table III. At early time points (5 and 10 min) a deficit in α -chain synthesis was clearly shown, yet with increasing time of incubation the α/β synthesis ratio approached unity. Synthesis of the α^{CS} -chain was detectable but at a level of only 2% of the total α -chain synthesis. In three separate incubations it was noticeable that the rate of globin chain synthesis remained linear for only 30 min, an unusually short time for samples that contain such a high reticulocyte count (21).

The increase in α/β ratio with increasing incubation time could be due to a preferential decline in β -chain synthesis with

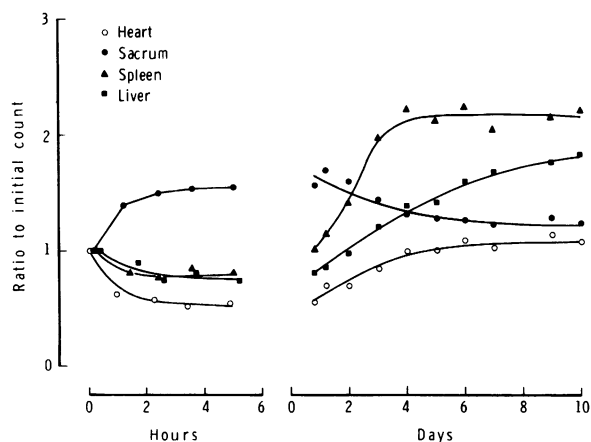


Figure 4. Surface counting of ^{59}Fe over various organs in the HbCS homozygote.

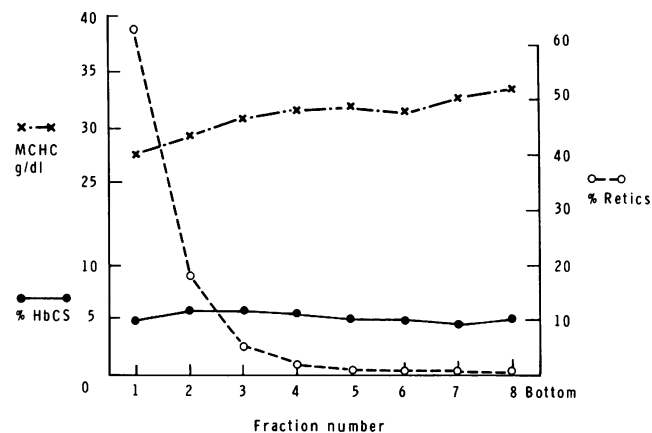


Figure 5. The distribution of HbCS, reticulocytes, and mean corpuscular Hb concentration in red cells from the HbCS homozygote, age stratified by centrifugation. Retics, reticulocytes.

time, or to degradation of β -chains (including those newly synthesized) during the incubation period. Evidence supporting the latter explanation was obtained from a pulse-chase experiment in which a 15-min pulse of $[^3\text{H}]$ leucine was followed by a 2-h chase in nonradioactive leucine. Whereas the specific activities of the α^{A} - and α^{CS} -chains remained unchanged during the chase, the β -chain specific activity decreased by 35%, resulting in an increase in the α/β ratio from 0.77 to 1.10. This β -chain degradation is probably due to proteolytic digestion, because when the protease inhibitor PMSF was included in the incubation medium, a much lower increase in the α/β ratio was observed over a 60-min incubation (Table III).

Further evidence for excess β -chain synthesis at short incubation times was obtained by gel filtration. When stroma-free lysate from a 10-min peripheral blood incubation was chromatographed through Sephadex G-75, two radioactive peaks eluted behind the Hb peak (Fig. 7). When carrier Hb was added to each of those peaks and the chains were separated, virtually all of the radioactivity eluted with the β -globin chains in each case; presumably these peaks corresponded to β -chain dimers and β -chain monomers. The Hb peak from this column had an α/β chain synthesis ratio of 2.32, further evidence that there must be a pool of free β -chains within these cells. When the overall α/β ratio was calculated from these three peaks, a value of 0.77 was obtained, compared with a ratio of 0.69 when the same sample was subjected to chain separation directly; this indicates that no substantial chain loss had occurred during removal of the membranes and gel filtration.

