Hemoglobin Hasharon ($\alpha_2^{47\ his}$(CD5)β_2): a hemoglobin found in low concentration

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Research Article

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Hemoglobin Hasharon $\left(\alpha_2^{47\ his}(CD5)\beta_2\right)$:

a Hemoglobin Found in Low Concentration

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INTRODUCTION

Human hemoglobins differ with respect to their con-
centrations within the red cell. Hemoglobin A_2 $(\alpha_2\delta_2)$, a
normal component, is found at a concentration only 1/40
that of Hb A $(\alpha_2\beta_2)$. A low rate of synthesis of the
δ-chain, relative to the β-chain, is considered to be re-
sponsible for that proportion, and several possible
mechanisms have been suggested (1–3). Most abnor-
mal hemoglobins vary from the normal in single amino
acid substitutions; the proportions in which they are
found in hemolysates vary widely. Hemoglobin J$_{Bangkok}$
and J$_{Baltimore}$ comprise more than 50% of the hemoglobin
in hemolysates of heterozygotes (4–7); hemoglobins
G$_{Accra}$ and K$_{Ibadan}$ comprise about 50% (8, 9), whereas
hemoglobins Nicosia (10), J$_{Oxford}$ (11), Caserta (12),
Köln (13), Torino (14), and Ube 1 (15) comprise 20%
or less.

These differences are not only thought to reflect vari-
ations in rates of protein synthesis, but variations in
rates of destruction of the abnormal proteins. In the first
case, change of a base in a codon not only may cause
substitution of one amino acid by another, but it may
also alter the level of the mutant protein being produced.
The mutant codon may alter the stability of RNA (16),
or it may have maximum affinity for a transfer RNA
that is in short supply (17). Anomalous folding of mes-
senger RNA, produced by the new codon, may alter
topographic relations between it and ribosomes in such
a manner that initiation or continuation of protein syn-
thesis may be affected (4). Steric properties of the in-
complete polypeptide chain, altered by the new amino
acid, may facilitate or inhibit further synthesis (1, 4).
The mutant polypeptide chain may have decreased affinity
for another subunit of the molecule, and completion of
synthesis may require binding of that subunit (18, 19).

On the other hand, observed differences in the concen-
tration of abnormal hemoglobins may reflect properties
of gene products rather than altered rates of synthesis.
In a heterozygote, red cells may vary in their content
of abnormal hemoglobin. If cells with more of that he-
moglobin are preferentially destroyed, hemolysates pre-
pared from cells that have survived the selection process
will contain less of the abnormal protein than was actu-
ally synthesized (20–22). Gabuzda, Nathan, and Gardner
(23), Reider, Zinkham, and Holtzman (24), and Rigas
and Koler (25) have extended this concept to include
unstable hemoglobins, which may denature and be re-
moved from red cells before the cell is destroyed. De-
naturation of hemoglobin, or its removal from the red
cell, shortens cell survival: the unstable hemoglobins
characteristically occur in relatively low concentration,
and they may produce hemolytic anemia.

Actually, alterations of hemoglobin synthesis and de-
struction probably operate in concert to produce ob-

served concentrations of abnormal hemoglobins. In the present study, members of a family of Ashkenazy Jews were found to be carriers of hemoglobin Hasharon, an abnormal hemoglobin first described in a Jewish family in Israel (26). Hemolysates contained only 16–19% of the abnormal protein. Investigation of the kindred and of the abnormal hemoglobin indicated that Hb Hasharon was produced in low yield. It was also found to be unstable, and heterozygotes were found to have an occult hemolytic process.

METHODS

Hematologic data, including measurement of red cell survival with ^{51}Cr, were obtained by standard laboratory methods; electrophoresis was performed as described in earlier publications from this laboratory (27). Chromatography of hemoglobin on DEAE-Sephadex was performed according to Huisman and Dozy (28); chromatography of globin on carboxymethyl-cellulose was performed by the procedure of Clegg, Naughton, and Weatherall, with the exception that the end buffer was 0.05 M phosphate (29). Analyses of isolated globin chains were carried out by Doctors Oestertag and Smith; a detailed description of their studies has been submitted for publication.[1] Haptoglobin in plasma was measured by an immunoassay, using commercial antiserum to human haptoglobin.[2] The effect of oxidant agents upon Hb Hasharon was determined by incubation of red cells with 5 volumes of 1% brilliant cresyl blue, 0.002 M primaquine diphosphate, or 0.01 M acetylphenylhydrazine for 3 or 24 hr at 37°C. Autohemolysis after sterile incubation at 37°C for 48 hr was measured by the method of Young, Izzo, Altman, and Swisher (30). Old and young red cells were separated by ultracentrifugation (31), or by the use of phthalate esters (32).

Denaturation by heat. Hemolysates from A-S and A-Hasharon heterozygotes were diluted to approximately 2.0 g/100 ml with 0.1 M PO₄ buffer, pH 7.4, and heated at 55°, 60°, or 65°C for 15, 30, or 60 min. Samples were centrifuged, supernatants were decanted, and their content of undenatured hemoglobin was measured as cyanmethemoglobin. Starch block electrophoresis was carried out on portions of the supernatants, and the proportion of each hemoglobin component that had been denatured was calculated from the difference between its concentration in the sample and that in an unheated control.

Hemoglobins A, S, C, and Hasharon were isolated from the blood of heterozygotes, and these fractions were concentrated by ultrafiltration and dialysis against 0.1 M PO₄, pH 7.4, to a concentration of about 2.8 g/100 ml. Aliquots of the fractions were heated at 55°, 60°, or 65°C for 15, 30, or 60 min, centrifuged, and the concentration of hemoglobin in the supernatant was measured as cyanmethemoglobin.

Alkali denaturation. Hemoglobins A, S, C, and Hasharon were separated from hemolysates of heterozygotes by starch block electrophoresis, and concentrated to 0.5–1.5 g/100 ml by ultrafiltration. After overnight dialysis at 4°C against 0.001 M PO₄ buffer, pH 7.4, alkali denaturation was measured by the method of Huisman and Meyering (33) and by the method of Singer, Chernoff, and Singer (34).

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[1] Ostertag, W., and E. W. Smith. 1968. Hb Sinai, a new α-chain mutant α_{47}^{His}. *Humangenetik.* 6: 377.

[2] Hyland Laboratories, Los Angeles, Calif.

Incorporation of ^{59}Fe into hemoglobins A and Hasharon in vivo. At the time that their red cells were labeled with ^{51}Cr, the propositus and a nephew also received intravenous doses of 5 μc of citrate-^{59}Fe. Blood samples were collected at intervals, and hemoglobins A and Hasharon were separated by starch block electrophoresis. Fractions were concentrated by ultrafiltration, and their specific activity (cpm/OD₅₄₀) was determined.

Hemoglobin synthesis in vitro. Bone marrow was aspirated into a large volume of heparinized cold NKM solution (35). No attempt was made to "enrich" reticulocytes in experiments with venous blood. Studies were performed according to Lingrel and Boorsook (36) with the following modifications: heparinized blood or bone marrow was washed three times with cold NKM solution, and then preincubated in capped vials for 10 min at 35°C. Dialyzed plasma was omitted from the reaction mixture. Total sample volume was 0.7 or 0.8 ml. After preincubation, equal amounts of labeled amino acid were added to each vial, and vials were removed from the shaking incubator at desired time periods. Incubation was stopped by addition of 2 ml of 0.01 M Tris-HCl (pH 7.5, containing 0.001 M KCN), and freezing in a dry ice–acetone bath; samples were stored at −25°C.

