Influence of Hemoglobin Precipitation on Erythrocyte Metabolism in Alpha and Beta Thalassemia

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ABSTRACT Certain aspects of the metabolism of centrifuged young and old erythrocytes in hemoglobin H disease have been examined and compared with similar studies of beta thalassemia and normal cells. Glycolysis, hexose monophosphate shunt activity (HMPS), potassium flux, and glutathione (GSH) content were measured. The distributions of hemoglobins H and F, as well as the activities of erythrocyte glucose-6-phosphate dehydrogenase (G6PD) and glutamic oxalacetic transaminase (GOT), were utilized for estimations of the relative ages of the cell samples. The young erythrocytes in hemoglobin H disease differed in several respects from older hemoglobin H cells. They contained more soluble hemoglobin H and GSH and, after splenectomy, fewer inclusions. HMPS activity was subnormal in hemoglobin H young cells and rose to normal activity in old cells. Potassium flux tended to increase in old cells when inclusions were present.

Beta thalassemia young cells contained less hemoglobin F and, after splenectomy, more inclusions than old cells. In addition, they had markedly increased glycolysis and HMPS activity. GSH was randomly distributed. Potassium flux was increased in younger cells and particularly increased when inclusions appeared in younger cells after splenectomy.

The results are interpreted to indicate that inclusion formation is associated with increased erythrocyte cation permeability in the thalassemia syndromes. This is not related to the level of intracellular GSH.

The decreased HMPS activity in young hemoglobin H cells may be due to the presence of the extra thiols of soluble hemoglobin H which can act as a reducing agent.

The substitution of hemoglobin H for glutathione in this capacity would then spare the NADPH-requiring glutathione reductase system. As a consequence, HMPS activity would decline. However, in older cells the oxidized hemoglobin H precipitates; these must rely upon GSH and glutathione reductase activity for thiol reduction capacity. Accordingly, HMPS activity increases to normal in the old cell population.

INTRODUCTION

The erythrocytes of patients with beta thalassemia and hemoglobin H disease can be separated by centrifugation into young and old cohorts (1-4). In beta thalassemia the young cells contain less hemoglobin F (2, 3) and, if the patient is splenectomized, more insoluble membrane-bound inclusions of precipitated alpha chains (4-7). In hemoglobin H disease, the young cells contain more soluble hemoglobin H, whereas inclusions of precipitated beta chains are detected in the older cells (1, 4, 8, 9). To examine some of the metabolic consequences of these hemoglobin inclusions, we evaluated the characteristics of the centrifuged cell cohorts with respect to glycolysis, HMPS activity, lactate production, GSH concentration, G6PD activity and potassium flux. These data were compared to similar studies of centrifuged normal cells. Since the inclusions were localized in cohorts of different ages in the two forms of thalassemia, a unique opportunity was provided for study of their age-dependent influences. The data confirmed that the development of hemoglobin precipitates in thalassemic erythrocytes is associated with alterations of membrane cation permeability characterized by enhanced potas-

Received for publication 9 February 1968 and in revised form 13 August 1968.

sium flux (4). These changes seem to be unrelated to cellular GSH (10).

A second purpose of this study was to evaluate the influence of soluble hemoglobin H on the HMPS in erythrocytes. A major role of the HMPS in red cells is to provide NADPH for glutathione reductase, an enzyme activity which maintains a renewable pool of reduced glutathione. The latter serves as an oxidant buffer for the thiol groups of red cell hemoglobin and membrane protein (11-13). When hemoglobin A is exposed to an oxidant strong enough to cause its denaturation and precipitation in vitro, glutathione is bound, presumably as a disulfide, to the precipitating protein (11). GSH may also be bound to certain abnormal hemoglobins which precipitate in vivo during the life span of the red cell (1, 10, 14). Among these abnormal hemoglobins is hemoglobin H (1, 8, 9), a tetramer of beta chains with eight rather than two exposed thiols per molecule (15). Recent studies have indicated that hemoglobin H may have a functional redox potential very close to that of glutathione (14). Therefore, it might substitute for glutathione and serve as an oxidant buffer for the cell until oxidative precipitation of this hemoglobin occurred. Oxidative precipitation of hemoglobin H is irreversible and the precipitates cannot be reduced by an NADPH-linked enzyme system. If hemoglobin H can act as a reducing agent, one might expect HMPS activity to be decreased in cells containing soluble hemoglobin H and to increase to normal when the hemoglobin H precipitates, because the NADPH-dependent glutathione reductase system would then be left to perform this essential buffering function. In these studies, it was found that in hemoglobin H disease, HMPS activity was indeed lower in the young cells, where soluble hemoglobin H predominated, than in the old cells, where inclusions predominated.