Bone marrow. A deficit of α -chain synthesis was also demonstrated after incubation of a bone marrow sample (Fig. 6). In this case, however, synthesis of the α^{CS} -chain accounted for 12% of the total α -chain synthesis at 25 min and its specific activity exceeded that of the α^{A} -chain (Table III).

The sample incubated for 60 min was also centrifuged

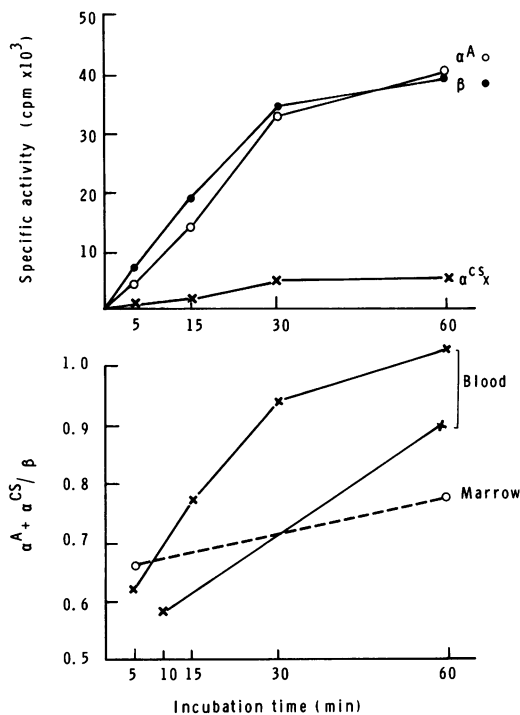


Figure 6. (Top) Specific activities (counts per minute per milligram) of separated globin chains isolated after incubation of peripheral blood from the HbCS homozygote with [³H]leucine for increasing periods of time. (Bottom) The change in globin chain specific activity ratios with time in two separate peripheral blood incubations and one bone marrow sample.

through a bovine serum albumin density gradient to fractionate the erythroblasts according to age. The separated cells showed more or less balanced globin synthesis (data not shown), presumably indicating continued proteolysis during the fractionation procedure, since an aliquot converted into globin immediately after incubation had an α/β ratio of 0.77. There was no obvious decline in the proportion of α^{CS} -chain synthesis from the most immature cell fractions that contained mostly basophilic erythroid cells to the mature, orthochromatic normoblast fraction. However, since the α^{CS}/α^A -specific activity ratio was <1.0 (0.7–0.9) in the fractions containing immature cells, some degradation of the newly synthesized α^{CS} -chains may have occurred during the fractionation.

Comparison with other forms of α -thalassemia

Time course experiments. Globin chain synthesis was also measured in two patients with HbH disease ($-\alpha^{3.7}/-$), two patients homozygous for α^+ -thalassemia ($-\alpha^{3.7}/-\alpha^{3.7}$), and one patient heterozygous for α^0 -thalassemia ($\alpha\alpha/-$); these diagnoses were proven by restriction enzyme mapping (27).

The globin synthesis ratios in these cases after various incubation periods are shown in Fig. 8 and compared with those

obtained for the HbCS homozygote. In all cases there was an increase in the α/β ratio with increasing incubation time, but it was not as marked as in the HbCS homozygote, not even in the two HbH disease cases where the β -chain excess was greatest. When pulse-chase experiments were performed in these samples, there was evidence of β -chain degradation in each case (Table IV). However, despite the lower α/β ratio and hence the greater proportion of excess β -chains in these cases, the amount of β -chain degradation was less than in P.P.