Later, samples were thawed and dialyzed for 48 hr at 4°C against large volumes of Drabkin's solution. After centrifugation, hemoglobin components were separated by chromatography on DEAE-Sephadex, and concentrated by ultrafiltration. The separated components were converted to globin by precipitation in cold 2% HCl-acetone after addition of a known amount of nonradioactive carrier hemoglobin. Recovery of carrier globin was assumed to be the same as that of labeled abnormal globin. Globin was dissolved in 0.1% formic acid, and optical density was measured at 280 mμ. Radioactivity was measured in a Packard Tri-Carb 3003 counter (with subsequent addition of internal standard) or in a Packard Tri-Carb 3375 counter with external standardization. Specific activity was expressed as cpm/optical density unit (after correction for dilution by carrier hemoglobin, and for quenching when appropriate).

CASE REPORT

R. L., a 70-yr old Ashkenazy Jewess, was born near Kovno, Lithuania. Her family migrated to Baltimore approximately 50 yr ago. Over the 20 yr before the study, she had been admitted to Sinai Hospital of Baltimore for a hysterectomy and for treatment of myocardial infarction and varicose veins.

In 1962 she was found to have an enlarged spleen; her hematocrit value was 35%. In 1966 her spleen extended 8 cm below the costal margin. The hematocrit value was 34%, red cells showed anisocytosis and poikilocytosis, and the reticulocyte count was 4.7%. The white blood cell count was 4700/mm³ with a normal differential count, but platelets were 38,000/mm³. Serum bilirubin was 0.8 mg/100 ml direct and 0.6 mg/100 ml, indirect. An intravenous cholangiogram revealed radiolucent calculi, and a bone marrow aspirate was cellular with erythroid hyperplasia. Electrophoresis of hemoglobin showed a slow component with the mobility of Hb S. The cause of hemolytic anemia was not established, and treatment with 40 mg of prednisone/day was instituted.

3 months later she was evaluated at the Johns Hopkins Hospital. She was not icteric, but the spleen was enlarged as previously described. The hematocrit value was 37.9%, and the reticulocyte count was 4.6%. Platelets appeared normal on smear. Therapy with prednisone was continued. The hematocrit value rose to 41%, but the reticulocyte count remained unchanged. In 1968 a bone marrow biopsy was normal, but large esophageal varices were demonstrated during fluoroscopy. A cholecystectomy was performed; the liver appeared cirrhotic, but was not biopsied.

FAMILY STUDY

24 family members were tested and nine were found to be carriers of the abnormal hemoglobin. Inheritance followed an autosomal co-dominant pattern (Fig. 1). Except for the propositus (II–10), no family members were found to be anemic. None had a history suggestive of hemolytic episodes or other hematologic abnormality, and none were known to have splenic enlargement. Physical examinations were performed on III–11 and III–13, and neither had splenomegaly. Hematocrit values, morphologic characteristics of red cells, and fetal hemoglobin levels were normal in all family members, but reticulocyte counts for heterozygotes were significantly higher than those of unaffected relatives ($P <$ 0.01) (Table I). Haptoglobin was absent from the plasma of III–9 and III–11.

SPECIAL STUDIES

Structure of the abnormal hemoglobin. When hemolysates containing the abnormal hemoglobin were subjected to starch gel electrophoresis at pH 8.6, the abnormal component had a mobility·equal to that of Hb S. Benzidine stains and chromatography on DEAE-Sephadex revealed a minor second abnormal component with approximately the mobility of Hb B_2. The major abnormal component could be separated from Hb A by agar electrochromatography at pH 6.0. The purified hemoglobin, prepared by starch block electrophoresis, moved between hemoglobins S and C at pH 7.0, suggesting that it differed by about $1\frac{1}{2}$ charge units from Hb A at that pH (Fig. 2).

Carboxymethylcellulose chromatography of globin prepared from the blood of the propositus revealed, in addition to normal components, a peak that was eluted more slowly than normal alpha chains (Fig. 3). Analysis of this peak[1] indicated that aspartic acid in position 47 of the alpha chain had been replaced by histidine, a substitution identical to that described for hemoglobin Hasharon (26). Partial ionization of histidine (pK$_2$ 6.1) can account for the apparent difference of $1\frac{1}{2}$ charge units between Hb A and Hb Hasharon at pH 7.0.

Effect of oxidants on Hb Hasharon. Equal numbers of inclusions were produced in red cells from the propositus and from a normal control after incubation with brilliant cresyl blue. Similar results were obtained after
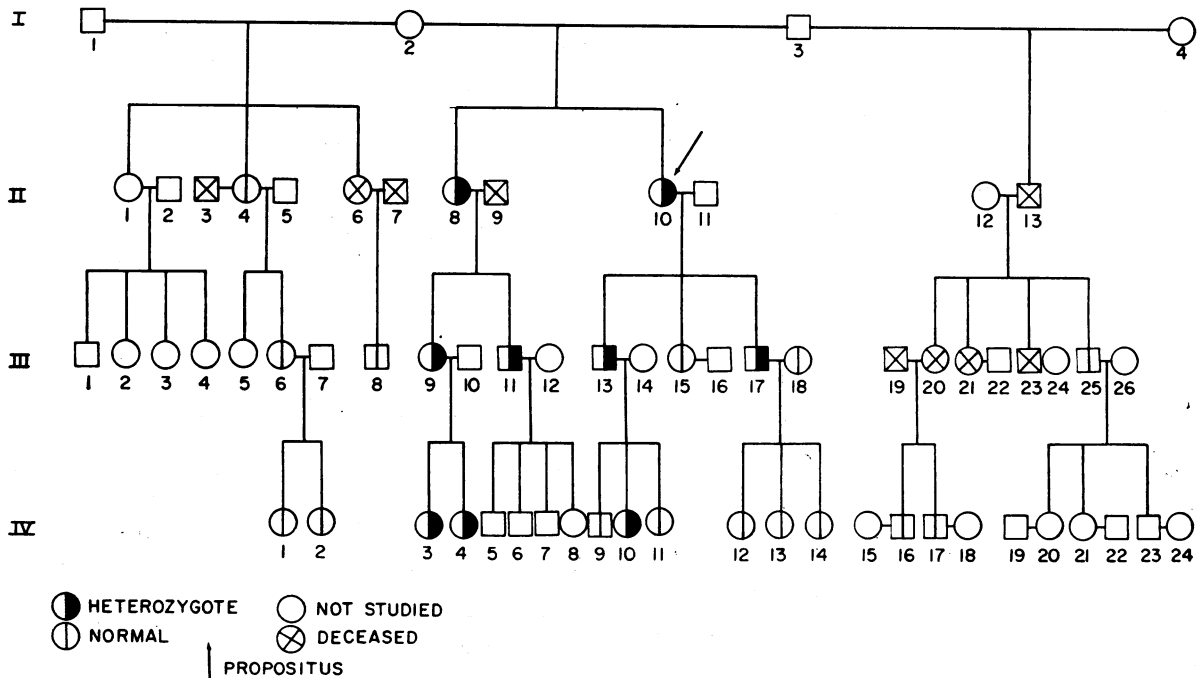

FIGURE 1 Pedigree of family with Hb Hasharon.