METHODS

Studies were performed on blood from five patients with hemoglobin H disease, six patients with Cooley's anemia, two patients with S-thalassemia and a group of eight normal controls. Percentages of hemoglobins A and H were determined by starch granule electrophoresis (16). None of the patients had received blood transfusions in recent years.

Venous blood was collected with preservative-free heparin ¹ (0.1 mg/ml blood) and studied immediately. The blood was separated into layers of young and old cells by centrifugation of 15 ml of whole blood in celluloid tubes at 20,000 g for 1 hr. After this procedure, the relatively younger large cells are found at the top of the tube, and the older smaller cells segregate at the bottom of the tube. The buffy coat and unavoidably the top 1% or 2% of erythrocytes were removed by aspiration. The remaining top (reticulocyte-rich or young) and bottom (reticulocyte-poor or old) 1.5-ml fractions were then collected and resuspended at hematocrits of approximately 40% in the autologous plasma. Thus, the

top and bottom fractions each contained approximately 20% of the initial volume of cells.

White blood cell counts, reticulocyte counts, inclusion body counts and measurement of red cell indices were performed on all fractions by standard methods (17). Inspection of reticulocyte smears revealed virtually absent platelets. The ratio of leukocytes to red cells was less than 2×10^{-4} in top layer cell suspensions and less than 10^{-4} in bottom layer suspensions.

Minimum HMPS activity by evolution of $^{14}\text{CO}_2$ from glucose-1- ^{14}C was studied in duplicate by modifications of the method of Murphy (18). After incubation in the presence of 1 μC of glucose-1- ^{14}C , a small piece of filter paper, which had been suspended from the inner edge of the serum cap of the incubation flasks before incubation, was soaked with KOH. The blood was acidified with 0.1 ml of 0.5 N H_2SO_4 . The acid and alkali additions were made with syringes and hypodermic needles. The filter papers and appropriate standards applied to filter paper were dried and counted for ^{14}C activity in a liquid scintillation spectrometer (19). The diffusion system for $^{14}\text{CO}_2$ was evaluated with $\text{Na}_2^{14}\text{CO}_3$ standards and found to be quantitative. Blanks were performed by acidification and diffusion immediately after the addition of glucose-1- ^{14}C to the incubate. Murphy's method of calculation was utilized to derive a value for shunt activity in terms of mm glucose/liter cells per hr.

The effects of stimulation of HMPS activity by glucose and methylene blue were measured by the methemoglobin reduction technique of Beutler and Baluda (20). Methemoglobin and hemoglobin were assessed by the technique of Evelyn and Malloy (21). The total hemoglobin in the hemolyzates remained constant even in samples containing hemoglobin H. Failure to recognize possible precipitation of hemoglobin H by methylene blue might have been because the molar hemoglobin to dye ratio was approximately 300 rather than the ratios approaching unity that have been utilized in other studies of hemoglobin H precipitation by such dyes (6). Because it is difficult to remove all nitrite from the cells (22), this technique indicates the *net rate of methemoglobin reduction* rather than absolute reduction rates. Therefore, only the periods of linear methemoglobin reduction were considered in the calculations of comparative reduction rates.

Glucose consumption and lactate production during incubation were measured on perchloric acid filtrates by the glucose oxidase (23) and the LDH:NADH (24) methods respectively.

GSH concentrations in top and bottom cell suspensions were measured by the method of Beutler, Duron, and Kelly (25). G6PD and GOT activities in hemolyzates were respectively measured by suitable modifications of the technique of Zinkham and Lenhard (26) and by the method of Karmen, Wróblewski, and LaDue (27).

