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Heme Synthesis in Normal and Leukemic Leukocytes

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SUMMARY

Incorporation of glycine-2-^{14}C and Δ-aminolevulinic acid (ALA)-4-^{14}C into heme was studied in normal and leukemic leukocytes. Activities of ALA synthetase, ALA dehydrase, and heme synthetase in these cells were also measured. ALA synthetase activity and/or incorporation of labeled glycine into heme were demonstrated in leukocytes from patients with acute and chronic myelogenous leukemias. Normal granulocytes, normal lymphocytes, and leukocytes from a patient with chronic lymphocytic leukemia could not utilize glycine for heme synthesis. When normal and leukemic leukocytes were incubated with labeled ALA, incorporation of the ALA into heme was observed in all kinds of leukocytes irrespective of their types and stages of maturation. ALA dehydrase and heme synthetase activities were observed in these cells. It is suggested that the leukocytes would lose enzymes for heme synthesis in the process of cellular differentiation, ALA synthetase being the first enzyme lost.

INTRODUCTION

Despite the fact that the presence of such heme proteins as catalase, peroxidase, and the cytochromes has been demonstrated in leukocytes (1, 3, 6, 9), studies on the heme synthesis in these cells have been few. Protoporphyrin isolated from rat chloroma cells was radioactive after *in vivo* administration of glycine-^{14}C (10). Vannotti and Jeunet (12) have shown that in leukocytes isolated from patients with acute leukemia, Δ-aminolevulinic acid (ALA) can be converted to porphyrins, whereas in leukocytes from other patients, ALA was converted only to porphobilinogen.

More recently, Walters *et al.* (13) have shown that immature leukemic cells from patients with acute leukemia possessed a capacity to utilize ALA and protoporphyrin for heme synthesis, whereas polymorphonuclear peritoneal exudates from rabbits, as well as leukocytes from healthy adult volunteers and patients with chronic leukemia, lacked this capacity. They could not demonstrate ALA synthetase activity nor utilization of glycine as a substrate for heme synthesis in acute leukemic cells.

This paper describes a summary of our data on heme synthesis in normal and leukemic leukocytes.

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MATERIALS AND METHODS

Isolation of Leukocytes

Blood was collected from healthy and leukemic patients by syringes rinsed with heparin. From 100 to 200 ml of blood were used to obtain normal leukocytes, and for leukemic leukocytes, the amounts of blood collected differed according to the number of peripheral leukocytes.

Heparinized blood was centrifuged for 5 min at 3000 rpm and the plasma was removed. Buffy coat layers and the upper part of the sedimented red cells were transferred to 5-10 volumes of isotonic saline solution containing 3% dextran. After mixing, they were allowed to settle for 30 minutes at 37°C. The supernatant was removed and centrifuged for 2 min, at 850 rpm. The sediment was used as the granulocyte-rich fraction, while the supernatant was centrifuged for 5 min at 3000 rpm to obtain the lymphocyte-rich fraction. In separating leukemic leukocytes, this differential centrifugation was not used except when the percentage of leukemic cells in the peripheral blood was below 50%. In these cases the supernatant was centrifuged at 850 rpm for 5 min and the sediment was used as the leukemic-cell fraction. To these leukocyte fractions, washed once with isotonic saline, was added 2 ml of distilled water, and mixed well to hemolyze contaminated red cells. After centrifugation at 2000 rpm for 5 min, the sediment was used as the leukocytes.

Incorporation of Glycine-2-^{14}C into Heme in Leukocytes

The leukocytes were suspended in a medium consisting of an equal volume of autologous plasma and solution consisting of 0.76% NaCl, 0.039% KCl, 0.15% $MgCl_2 \cdot 6H_2O$, 100 mg% glucose and 0.02 M potassium phosphate buffer (pH 7.4). Glycine-2-^{14}C (specific activity 21.5 mc/mmole) was added at a concentration of 1-4 microcuries/ml. One half ml of the leukocyte suspension was added to each roller tube and incubated at 37°C for 4 hours under atmospheric conditions of 95% air and 5% carbon dioxide. After incubation, cells were washed 3 times with cold isotonic saline solution. From 100 to 200 mg of hemoglobin solution was added to each tube as the carrier hemoglobin. Hemin crystals were prepared from each tube according to the method of Chu and Chu (2). The crystallized hemin dissolved in 3% ammonia water was plated onto a planchet, and its radioactivity was counted with a windowless gas-flow counter. Amounts of hemin obtained were measured colorimetrically by the cyanmethemoglobin method (14). Amounts of glycine-2-^{14}C incorporated into heme during the incubation were calculated from the specific activity and recovery percentage of the crystallized hemin

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and expressed as moles of glycine incorporated per 10^8 leukocytes.