TABLE I
Hematologic Data of Kindred with Hemoglobin Hasharon

Pedigree No.	Hb Hasharon carrier	Hct	Reticulocyte count	Starch block electrophoresis			Fetal Hb
				A	Hasharon	A₂	
				%			
II-8	+	45.7	2.1	80.0	18.0	2.0	0.55
II-10	+	37.0	3.9	80.1	18.6	1.3	0.56
III-6	−	44.0	1.2				
III-8	−	46.2	1.2				
III-9	+	42.7	3.7	77.7	20.0	2.3	0.59
III-11	+	48.9	2.4	81.3	16.8	1.9	0.74
III-13	+	48.0	2.3	81.4	16.6	2.0	0.30
III-15	−	43.0					
III-17	+	51.0	3.0	80.7	17.2	2.1	
III-25	−						
IV-1	−	44.3	1.6				
IV-2	−	44.3	1.5				
IV-3	+	43.8	1.2	81.9	16.3	1.8	0.69
IV-4	+	45.0	1.6	78.8	19.2	2.0	0.88
IV-9	−	43.0	1.4				
IV-10	+	40.0		81.7	16.0	2.3	
IV-11	−	40.0					
IV-12	−	30.0	0.7				
IV-13	−	45.0	1.7				
IV-14	−	45.0	1.2				
IV-16	−	43.0					
IV-17	−	46.0	1.2				

incubation with acetylphenylhydrazine or primaquine. The proportion of hemoglobin Hasharon in hemolysates which had been incubated with these drugs did not appear to differ from that in control samples. Autohemolysis was normal.

Denaturation by heat. When hemolysates from carriers of Hb Hasharon were heated to 55°C for 1 hr, there was little precipitation of either Hb A or the abnormal component. At higher temperatures more Hb Hasharon than A precipitated. Hemoglobin S precipi-
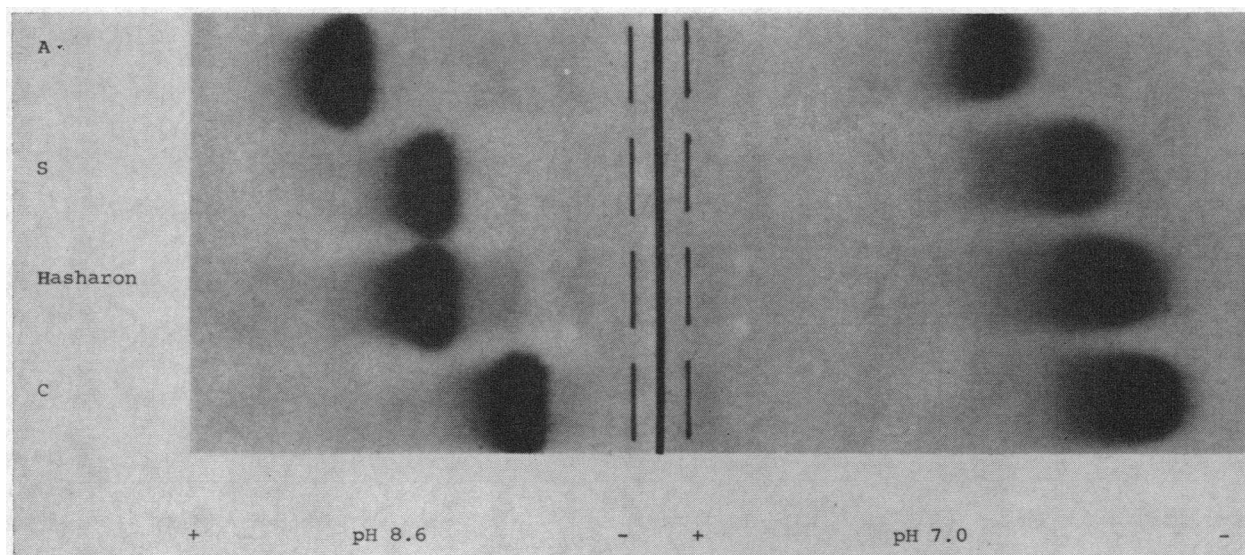

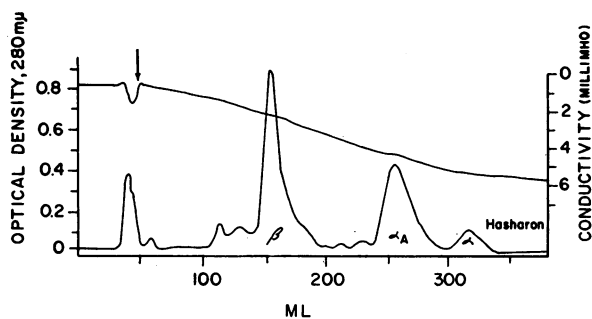FIGURE 2 Electrophoresis of purified solutions of Hb A, S, C, and Hasharon, at pH 8.6 and 7.0.

FIGURE 3 Chromatography of globin from a carrier of Hb Hasharon, in 8 M urea on carboxymethyl cellulose.

tated from an A-S hemolysate under the same conditions, but to a lesser degree than did Hb Hasharon. When purified solutions of hemoglobins A, S, C, and Hasharon were heated, Hb Hasharon was more readily precipitated than the other three hemoglobins tested (Fig. 4). Conversion to cyanmethemoglobin before heating prevented heat precipitation of all hemoglobins tested.

Alkali denaturation. Purified Hb Hasharon was somewhat more resistant to denaturation by alkali than Hb A eluted from the same starch block, or the same column of DEAE-Sephadex, using either of two techniques (Table II). Alkali-resistance of all samples studied was higher than anticipated, particularly if they had

been converted to cyanmethemoglobin before chromatography or electrophoresis. Hb Hasharon was consistently more alkali-resistant than other hemoglobins tested; the generalized increase in resistance was not pursued further. Hemolysates of carriers of Hb Hasharon did not contain an increased proportion of alkali-resistant hemoglobin.

Red cell survival. Red cell survival was measured in two carriers of Hb Hasharon (III–11 and III–13) using ^{51}Cr. The half-lives were 25 and 19 days, respectively (normal, 25–35 days). Reticulocyte counts of these individuals were 2.3 and 2.4%.

Hemoglobin synthesis in vivo. Citrate-^{59}Fe was administered to the propositus and a nephew (III–11), and the specific activities of Hb A and Hasharon were measured at intervals thereafter. The specific activity of the Hb Hasharon fraction was consistently higher than that of Hb A (Fig. 5). Approximately 27% of the incorporated iron appeared in the Hasharon fraction of the propositus' hemolysate, while 20% appeared in that fraction of her nephew's hemolysate. In the propositus, who was followed for a longer time, the specific activity of Hb Hasharon appeared to fall with the passage of time; specific activity of Hb A remained constant.