Potassium fluxes were measured by micromodifications of the methods of Solomon (28).

The top and bottom layer cells were of different size distribution, the top cells having a larger mean volume than the bottom. To develop meaningful comparisons between the layers the measurements described above (with the exception of G6PD and GOT activities which were expressed as changes of optical density at $340 \mu/10^{10}$ erythrocytes), were modified in terms of a standard cell volume of $82 \mu^3$. For example, the measured value for glucose consumption per liter of bottom layer cells was multiplied by the ratio of (MCV bottom layer cells)/(MCV standard cells) in which MCV stands for mean corpuscular volume. The corrections were not entirely suitable for statistical comparison.

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sons of normal cells to the cells of beta thalassemia. Size distribution studies showed that the cells of splenectomized beta thalassemia patients were not merely smaller than normal cells. The variances of their mean volumes were considerably greater than those of normal cells so that simple statistical comparisons on the basis of mean volume were of limited significance.² On the other hand, the variances of the mean volume of beta thalassemia and hemoglobin H disease top and bottom layer cells were not as widely different. Nor were the variances of normal and hemoglobin H cells sufficiently dissimilar to limit statistical comparisons of their metabolic functions on the basis of a correction for cell volume. With these limitations considered, the corrected data were recorded in the tables and figures.

RESULTS

The adequacy with which centrifugation separated cells into young and old populations is illustrated in Table I where the substantial differences in reticulocyte counts and GOT and G6PD activities between top and bottom fractions are tabulated. As expected (1, 2, 4, 29), the young cells were in the top layers and the older cells in the bottom fractions.³

Glucose metabolism in young and old cells. The rates of total glucose consumption, lactate production, glucose oxidation by the HMPS, and reduction of methemoglobin with methylene blue and glucose are shown in Table I.

² The authors are grateful to Dr. Frederick Stohlman, Jr. for performing some of these size distribution studies.

³ As previously noted (4), we have further evaluated the validity of the separation method by administration of ⁵⁹Fe to one adult patient with Cooley's anemia. 5 days after isotope administration nearly all of the radioactivity was localized in the large hypochromic cells of the upper layer which were rich in reticulocytes and poor in hemoglobin F.

As anticipated (30-33), total glucose consumption and lactate production in normal young cells was greater than in normal old cells. In beta thalassemia erythrocytes the same pattern was detected, an expected finding in view of the large number of reticulocytes and nucleated red blood cells in the top layer cells of these patients. However, in four of the five studies of hemoglobin H, glucose consumption and lactate production were greater in old cells than in young cells, despite relatively large numbers of reticulocytes in the top cell layers. HMPS activity in young normal cells, measured either by the ¹⁴CO₂ or by the methemoglobin reduction methods, was greater than HMPS activity in old normal cells (31-33). The same was true to an even greater extent of the cells of patients with beta thalassemia. On the contrary, the HMPS activity of old cells from patients with hemoglobin H disease was equal to or greater than that of young cells from these patients. These differences in the cellular localization of shunt activity are demonstrated in Fig. 1 in which the top and bottom cell ratios for HMPS activity are graphically shown. This figure shows that the differences in shunt activity between young and old cell fractions were easily demonstrable by the glucose-1-¹⁴C technique whereas they were barely if at all discernible by the methemoglobin reduction technique since the latter only measures the potential maximal activity of the shunt based largely upon available glucose, NADP, G6PD, and NADPH:methylene blue reductase activity in the bottom and top layer cells.

G6PD activity and GSH concentrations in young and old cells. The relative youthfulness of all cells from

FIGURE 1 Relative predominance of total HMPS activity in top and bottom cell layers measured in patients with hemoglobin H disease (H), beta thalassemia (β^t), and normals (N).