Gel filtration. Lysates from incubated reticulocytes were also subjected to gel filtration to estimate the size of the free β -chain pool in several of these cases (Fig. 9). In both the $\alpha\alpha/-$ and $-\alpha/-\alpha$ patients, no clear peak of β -chains could be detected (four separate experiments, three patients) at any time between 5 and 60 min of incubation. In contrast, in the HbH disease patient ($-\alpha/-$) the majority of the incorporated radioactivity migrated in a peak after the hemoglobin peak and was shown to be free β -chains. The relative proportions of counts under the hemoglobin and free β -chain peaks did not alter appreciably between 5-, 30-, and 60-min incubations.

It seems clear, therefore, that in HbH disease the newly synthesized β -chains enter a large β -chain pool and are not incorporated appreciably into HbA, even after a 1-h incubation; thus the Hb fraction eluted from the gel filtration column has an α/β radioactivity ratio >4 (a more precise estimate could not be obtained because of the incomplete separation of the two peaks). In the conditions where two of the four α -genes are functional, no free β -peak can be detected at any time, and the α/β radioactivity ratio in the hemoglobin fraction shows the same deficit of α -chains as does the unprocessed total globin. In these cases, the excess β -chains seem to be in the form of tetramers, which contrasts completely with the results obtained in the HbCS homozygote, where, with an overall deficit in α -chain production similar to the $-\alpha/-\alpha$ and $\alpha\alpha/-$ conditions, the pattern on gel filtration more closely resembled that of the HbH disease, with most of the β -chains in a nontetramer form.

Bone marrow incubation in $-\alpha/-\alpha$. Bone marrow cells from one patient with the $-\alpha/-\alpha$ genotype gave α/β ratios of 0.66 and 0.93 after 5 and 60 min of incubation, respectively. This is higher than the ratio obtained in peripheral blood cells of the same patient ($\alpha/\beta = 0.42$ – 0.53 over a 15–60-min time course), possibly reflecting greater proteolytic activity in nucleated cells.

When the 5-min marrow incubation was subjected to gel filtration, no pool of free β -chains was observed and the α/β ratio of the Hb fraction was 0.65, identical with the ratio obtained from the whole globin sample. Similarly, after the addition of nonradioactive HbH, another aliquot chromatographed on Amberlite IRC 50 produced no radioactive globin chains in the HbH peak eluted at 4°C and an α/β ratio of 0.66 in the HbA peak eluted at room temperature.

It is not clear how an excess of newly synthesized β -chains can occur in these cells yet not be detected either as β -chain dimers/monomers or as β_4 -tetramers (HbH), unless there is

Table III. Globin Chain Synthesis Results in Three Separate Blood Samples from the HbCS Homozygote

Experiment	Incubation period	Total counts		Specific activities				
		$(\alpha^A + \alpha^{CS})/\beta$	% α^{CS}	α^A	α^{CS}	β	α^A/β	α^{CS}/α^A
	min			cpm/mg	cpm/mg	cpm/mg	cpm/mg	cpm/mg
PB I	10	0.58	2.5	7,128	1,072	11,401	0.63	0.15
	60	0.91	1.3	20,400	2,906	21,043	0.97	0.14
PB II	5	0.62	3.0	4,478	966	6,646	0.68	0.21
	15	0.77	2.0	14,011	1,854	18,771	0.75	0.13
	15 + 2-h chase	1.10	1.7	14,227	1,821	12,180	1.17	0.13
	30	0.94	1.6	32,740	5,941	34,465	0.95	0.18
	60	1.03	2.0	40,328	6,572	39,705	1.01	0.16
PB III	10	0.69	1.5	11,360	2,512	14,968	0.76	0.22
	60 + PMSF	0.78	2.0	32,691	9,775	37,958	0.86	0.30
BM I	5	0.64	12.3	23,000	31,651	38,914	0.59	1.38
	60	0.77	8.1	159,600	170,512	217,008	0.76	1.06

PB, peripheral blood; BM, bone marrow.

considerable exchange of subunits with preformed HbA tetramers.