Hemoglobin synthesis in vitro. Bone marrow from the propositus was incubated with ^{3}H- or ^{14}C-labeled amino acids. Results differed from those obtained with ^{59}Fe in vivo; specific activities of the normal and ab-
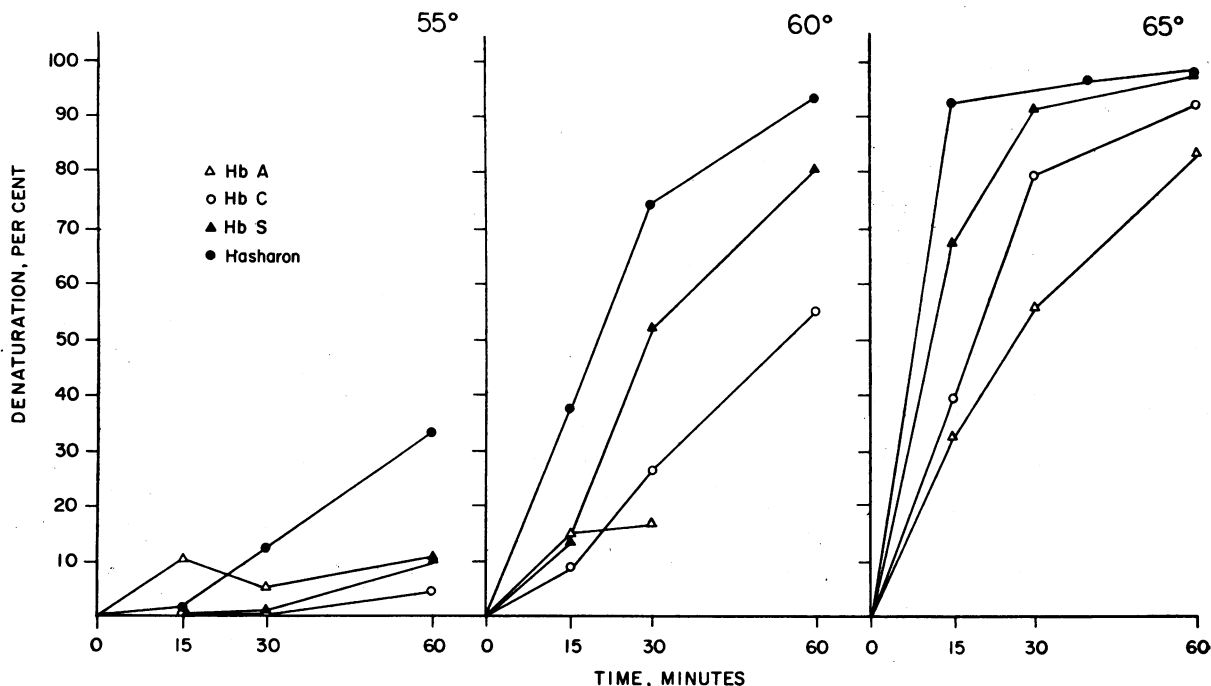

FIGURE 4 Heat denaturation of Hb A, S, C, and Hasharon.

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TABLE II

Alkali Denaturation of Dilute Solutions of Purified Hemoglobin

	Alkali-resistant hemoglobin	
Hemoglobin	Oxyhemo-globin	Cyanmethemo-globin
	%	%
A (AS)	—	20
S (AS)	—	21
A (AC)	4.5	15
C (AC)	5.0	14
A (A—Hasharon)	4.0	16
Hasharon (A—Hasharon)	8.5	33

Hemoglobins were prepared by starch block electrophoresis from hemolysates of heterozygotes, and dialyzed against 0.001 M PO$_4$ buffer, ph 7.0, before exposure to alkali.

normal fractions were equal in all studies (Fig. 6). Similar experiments, using marrow from hematologically normal persons with either Hb S or Hb C trait, yielded the same result. Incorporation of labeled amino acids into hemoglobin was studied with reticulocytes from II–10, III–9, and III–11. In contrast to results of the incubation of bone marrow, specific activity of Hb A was higher than that of Hb Hasharon (Fig. 6). Studies carried out with reticulocytes from *hematologically normal* individuals with sickle-cell trait (Fig. 6), or Hb C trait, also showed the specific activity of Hb A to be greater than that of the abnormal hemoglobin.

Separation of old and young red cells. Old and young red cells were separated from the blood of two heterozygotes by ultracentrifugation. Reticulocyte counts in the "young" fraction were from two to three times that of whole blood, whereas reticulocytes in the "old" fraction were decreased by 20–80%. The proportion of Hb Hasharon in old and young cells did not differ significantly. Similar studies, using the phthalate ester technique, were carried out with blood from III–9, and carriers of Hb S, C, and Zürich (Table III). Reticulocytes were considerably enriched with blood from AS and AC donors, and to a lesser degree with blood from III–9. Little or no enrichment could be obtained with blood from two carriers of Hb Zürich. Proportions of
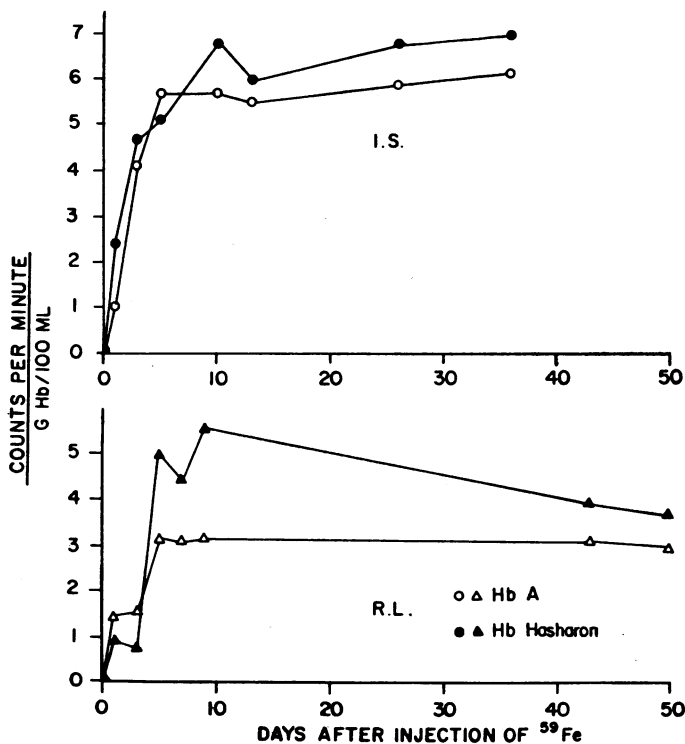