TABLE I
Metabolic

Patient	Diagnosis*	Sple- nec- tomy	Cell fraction	MCV	Hemo- globin inclu- sions	Hemo- globin H or F	Reticu- locytes	Lactate production	Glucose consumption
				μ^3	%	%	%	mM/liter	cells per hr†
V. J.	H (9%)	Yes	Top	75	1.6	14 H	12.2	10.5	4.6
			Bottom	53	6.6	6.2 H	1.0	12.3	5.3
L. D.	H (8%)	No	Top	75	0	18 H	10.0	8.5	3.4
			Bottom	50	0.1	5 H	2.3	8.6	4.3
M. L.	H (10%)	Yes	Top	84	3.8	15 H	22.6	10.0	4.2
			Bottom	54	12.8	4 H	8.0	12.0	5.2
A. R.	H (15%)	Yes	Top	75	3.4	30 H	16.2	7.0	3.7
			Bottom	53	11.8	8 H	2.3	10.0	4.4
W. D.	H (8%)	No	Top	81	0	12 H	10.8	11.8	4.9
			Bottom	63	0.2	2 H	1.8	8.8	3.6
S. D.	CA	Yes	Top	69	50	7 F	18.6	—	5.0
			Bottom	47	5	32 F	1.0	—	3.8
J. C.	CA	No	Top	65	0	8 F	7.2	—	3.5
			Bottom	51	0	34 F	0.3	—	2.0
C. Z.	CA	Yes	Top	113	45	22 F	30.0	10.6	5.3
			Bottom	71	7	56 F	5.0	4.6	2.7
G. P.	CA	No	Top	75	0	20 F	15.0	—	4.5
			Bottom	64	0	40 F	4.0	—	1.3
P. M.	CA	Yes	Top	89.5	38	22 F	28.0	—	—
			Bottom	74	3	37 F	1.4	—	—
P. F.	CA	Yes	Top	102	47	30 F	25.0	—	—
			Bottom	68	5	44 F	1.2	—	—
S. G.	S-T	Yes	Top	95	15	13 F	14.4	12.1	6.1
			Bottom	75	2	25 F	0.9	—	3.4
A. G. R.	S-T	No	Top	75	0	7 F	10.0	—	5.0
			Bottom	63	0	13 F	2.0	—	3.0
Normal controls (8)		No	Top	91 $\pm$ 3.0§	0		3.1 $\pm$ 0.8§	5.8 $\pm$ 0.7§	3.1 $\pm$ 0.5§
			Bottom	82 $\pm$ 3.9	0		0.1 $\pm$ 0.1	2.8 $\pm$ 0.4	1.5 $\pm$ 0.3

* H, hemoglobin H; CA, Cooley's anemia; S-T, S-thalassemia

† Corrected to a standard cell volume of 82 μ^3 .§ Mean $\pm$ SD of eight separate controls.

|| Range of three determinations.

patients with hemoglobin H disease and beta thalassemia is shown by their high G6PD and GOT activities compared with those of normal cells (Table I and II). The G6PD and GOT activities of young cells from all subjects were greater than those of old cells.

As described previously (14), the GSH content of normal old cells was only slightly less than that of

young cells; but the mean GSH concentration of old hemoglobin H erythrocytes was only 47.8 $\pm$ 7.5 mg/100 ml cells, significantly less GSH than was measured in normal old cells ($P < 0.01$) and 32% less GSH than was found in young hemoglobin H cells ($P < 0.05$).

The distribution of glutathione in young and old cells in the beta thalassemia patients was quite widely scat-