Discussion

The various phenotypes associated with different molecular variations of α -thalassemia have been reviewed recently (28).

α^+ -Thalassemia carriers, who have a single α -gene deletion ($-\alpha/\alpha$) have, at most, a very mild reduction in their MCH and MCV values; α^0 -thalassemia heterozygotes ($-\alpha/\alpha$) and α^+ -thalassemia homozygotes ($-\alpha/-\alpha$), who each have two deleted α -globin genes, have mild anemia with reduced MCH and MCV values and are phenotypically indistinguishable. The effect of the α^{CS} -gene is to reduce the output of α -globin chains from

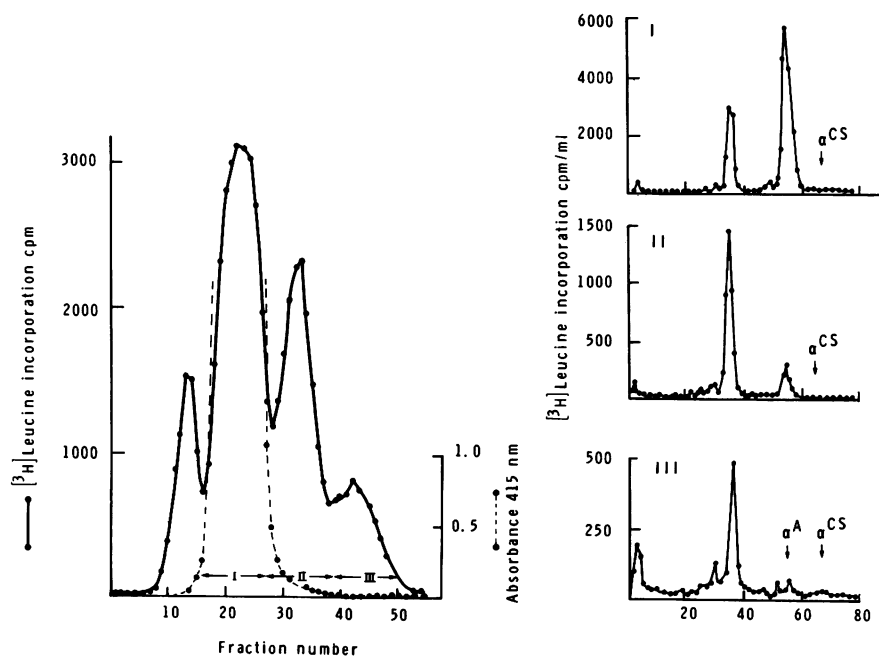


Figure 7. (Left) Gel filtration chromatography of hemolysate from homozygous HbCS reticulocytes incubated with $[^3\text{H}]$ leucine. Peak I corresponds to hemoglobin tetramers; peaks II and III are of lower molecular weight. (Right) Globin chain separations of the pooled fractions of peaks I, II, and III after the addition of carrier hemolysate from the HbCS homozygote. In both peaks II and III, the majority of the radioactivity elutes with the β -chains.

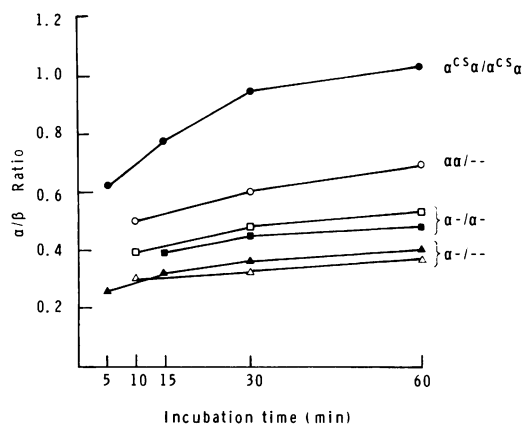


Figure 8. Globin chain synthesis ratios after various incubation periods in the HbCS homozygote compared with various types of deletion α -thalassemia.