FIGURE 5   Incorporation of ^{59}Fe into hemoglobins of two carriers of Hb Hasharon, in vivo.
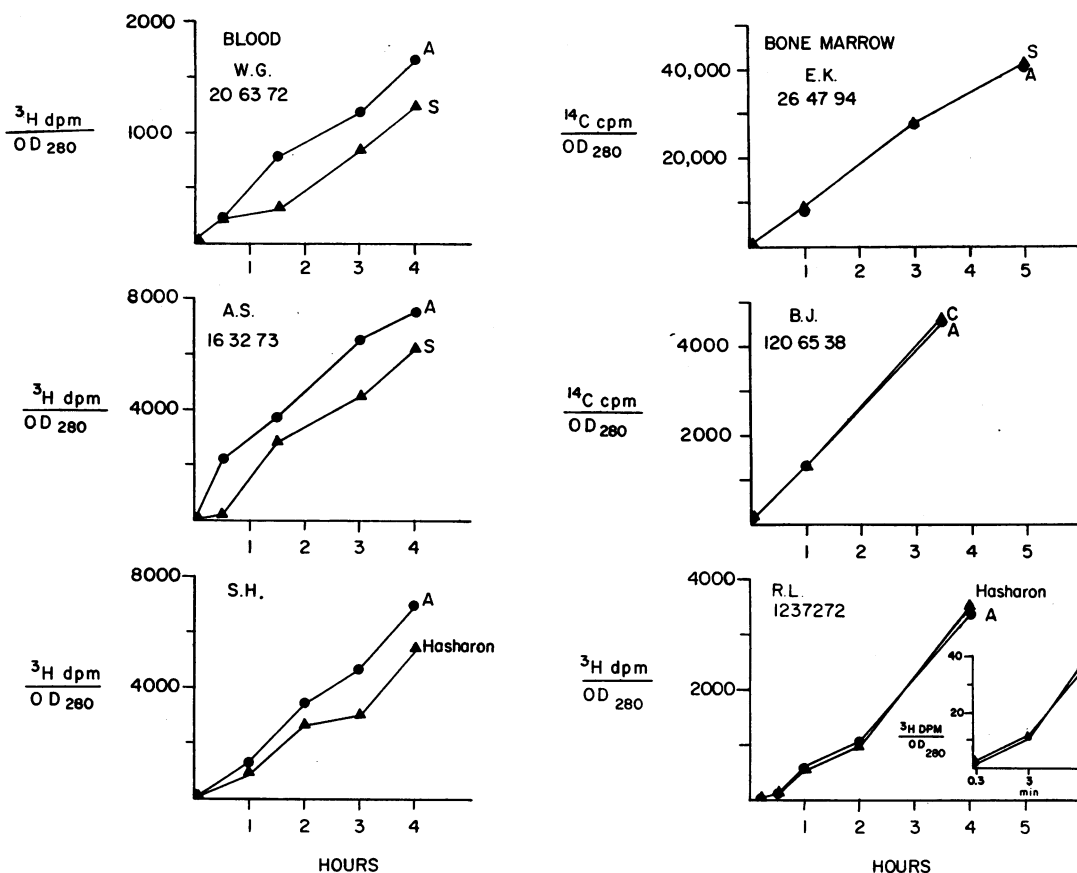
FIGURE 6 Incorporation of labeled amino acids into normal and abnormal hemoglobins,
by reticulocytes and bone marrow, in vitro.

TABLE III

Centrifugation of Red Cells with Phthalate Esters

Hemoglobin type	Fraction	Reticulocyte count	Abnormal Hb
AS	Top layer	6.4	37.1
	Whole blood	1.6	41.8
	Bottom layer	0.1	42.7
AC	Top layer	12.0	33.4
	Whole blood	1.4	34.7
	Bottom layer	0.1	32.0
A-Hasharon	Top layer	14.5	19.6
	Whole blood	2.3	20.6
	Bottom layer	0.4	18.7
A-Zürich	Top layer	3.9	33.7
	Whole blood	1.9	33.6
	Bottom layer	1.5	37.0
A-Zürich	Top layer	1.6	32.4
	Whole blood	3.9	35.1
	Bottom layer	2.0	36.7

normal and abnormal hemoglobin differed between top and bottom fractions in only one specimen, from a patient with sickle-cell trait.

DISCUSSION

In hemoglobin Hasharon, histidine is substituted for aspartic acid in position α^{47} (26). The hemoglobin was initially described in an Israeli family, and has also been detected in Texas (37). In both of those reports the fetal analogue of Hb Hasharon ($\alpha_2^{47 \text{ his}} \gamma_2$) was detected in a survey of umbilical cord bloods. Affected parents in both instances were Ashkenazy Jews. The family from Texas initially came from Galicia [3] and could conceivably have had an ancestor in common with the family from Baltimore.

Red cell survival was normal in one member of the Texan kindred,[3] but survival was shortened in III–13 and at the lower limit of normal in III-11. Low proportions of abnormal hemoglobin were present in the

[3] Alperin, J. B., and R. G. Schneider. Personal communication.

Texas family, and reticulocytosis was present in affected individuals.

Our attention was directed to Hb Hasharon because of its low concentration in hemolysates, only 16–19% of the total. Three observations suggested a possible explanation: (a) heterozygotes had slightly elevated reticulocyte counts, and red cell survival was slightly shortened or at the lower limit of normal in two of these carriers; (b) specific activity of Hb Hasharon was consistently higher than that of Hb A after administration of ^{59}Fe to two heterozygotes, suggesting that a greater proportion of Hb Hasharon was synthesized than one would anticipate from its concentration in peripheral blood; and (c) Hb Hasharon was more heat-labile than Hb A, S, or C. A similar constellation of abnormalities has been described for most of the hereditary hemolytic anemias produced by unstable hemoglobins.

Sufficient data are available to divide those disorders into three categories. In the first of these, typified by Cooley's anemia, red cell survival is markedly shortened. Large *preformed* inclusions, composed of precipitated α-chains, are demonstrable primarily in young red cells. The precipitates tend to disappear during red cell maturation, leaving hypochromic distorted red cells with extremely short survival (23, 38). In the second category, hemolysis is only moderately severe. Examples are the disorders found in carriers of Hb Köln, Torino, Ube 1, H, Hammersmith, Genova, and Sydney (13–15, 25, 39–41). Preformed inclusion bodies are found in the circulating red cells of splenectomized subjects. The mean corpuscular hemoglobin is low, and red cells show mild to moderate morphologic abnormalities. In the third category, accelerated erythropoesis can compensate for hemolysis. Carriers of Hb Zürich and Gun Hill are not anemic, and they have not been subjected to splenectomy (24, 42, 43). Preformed inclusion bodies are not found, but can readily be produced by incubation with oxidant dyes. Red cells are normal in appearance.

Detailed studies have been carried out with Hb H, Zürich, Gun Hill, Hammersmith, and Köln (23, 24, 43–46 and footnote 4). Those hemoglobins comprise, respectively, 20, 26, 30, 30, and 15% of hemolysates. During in vitro studies of synthesis of these hemoglobins, specific activity of the abnormal fraction was higher than that of Hb A. More isotope was incorporated into the unstable protein than could be anticipated from its concentration in hemolysates. In vivo studies with Hb H, Zürich, and Gun Hill yielded similar results (23, 24, 43). In vivo studies with hemoglobin Köln are compatible with in vitro studies, but the data may be interpreted in a different fashion (46).

In all of these disorders the abnormal hemoglobin is precipitated by mild heating in vitro, generally to 50°C.

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4 White, J. M. Personal communication.

Denaturation is thought to occur in vivo as well, producing a lesion which accelerates red cell destruction, even in the absence of spontaneous Heinz body formation. A hypothetical model, consistent with data for Hb H, Zürich, Hammersmith, and Gun Hill, is presented in the Appendix. In brief, the degree of denaturation of an unstable hemoglobin is assumed to be a function of cell age. Red cells containing denatured hemoglobin undergo premature destruction because of altered physical or physiological properties, when denaturation has proceeded to a "lethal" level. Denatured hemoglobin does not appear in hemolysates, either because it is lost during their preparation in the laboratory or because it is removed from red cells in vivo. As a result, aged red cells appear to contain less abnormal hemoglobin than do young cells. If the unstable hemoglobin is nonhomogeneously distributed among cells of the same age, those red cells which initially contain it in highest concentration have the shortest survival times.