Hexose monophosphate shunt activity		Enzyme activity			Potassium flux		
Consumption of ^{14}C -1-glucose	Methemoglobin reduction by nitrated erythrocytes with methylene blue	G6PD	GOT	Glutathione	in	out	net
<i>mM/liter cells per hr†</i>	<i>% methemoglobin reduced/min†</i>	<i>units/10¹⁰ cells</i>		<i>mg/100 ml cells‡</i>	<i>mEq/liter cells per hr‡</i>		
0.21	1.46	3.32	7.32	65.3	3.4	4.1	-0.7
0.43	1.84	2.21	2.28	45.9	3.6	4.4	-0.8
0.05	3.15	1.74	—	61.9	2.3	2.9	-0.6
0.18	2.83	1.10	—	36.0	2.1	2.3	-0.2
0.19	2.06	3.66	—	71.2	3.2	3.4	-0.2
0.18	2.33	2.16	—	47.0	3.6	3.8	-0.2
0.15	1.70	4.09	—	82.4	2.5	2.7	-0.2
0.18	1.80	2.54	—	58.9	3.5	3.2	+0.3
0.19	1.25	3.80	3.97	70.4	3.5	3.4	+0.1
0.24	1.25	1.62	0.86	51.4	2.2	2.1	+0.1
1.09	2.90	2.30	—	52.4	7.5	8.1	-0.6
0.96	2.30	1.74	—	60.7	2.9	3.4	-0.5
0.25	1.50	3.56	—	54.4	5.5	5.8	-0.3
0.10	1.00	1.82	—	65.9	2.7	2.5	+0.2
0.55	—	—	—	—	12.7	13.9	-1.2
0.13	—	—	—	—	5.4	4.4	+1.0
—	—	—	—	—	8.3	9.0	-0.7
—	—	—	—	—	5.1	5.5	-0.4
—	—	—	8.3	90.0	—	—	—
—	—	—	3.1	60.0	—	—	—
—	—	—	12.0	85.0	—	—	—
—	—	—	4.0	60.0	—	—	—
0.35	2.16	7.12	—	65.0	8.5	8.7	-0.2
0.17	2.43	4.51	—	56.6	3.2	2.6	+0.6
0.42	—	—	—	66.3	2.9	3.0	-0.1
0.15	—	—	—	58.9	1.8	2.0	-0.2
0.27 ± 0.14§	0.90 ± 0.18§	2.32 ± 0.50§	2.1-3.1	87 ± 5.0§	1.8 ± 0.2§	1.7 ± 0.2§	0.1 ± 0.01§
0.17 ± 0.07	0.75 ± 0.25	1.47 ± 0.50	0.6-0.9	77 ± 7.0	1.9 ± 0.2	1.7 ± 0.2	0.2 ± 0.1

tered and exceeded the range observed in the cells of normal individuals. The mean value was not distinguishable from that of normal. Certainly the uniform reduction of the GSH concentration of old cells in hemoglobin H disease was not regularly observed in the beta thalassemia patients. These data are graphically presented in Fig. 2.

Relationship between cellular GSH, G6PD activity and HMPS activity with special reference to hemoglobin H disease. Fig. 3 reveals that the distribution of glutathione among top and bottom (young and old) cells was strongly associated with the relative rate of shunt activity, the shunt activity being higher as glutathione declined. However, Table II shows that the

TABLE II
Hemoglobin H Disease, Relationship of HMPS Activity to Cell Age and G6PD Activity

	Hemoglobin H disease		Normal	
	Top	Bottom	Top	Bottom
HMPS activity, mM/liter cells per hr	0.16 $\pm$ 0.06 $P = 0.02-0.01$	0.24 $\pm$ 0.096	0.27 $\pm$ 0.14 $P = 0.1-0.05$	0.17 $\pm$ 0.07
G6PD units	3.32 $\pm$ 0.82 $P < 0.001$	1.92 $\pm$ 0.5	2.32 $\pm$ 0.40 $P = 0.02-0.01$	1.47 $\pm$ 0.50
Ratio of $\frac{\text{HMPS}}{\text{G6PD}}$	$P < 0.001$ $P = 0.02-0.01$			
	0.049 $\pm$ 0.03	0.129 $\pm$ 0.04	0.116 $\pm$ 0.037	0.115 $\pm$ 0.046

influence of glutathione content on shunt activity may be quite indirect at least in hemoglobin H disease. This table compares shunt activity and G6PD activity in top and bottom cell layers in hemoglobin H disease and in normals. It is notable that the utilization of glucose via the hexose monophosphate shunt was absolutely lower in hemoglobin H disease top layer cells than in normal top layer cells. When an attempt was made to normalize the potential shunt activity of normal and hemoglobin H top layer cells by construction of a ratio of shunt activity to G6PD activity, the difference between the hemoglobin H and normal top layer cells was even more marked. On the other hand, bottom layer (old) hemoglobin H cells had a somewhat higher shunt activity than bottom layer normal cells. When normalized to a constant G6PD activity, the shunt rates in bottom layer hemoglobin H cells and normal bottom layer cells

were the same. Thus, it would appear that the falling GSH concentration in hemoglobin H cells was associated with an increase of HMPS activity from below normal toward normal rather than an increase to levels above normal. Table I shows that the decline of glutathione in aging hemoglobin H cells was also associated with a reduction in soluble hemoglobin H, a fact expected from the previous investigations of Rigas and Koler (1).