the $\alpha 2$ -locus to ~ 1 –2% of normal. For this reason, HbCS heterozygotes should be phenotypically similar to α^+ -thalassemia heterozygotes, and this is the case; i.e., they have mild hematological changes and a slightly elevated level of Hb Barts at birth (7). However, HbCS homozygotes are not phenotypically the same as α^+ -thalassemia homozygotes. The findings in the HbCS homozygote described here are identical to those previously described (6, 8, 9). The patient had a moderate degree of anemia with an elevated reticulocyte count, marked basophilic stippling of the red cells, an almost normal MCH value, a normal MCV value, and an elevated level of Hb Barts. These findings, together with the increasing evidence that $--/\alpha^{CS}\alpha$ individuals have a more severe form of HbH disease than those with the $--/\alpha$ arrangement (10, 11) indicate that the pathophysiology of the HbCS mutation is different from that of the deletion forms of α -thalassemia.

Previous work on the synthesis of HbCS has shown that, unlike other forms of α -thalassemia, both heterozygotes and homozygotes seem to have excess α -chains (5, 6), despite a deficit of α -mRNA (3). The present studies have resolved this anomaly, clearly demonstrating a deficit of α -chain production when erythroid cells were incubated for short periods. With increasing periods of incubation, the excess β -chain radioactivity was gradually lost and the α/β globin chain synthesis ratios approached those reported in the literature. Furthermore, the rate of globin chain synthesis remained linear in the cells of the HbCS homozygote for only 30 min, an unexpectedly short time for samples with such a high reticulocyte count. This fact, together with the rapid removal of the excess β -chains, presumably accounts for the high α/β ratios observed by other workers in their 2–3-h incubations of the cells of HbCS homozygotes.

Thus, the clinical and hematological findings in the HbCS homozygotes seem to arise from the following series of events.

After transcription of the α^{CS} -gene (whether this is at a normal or subnormal level is not yet known) there is instability of the α^{CS} -mRNA, which decreases during erythroid maturation and which is virtually absent from reticulocytes (3, 30). The synthesis of α^{CS} -chains follows the same pattern, decreasing from bone marrow to reticulocytes (2, 31); but once incorporated into a hemoglobin tetramer, the α^{CS} -chains seem to be quite stable in vivo. As a result of the deficit of α^{CS} -chain synthesis, excess β -chains accumulate in the red cells and are detectable as a pool of β -chain dimers and monomers. However, this pool seems to turn over rapidly, probably as a result of proteolysis, and is destroyed at a rate greater than that seen in other forms of α -thalassemia with a comparable or even greater deficit of α -chain synthesis. The net result of these events is that many of the erythrocytes emerging from the bone marrow are recognized as abnormal by the spleen and are rapidly removed from the circulation, resulting in hemolysis and splenomegaly. It seems that those cells that survive this early cull have a more normal lifespan.

This course of events does not explain why the phenotype of homozygous HbCS differs to such a degree from deletion forms of α -thalassemia with comparable deficits in α -chain production. In many ways the findings in our patient homozygous for HbCS are similar to those in HbH disease, albeit the overall degree of chain imbalance is less in HbCS. The presence of a short-lived red cell population has been described in HbH disease (29), while the electron-microscopic appearances of the HbCS red cell precursors are closer to those of HbH disease than to the different α -thalassemia carrier states. Similarly, the gel filtration experiments show that, as in HbH disease, HbCS ho-

Table IV. Globin Chain Specific Activities in Pulse-Chase Experiments Using Peripheral Blood from the HbCS Homozygote and from Two Individuals with Deletion Forms of α -Thalassemia

Case	Pulse min	Chase min	SA		SA _α / SA _β	% Decrease in SA _β
			α ^A cpm/mg	β cpm/mg		
$\alpha^{CS}\alpha/\alpha^{CS}\alpha$	15	—	14,011	18,771	0.75	35.1
	15	120	14,227	12,180	1.17	
$\alpha-/ \alpha-$	10	—	231	669	0.35	20.4
	10	120	250	532	0.47	
$\alpha-/ \alpha-$	30	—	792	1,666	0.48	25.0
	30	120	751	1,250	0.60	
$\alpha-/--$	15	—	10,678	28,149	0.38	23.1
	15	120	10,456	21,651	0.48	

SA, specific activity.