It is evident that Hb Hasharon has some relation to previously described unstable hemoglobins, particularly Gun Hill and Zürich. Lack of precipitation at 50°C and absence of formation of Heinz bodies by oxidant dyes suggested that it might *not* be unstable in vivo. Two predictions of the hypothetical model were examined, to determine if there were other differences between Hb Hasharon and the majority of other unstable hemoglobins. An attempt was made to separate young and old red cells, and hemoglobin synthesis was studied in vitro.

Repeated efforts did not detect a difference in content of Hb Hasharon between "young" and "old" cells separated by centrifugation. Further experiments indicated that those results must be interpreted with caution (Table III). Centrifugation did increase the proportion of reticulocytes in the "top layer" of blood from carriers of Hb Hasharon, but a greater degree of enrichment was found with A-S and A-C cells, and *little or none was found with red cells containing Hb Zürich*. These findings suggest that, if abnormal hemoglobin is removed from cells, its removal may lower the specific gravity of old cells enough to interfere with their separation by centrifugation. Flotation of reticulocytes in carriers of Hb Hasharon, but not Zürich, may reflect more significant denaturation in the latter disorder. Enrichment of reticulocytes, without enrichment of old cells, is known to occur in other situations (47, 48). An increased proportion of Hb S was found in "old" red cells from one patient with sickle-cell trait. That finding was not true of other patients, and was not pursued further.

Studies of hemoglobin synthesis in vitro were initially carried out with reticulocytes obtained from carriers with nearly normal reticulocyte counts. In three studies the specific activity of Hb Hasharon was less than that of Hb A. Similar results were obtained using reticulo-

cytes from hematologically normal carriers of hemo-globins S or C, suggesting that we were observing synthesis by a nearly exhausted manufactory.

Similar results have not been obtained by all other investigators. In some studies, reticulocytes were obtained from patients with reticulocytosis produced by bleeding, or by treatment of iron or folate deficiency. The latter conditions are known to alter synthesis of abnormal hemoglobins (49, 50), and young reticulocytes produced in response to hemorrhage cannot be compared with more mature forms from nonanemic donors (16). The studies of Heywood, Karon, and Weissman, and of Boyer, Hathaway and Garrick can be compared with the data reported here (51, 16). Specific activities of hemoglobins A and S were found to be equal in the former study, while Boyer and coworkers found a higher specific activity in the normal component. That finding was attributed to instability of the abnormal messenger RNA, for a progressive decrease in Hb S synthesis was observed when marrow cells, young reticulocytes, and old reticulocytes were compared (16).

When in vitro synthesis by bone marrow of heterozygous carriers was investigated, the specific activity of Hb S, C, or Hasharon was equal to that of Hb A, even after very brief periods of time (Fig. 6). Few studies of hemoglobin synthesis by marrow cells are available for comparison, but studies carried out in vivo might be expected to yield similar data. Ranney and Kono found the specific activity of normal and abnormal hemoglobins to be equal, after administration of ^{59}Fe to persons with Hb S or Hb C trait (52). In contrast, Levere and Lichtman found unequal specific activities of hemoglobins A and S in persons with sickle-cell trait. They suggested that the two hemoglobins might be "turned over" at different rates, due to heterogeneous distribution between cells (53). A similar explanation has been suggested for the unequal specific activities of Hb S and F found in patients with sickle-cell anemia (20–22, 54, 55). The anticipated effects of a heterogeneous distribution are outlined in the Appendix.

Our in vitro experiments suggest that the proportion of isotope incorporated into Hb Hasharon was the same as the proportion of that hemoglobin in the nonsynthesizing red cells which contaminated the marrow aspirate, about 18%, rather than the 20–27% of ^{59}Fe incorporated in vivo. Discrepancy between the in vivo and in vitro studies could be explained if some newly synthesized Hasharon globin or hemoglobin precipitated in the latter experiments during the preparation of hemolysates for chromatography, despite precautions taken to avoid denaturation. It is unlikely that denaturation of hemoglobin occurred during the incubation per se, for specific activities of Hb A and Hasharon were equal after even very brief periods of incubation (Fig. 6). Exchange of

hemes (56) or globin chains,[4] may have introduced uncontrolled artefacts in one or in both studies. The difference between the studies is small, however, and it seems most likely that both in vivo denaturation and decreased synthesis are responsible for the low proportion of abnormal hemoglobin in hemolysates, with the latter mechanism predominant.

It should be noted that, if an appreciable proportion of newly formed $\alpha^{Hasharon}$ chains precipitated before their incorporation into hemoglobin, they would not be detected in our experiments. Synthesis of the abnormal globin could then be considered "ineffective," rather than decreased. If 50% of the nascent chains precipitated, synthesis of $\alpha^{Hasharon}$ subunits would be equal to that of many other abnormal hemoglobins. Excess β-chains would not be likely to accumulate, if preformed α-chains are a prerequisite for their release from ribosomes (19). It is expected that normoblasts in the bone marrow would contain inclusions composed of the precipitated chains; in our studies, appropriate stains were not utilized to rule out that possibility. We would expect red cell morphology to be abnormal (38, 61, 62); it was not.

The normal appearance of erythrocytes, the relatively poor yield of reticulocytes in flotation experiments, and the mildness of hemolytic anemia in carriers are consistent with a relatively small amount of denaturation of Hb Hasharon in circulating red cells. As outlined in the Appendix, mean red cell life span is inversely related to the proportion of an unstable hemoglobin synthesized, and it may be of some evolutionary advantage for an unstable hemoglobin to be synthesized in smaller amounts than are the more stable proteins. A similar suggestion has been advanced by Beretta, Prato, Gallo, and Lehmann, in discussing the very low proportion of Hb Torino (14).

Abnormal hemoglobins involving mutations at the α-47 locus have been described in widely divergent ethnic groups; all of these hemoglobins appear to have certain properties in common. In hemoglobins Kokura, Umi, and L$_{Ferrara}$, residue α-47 is glycine rather than the histidine found in Hb Hasharon (57, 58). The former hemoglobins are also found in low proportion, and carriers have mild reticulocytosis (Table IV). Hemoglobin Columbia also has the formula $\alpha\alpha^{47\ Gly}\beta_2$, and it displays heat precipitability very similar to that observed with Hb Hasharon.[5] The molecular alterations involved in heat precipitation are unclear, but they may involve dissociation of the hemoglobin molecule, with precipitation of relatively unstable free globin chains (64). The process is quite sensitive to changes in pH, and cyanmethemoglobin derivatives are very much more resistant to denaturation than are oxyhemoglobins. Residue α-47, which corresponds to position CD5 in the Perutz model of

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[5] Nagel, R. L. Personal communication.