Potassium flux in young and old cells. There were no detectable differences in potassium flux among top and bottom cell layers of normal erythrocytes. In patients with either beta thalassemia or hemoglobin H disease who were not splenectomized and whose red blood cells did not contain inclusions, the young red cells had a higher rate of flux than bottom layer cells (Fig. 4). This result was expected in view of the predominance of young cells in the upper layers (34). Splenectomized patients with the two forms of thalassemia had definite differences in red cell potassium fluxes. In splenectomized patients with beta thalassemia, potassium flux was considerably higher in the young cells in which inclusions were present. In hemoglobin H disease, the fluxes tended to be equal in top and bottom layer cells with a slight predominance in bottom layer cells where inclusions were present.

DISCUSSION

The role of unbalanced hemoglobin synthesis and hemoglobin subunit precipitation in the erythrocyte destruction which characterizes thalassemia has attracted increasing attention. In hemoglobin H disease, the β_4 tetramer slowly precipitates from the cell during its life span (1) although some precipitates may also be found in the bone marrow (35). In homozygous beta thalassemia very large precipitates are easily detected in many marrow cells (5). These either are evulsed from the cells by the spleen or the cells containing them

FIGURE 2 Relative predominance of glutathione concentration in top and bottom cell layers.

FIGURE 3 Linear relation of GSH concentration to total HMPS activity in top and bottom cell layers. The symbol N represents the mean of eight determinations in normal subjects.

are destroyed in the spleen within hours after they are newly delivered to the circulation (4). Thus inclusions are found in young cells in beta thalassemia and old cells in hemoglobin H disease. These differences in precipitate localization contribute to the differences in severity of the two syndromes. In hemoglobin H disease, there is less destruction of developing erythroblasts within the marrow, and the rate of removal of newly formed peripheral blood cells, though increased above normal, is not extreme (36). In beta thalassemia, however, massive destruction of newly formed cells in the marrow (38) as well as in the peripheral blood (3) constitutes one of the major clinical problems in the management of this disorder.

The pathophysiological consequences of unbalanced hemoglobin synthesis in thalassemia also provide a mechanism for evaluation of age-dependent separations of thalassemic erythrocytes. In beta thalassemia hemoglobin F has a longer life span than hemoglobin A (3). Hence, any population of thalassemic erythrocytes which contains a higher hemoglobin F concentration than that of another sample of cells from the same individual must have an older mean age. Advantage was taken of this fact by Loukopoulos and Fessas (2) in their studies of the nature of hemoglobin distribution in beta thalassemia. It has been further substantiated by the present studies of the distributions of these hemoglobins and of G6PD and GOT activities in centrifuged beta thalassemic blood samples. In hemoglobin H disease, the β_4 tetramer has a higher rate of turnover than that of hemoglobin A (37), as was predicted from the studies of Rigas and Koler (1). For this reason a sample of cells containing more soluble hemoglobin H than that of another sample from the same individual must be a younger cell population. This fact was also confirmed by the present hemoglobin and enzyme distribution studies.