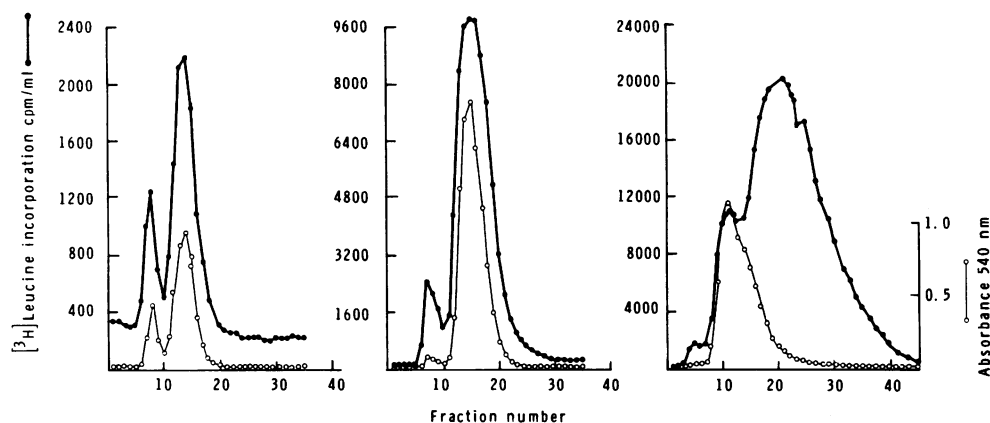


Figure 9. Gel filtration chromatography of incubated reticulocyte lysates from an α^+ -thalassemia homozygote, α^+/α^- (left); an α^0 -thalassemia heterozygote, α^0/α^- (middle); and an individual with HbH disease, α^-/α^- (right). Note that only in the case of HbH disease is there a significant amount of free β -chains; compare Fig. 7 (left).

mozygotes have a large pool of free β -chains in their red cells that could not be demonstrated in the cells of α^0 -thalassemia heterozygotes or α^+ -thalassemia homozygotes. Is it possible, therefore, that there is a greater deficit in α -chain synthesis in HbCS than is revealed even by short time point incubations? The α^{CS} -mutation occurs in the $\alpha 2$ -globin gene (28, 30, 32) and, while mRNA quantitation suggests a twofold excess of $\alpha 2$ -over $\alpha 1$ -mRNA in normal individuals (28, 30, 32), this difference appears to be compensated for by differential translation, producing equal proportions of α -chain from each gene (33). There is no evidence from quantitation of other abnormal α -chain variants that the output of α -chains directed by each gene differs significantly, and an individual homozygous for an $\alpha 2$ -gene deletion ($-\alpha^{4.2}/-\alpha^{4.2}$) is hematologically indistinguishable from any other individual with an α -gene deletion condition in which only two genes remain active (unpublished observation). It seems unlikely, therefore, that in HbCS the overall degree of α -chain production is significantly less than that in the deletion conditions in which only two α -genes remain; this suggests that the abnormal pattern of translation of the α^{CS} -mRNA may itself be responsible for the other abnormalities in homozygous HbCS cells, although how this might affect the rate of proteolysis is not at all clear.

Whatever the mechanism, these studies provide clear evidence for a significant overall deficit of α -chain production in the red cell precursors of an HbCS homozygote and show that there is both precipitation and rapid destruction of excess β -chains. These processes probably lead to damage to the red cell membrane, shortened red cell survival, and the marked reticulocytosis and splenomegaly that characterize this particular α -thalassemia syndrome.

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