TABLE IV

Abnormal Hemoglobins Produced by Mutations at α^{47} (CD5)

Hemoglobin	Reference	Hct. value	Reticu- locyte count	Proportion in hetero- zygotes
				%
Hasharon	—	37–51	1–4	16–19
	(37)*	38–55	2–4	14–18
	(26)	No anemia	—	low
Kokura	(57)	37	3	15–17
Umi	(57)	34	3	14–16
L Ferrara	(58)	(76–88)‡	—	22–38

* Alperin, J. B., and R. G. Schneider. Personal communication.
‡ Hemoglobin % Sahli.

hemoglobin (59), is on the external surface of the molecule,[6] and it would be expected to have interactions with other hemoglobin molecules, as well as with solvent molecules. No convincing theory relating the charge difference of Hb Hasharon at that position to the properties of the molecule can be advanced at present. Hemoglobins Hammersmith and Torino, which are quite heat-labile, involve mutations at position CD1; this is an important point of heme contact, and the mechanisms producing instability are probably quite different from that which exists in Hb Hasharon (60).

The data presented here are compatible with the hypothesis that the low proportion of Hb Hasharon in hemolysates is produced by a combination of decreased synthesis and increased destruction. Synthesis is not as low as that of Hb A₂, and destruction is not as rapid as that seen with more unstable hemoglobins. It is probable that, if Hb Hasharon were synthesized in greater amounts, the red cell lesions produced by the increased amount of denatured hemoglobin might shorten red cell survival even further, and would produce overt hemolytic anemia.

APPENDIX

The two types of hemoglobin contained in the red cells of any heterozygote are called X, the abnormal hemoglobin, and Y, the normal hemoglobin. Generally, they are synthesized at different rates. If the fraction of X in the newly formed cell is α, the ratio of X to Y is $\alpha(1 - \alpha)$. Initially, α may vary from cell to cell according to $f(\alpha)$, to which the usual properties of a probability density function are assigned. For simplicity, we can let the distribution be Gaussian, centered at $\bar{\alpha}$ and with a variance of σ^2. If the cells of a heterozygote show "uniform distribution" of X, σ becomes zero (i.e., $\alpha = \bar{\alpha}$ for all cells).

If X is an unstable hemoglobin, the life span of the cells under discussion generally is shortened. During their life span, X becomes denatured. Denaturation can be approximated by an exponential function, defined for the entire life span of a

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[6] Perutz, M. F. Personal communication.

red cell once it has appeared in peripheral blood. Denaturation of X may be assumed to be continuous in each cell, proceeding at a rate proportional to the amount of nondenatured X in the cell. Furthermore, it is assumed that:

(a) A radioactive "pulse" label incorporated into normal and abnormal hemoglobins is neither exchanged between molecules during the cell's life nor reincorporated after destruction of the original cohort.

(b) Denatured abnormal hemoglobin is either removed from the cell or is altered in such a way that it does not appear in hemolysates. Coprecipitation of the normal hemoglobin is negligible during the life of the cell.

(c) Red cells are destroyed as soon as a "lethal" amount of X has denatured. Destruction may be due to altered physical properties of the cell, functional lesions of its membrane, or some combination of these factors (38, 61, 62).

Applications of the model to studies of hemoglobin synthesis proceed immediately from a formal statement. If a labeled cohort appears in the peripheral blood at $t = 0$, then at any time t, the amount of remaining abnormal hemoglobin in a cell that initially contained X in the proportion α, is

$$X(t) = h\alpha e^{-t/\tau} \qquad [1]$$

where τ is the time constant of denaturation and h is the total initial amount (in $\mu\mu g$) of hemoglobin in a cell. (h is assumed to be a constant.) The cell described by equation 1 obviously contained initially an amount $h\alpha$ of the component X, and it will be destroyed at that time t at which the amount of denatured X has reached a "lethal" level k:

$$h\alpha(1 - e^{-t/\tau}) = k \qquad [2\,a]$$

In order to express the fact that α in equation 2 a is no longer an independent variable we restate this relation as follows: at any time t those cells are being destroyed which initially contained α_k such that

$$h\alpha_k(1 - e^{-t/\tau}) = k \qquad [2\,b]$$

Conversely, at time t those cells are still circulating whose initial α was in the range of

$$0 \leqslant \alpha < \alpha_k$$

where, from equation 2 b

$$\alpha_k = \frac{k}{h(1 - e^{-t/\tau})} \qquad [3]$$

Hence, the total number $N(t)$ of labeled cells circulating is

$$N(t) = N_0 \int_0^{\alpha_k} f(\alpha) d\alpha = \int_0^{k/h(1-e^{-t/\tau})} f(\alpha) d\alpha \qquad [4]$$

where N_0 is the original number of cells in the cohort, and the total amount of X in the labeled cohort becomes

$$[X(t)]_{tot} = h e^{-t/\tau} \int_0^{\alpha_k} \alpha f(\alpha) d\alpha \qquad [5]$$

The total radioactivity of X at any time is proportional to equation 5.

The level of total circulating hemoglobin (unlabeled and labeled) can be found by averaging all $[X(t)]_{tot}$'s of equation 5 from zero time to the longest possible life span:

$$[\bar{X}] = \frac{h}{T_{max}} \int_0^{T_{max}} e^{-t/\tau} \int_0^{\alpha_k} \alpha f(\alpha) d\alpha \, dt \qquad [6]$$

T_{max}, the maximum life span, is obtained from

$$h\alpha_{min}(1 - e^{-T_{max}/\tau}) = k \qquad [7]$$

where $h\alpha_{min}$ is the smallest amount of X synthesized in any of the cells. Equation 7 assumes that $k/h < \alpha_{min}$, i.e., that there

are no cells that initially contain so small an amount of X that they are never destroyed by the process described here. This appears to be a reasonable assumption since the few known $f(\alpha)$'s show quite small standard deviations (54).

Specific activity of a labeled cohort on day t is proportional to:

$$\frac{e^{-t/\tau}\int_0^{\alpha_k}\alpha f(\alpha)d\alpha}{\frac{1}{T_{max}}\int_0^{T_{max}}e^{-t/\tau}\int_0^{\alpha_k}\alpha f(\alpha)d\alpha} \qquad [8]$$

This equation predicts that specific activity of an unstable hemoglobin (X) would initially be higher than that of hemoglobin Y, for the radioactive label would be mostly in young cells, which contain a higher proportion of X than does the hemolysate that is prepared from cells of all ages from 0 to T_{max}.