Glutathione may be bound to hemoglobin as it precipitates in red cells (1). Since it has been proposed that the level of glutathione may influence to some extent the activity of the hexose monophosphate shunt (39), it was of interest to examine the relationship of HMPS activity to cell age and glutathione concentration in the normal individuals and in the two forms of thalassemia. In fact, when ratios were established be-

FIGURE 4 Relative predominance of K⁺ efflux in top and bottom cell layers in splenectomized and nonsplenectomized patients with hemoglobin H disease and beta thalassemia.

tween the top and bottom cell layers, the data suggested that HMPS activity is inversely related to the level of glutathione in the cells, but the relationship may be coincidental and not causal. The more interesting fact was that the level of HMPS activity was actually low in young cells in hemoglobin H disease. The activity rose to normal as the cells aged and glutathione concentration fell. An explanation for this phenomenon which appears more reasonable than that of glutathione loss is to be found by consideration of the peculiar sensitivity of hemoglobin H to oxidative precipitation. The additional reactive thiols in soluble hemoglobin H may well participate in cellular reduction reactions and substitute in this regard for glutathione. As mentioned in the introduction to these studies, HMPS activity would be expected to diminish where the oxidative burden on glutathione is less because the demand for NADPH by glutathione reductase would be commensurately reduced. HMPS activity would be expected to increase to normal in old hemoglobin H cells containing less soluble hemoglobin H, perhaps in part because of loss of glutathione in coprecipitates with hemoglobin H, but more likely because of loss of the reducing substitute for glutathione, soluble hemoglobin H.

HMPS activity was consistently higher in young than in old red cells in beta thalassemia. The influence of the inclusions, which predominated in the young cells, on HMPS activity could not be evaluated since the immature cells would be expected to maintain a considerably higher rate of HMPS activity than old cells whether inclusions were present or not (32). Since alpha chains do not contain increased exposed thiol groups and in addition precipitate extremely rapidly even without the influence of oxidative stress, it is unlikely that they serve as a useful substitute for glutathione and, hence, it seems as unlikely that their presence would significantly influence HMPS activity.

Another result of hemoglobin precipitation in thalassemia may be an increase in membrane cation permeability. Such an increase was associated with cell aging in hemoglobin H disease and was present in the young cells in the beta thalassemia syndromes. The enhanced permeability was not related to glutathione content. Although it is tempting to relate the increased potassium efflux to the presence of hemoglobin precipitates in beta thalassemia, we cannot be sure of this interpretation. The markedly increased flux observed in inclusion bearing upper layer cells of splenectomized patients with beta thalassemia could be related to the large number of nucleated erythrocytes and reticulocytes in these fractions as well as to the inclusions themselves (34). That nucleated erythrocytes do not necessarily obscure these studies is suggested by the results of one experiment performed in a splenectomized patient with polycythemia vera and iron deficiency (4)

in whose cells inclusions were not present and in whom more nucleated red cells were detected in lower layer cells than in upper layer cells. The upper layer potassium flux was 20% higher than that of the bottom layer cells (4).⁴ More striking was the fact that in splenectomized patients with hemoglobin H disease the older cells contained inclusions and tended to have a higher rate of potassium flux than the young cells. These data provide firmer support for the concept that inclusions of hemoglobin may alter membrane permeability. On the other hand it does not appear likely to us that enhanced cation flux constitutes the most serious threat produced by intra-erythrocyte hemoglobin subunit precipitation. Rifkind (40), Slater, Muir, and Weed (41), and Wennberg and Weiss (42) have clearly demonstrated that large intra-erythrocyte inclusions enhance splenic sequestration, erythrocyte pitting and destruction of erythrocytes by mechanical interference with red cell passage through tortuous channels. This seems to us to constitute a much more important pathophysiological consequence of inclusion formation which results from unbalanced hemoglobin subunit synthesis in the thalassemia syndrome.

ACKNOWLEDGMENTS

We are grateful to Drs. William Beck and Barbara Rosen, Massachusetts General Hospital, and Thomas B. Necheles, Boston Floating Hospital, for encouraging some of their patients to participate in these studies. The technical assistance of Lynne Greer Esler and Jeanne Forgiione is deeply appreciated.

This work was supported by U. S. Public Health Service Grants HD-02777-01, T1 HE 5255, and AM 01975, and by a grant from the John A. Hartford Foundation. David G. Nathan is the recipient of U. S. Public Health Service Career Development Award K3 AM 35361. Some of these clinical studies were performed in Clinical Research Centers at the Peter Bent Brigham Hospital and Children's Hospital Medical Center supported by U. S. Public Health Service Grants 5MO1-FR3 and FR-00128.

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