Detailed descriptions of the time course of specific activity can be obtained most easily by studying cells containing initially equal amounts $(h\bar{\alpha})$ of X (or cells for which $f(\alpha)$ has a very small σ). Then we have in each and every cell:

$$X(t) = h\bar{\alpha}e^{-t/\tau} \qquad [9]$$

such that the average amount of abnormal hemoglobin in all cells is

$$\bar{X} = \frac{h}{T}\bar{\alpha}\int_0^{\bar{T}}e^{-t/\tau}\,dt \qquad [10]$$

where $\bar{T}$ is the life span of these cells given by

$$\bar{\alpha}h(1 - e^{-\bar{T}/\tau}) = k \quad \text{or} \quad \bar{T} = \tau\ln\left[\frac{1}{1 - (k/h\bar{\alpha})}\right] \quad [11]$$

Hence

$$\bar{X} = \frac{-k}{\ln[1 - (k/h\bar{\alpha})]} \qquad [12]$$

With equations 9 and 12 specific activity is found as

$$SA = \frac{\ln[1 - (k/h\bar{\alpha})]}{-(k/h\bar{\alpha})}e^{-t/\tau}\ldots\text{for} \quad 0 < t < T_{max}$$
and
$$= 0 \qquad\qquad \ldots\text{for} \quad T_{max} \leqslant t \qquad [13\,a]$$

Note that further algebraic manipulations of equations 11 and 13 a yield an expression for the SA independent of τ:

$$SA = \frac{h\bar{\alpha}}{k}\ln\left[1 - \frac{k}{h\bar{\alpha}}\right]\left(1 - \frac{k}{h\bar{\alpha}}\right)^{t/\bar{T}}\ldots 0 < t < T_{max} \quad [13\,b]$$
$$= 0 \qquad\qquad \ldots T_{max} \leqslant t$$

Several examples of this theoretical prediction of specific activity are shown in Fig. 7 where SA is plotted vs. time (as measured in time constants τ of denaturation). Unity on the ordinate is the SA that would be observed if all of a labeled hemoglobin fraction appeared in the hemolysate. This is the specific activity of normal hemoglobin, which does experimentally appear to be constant with time (Fig. 5). Specific activity of the unstable component X is initially higher, as predicted in equation 8. The model also accounts for decay of radioactivity, so that eventually SA of X becomes less than that of Y.

The "lethal" level (k) could conceivably be the same for all hemolytic anemias in which red cell destruction is produced by denaturation of hemoglobin. If an unstable hemoglobin is uniformly distributed ($\sigma = 0$), and if the proportion in hemolysates ($\bar{X}/h$) and the proportion synthesized ($\bar{\alpha}$) are known, k can be estimated from equation 12. Using data collected in vivo from carriers of unstable hemoglobins, there is some support for that hypothesis (Table V). Data collected from in vitro experiments yield values of k that are quite different, but most of those experiments were carried out with reticulocytes. The one study carried out with bone marrow cells yielded data similar to those obtained in vivo.

The value of k could be predicted from equation 13 a if
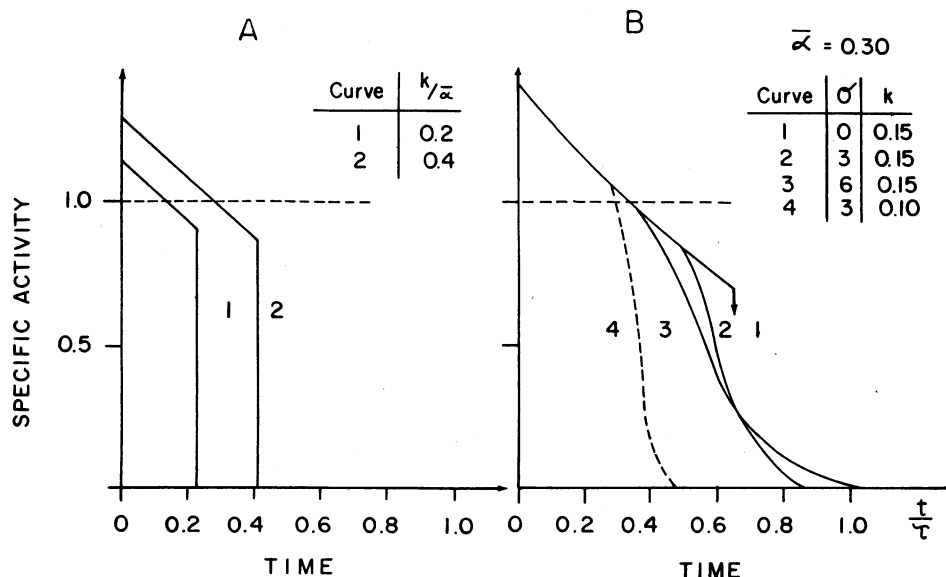

FIGURE 7 (A) Specific activity of a "uniformly distributed" unstable hemoglobin. (B) Specific activity of a "nonuniformly distributed" unstable hemoglobin. Day of peak radioactivity in blood = day 1; time expressed in units of the time constant of denaturation. Specific activity of normal hemoglobin from the same person = 1. See text.

TABLE V

Estimates of the "Lethal Amount" (k) of Denatured Hemoglobin

Hemoglobin	Reference	h	$\bar{X}$	$h\bar{X}$	$\bar{\alpha}$	k
		μμg		μμg		μμg
In vivo studies						
H	(23)	17	0.20	3.4	0.28	2.4
Zürich	(24)	29	0.26	7.5	0.31	2.8
Gun Hill	(43)	28	0.30	10.2	0.53	8.0
Hasharon		30	0.18	5.4	0.23	2.7
In vitro studies						
H*	(23)	17	0.14	2.4	0.40	6.3
Zürich	(24)	29	0.26	7.5	0.58	14.2
Gun Hill	(43)	28	0.30	10.2	0.65	13.2
Hammersmith	(44)	35	0.30	10.5	0.47	10.2

*Bone marrow cells.

initial and final specific activity were known. The ratio of these two measurements depends directly on the initial concentration ($\bar{\alpha}$) of X in the cell, and on k:

$$\frac{SA(t = 0)}{SA(t = T - \epsilon)} = 1 - \frac{k}{\alpha} \quad \text{(where } \epsilon = \text{a short time)} \quad [14]$$

Data of this type are not available for Hb Hasharon, and k cannot be estimated from equation 14.

In theory (Fig. 7 A), the time constant of denaturation (τ) may be quite long, whereas the life span of the red cell may be significantly less than 120 days. If small amounts of denatured X can lead to cell destruction, the critical level k is reached long before the initial concentration of X has decreased significantly. (This fact also causes the decay curve to appear almost linear.) Specific activity of X may appear to be "unexpectedly" large during the 1st 30–50 days, especially if the life span of the cells is close to 120 days. Curve 2 of Fig. 7 A represents such a case, in which $\bar{\alpha} = 30\%$, $k = 12\%$ were assumed.

Conflicting evidence has been presented regarding the distribution of abnormal hemoglobins between cells (53, 63). Data for fetal hemoglobin are most satisfactory: it is nonuniformly distributed in thalassemia and sickle-cell anemia, but distribution is uniform in hereditary persistence of fetal hemoglobin (20, 21, 54, 55). Some of the considerations given above for an infinitely narrow $f(\alpha)$ take on a more complex nature if the unstable hemoglobin is distributed more broadly over all cells ($\sigma \neq 0$). The phenomena encountered, in addition to those above, are illustrated in Fig. 7 B. Those cells that contain the most X($\alpha > \bar{\alpha}$) are destroyed before the majority, so that radioactivity begins to deviate from the exponential decay curve at times considerably less than the average life $\bar{T}$. Two of the introduced quantities determine how soon that deviation may occur: the spread of the distribution curve (σ), and the lethal level k. If σ is large, an early deviation of specific activity from exponential decay would be easily detectable. If σ is not large, its experimental determination during a short period of observation would be difficult if data were scattered. In the case of Hb Hasharon, observation over a period of at least 100 days would be necessary to estimate the distribution of the abnormal hemoglobin. Such a study could not be performed, which was doubly unfortunate, for it might have resolved differences between the in vivo studies that *were* carried out and the in vitro bone marrow studies.

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