Incorporation of ALA-4-^{14}C into Heme in Leukocyte Homogenate

Leukocytes were suspended in the saline solution described above and homogenized well with a Potter-Elvehjem homogenizer. ALA-4-^{14}C (specific activity 27 mc/mmole) was added to the homogenized leukocyte preparation at a concentration of 1 microcurie/ml. One-half ml of the homogenate was incubated under the same conditions as the leukocyte suspension. After incubation, carrier hemoglobin was added, and the specific activity of the hemin crystal was measured as described above. Amounts of ALA-4-^{14}C incorporated into heme were similarly expressed as moles of ALA incorporated per 10^8 leukocytes.

Preparation of Enzyme Solution of ALA Synthetase, ALA Dehydrase, and Heme Synthetase

Leukocytes were homogenized with a Potter-Elvehjem homogenizer in 0.25 M sucrose. The homogenate was centrifuged in the cold at 9000 × *g* for 15 min. The supernatant was assayed for ALA dehydrase activity. The sediment, washed 3 times with 2 mM potassium phosphate buffer (pH 7.4) containing 1.5 M potassium chloride, was used for the assay of ALA-synthetase and heme synthetase.

Assay for ALA Synthetase Activity

ALA synthetase activity in the mitochondrial fraction of leukocytes was measured by essentially the same method as Lavers *et al.* (3, 7). The incubation mixture contained glycine, 50 μmoles; α-ketoglutarate, 3 μmoles; ethylenediaminetetraacetic acid 1 μmole; $MgCl_2$, 2 μmoles; pyridoxal phosphate, 0.2 μmoles; Coenzyme A (Sigma), 0.25 μmole; potassium phosphate buffer (pH 7.4), 25 μmoles; 0.3 ml of mitochondrial solution suspended in 2 mM potassium phosphate buffer (pH 7.4); and water to make up final 0.5 ml. This mixture was incubated under air at 37°C for 2 hours. Incubation was stopped by adding 1 ml of 5% trichloracetic acid and the supernatant was applied to Dowex 1 and IRC column as described by Urata and Granick (11). Amounts of ALA pyrrole (2-methyl-3-acetyl-4-propionic acid pyrrole), which were eluted from the Dowex 1 column with methanol:acetic acid (2:1), was measured spectrophotometrically at 553 mμ after addition of Ehrlich reagent. The amounts of ALA formed during the 2 hours incubation showed the activity of ALA synthetase.

Assay for ALA Dehydrase Activity

ALA dehydrase activity of the supernatant of the leukocyte homogenate was assayed in 0.43 ml of a medium consisting of leukocyte supernatant, 0.3 ml; reduced glutathione, 1 μmole; ALA, 1 μmole; and potassium phosphate buffer (pH 7.0), 10 μmoles. The medium was incubated at 37°C in air for 1 hour. Incubation was stopped by adding a volume of 5% trichloracetic acid. Amounts of porphobilinogen formed during the incubation were measured according to the method of Gibson *et al.* (4). The amounts of porphobilinogen showed the activity of ALA dehydrase.

Assay for Heme Synthetase Activity

Heme synthetase activity of leukocyte mitochondria was assayed in 0.8–1.0 ml of a medium consisting of $^{59}FeSO_4$ (specific activity 5 mc/mmole), 0.02 μmole; Tris buffer (pH 8.0), 100 μmoles; reduced glutathione, 40 μmoles; protoporphyrin IX, 0.02 μmole, and leukocyte mitochondria, 0.3–0.5 ml (8). The medium was incubated under nitrogen at 37°C for 2 hours. After incubation, 100–200 mg of carrier hemoglobin was added. Crystallization and measurement of the specific activity of the hemin from the incubated tube were performed according to the method previously described. Amounts of radioiron incorporated into heme during the above 2 hours incubation showed the activity of heme synthetase. All the enzyme assays and incorporation of labeled precursors were performed in duplicate. Mean values of the duplicates are presented in the following charts and tables.

RESULTS

The optical absorption of the ALA pyrrole formed from glycine and α-ketoglutarate in mitochondria of leukocytes of an acute myelogenous leukemia is shown in Chart 1. The absorption curve was identical to that of the authentic ALA pyrrole, indicating an existence of ALA synthetase activity in the mitochondria of acute myelogenous leukemia leukocytes. Similar ALA synthetase activity was demonstrated in leukocytes from another case of acute leukemia, whereas by using almost equal numbers of peripheral leukocytes, we could not

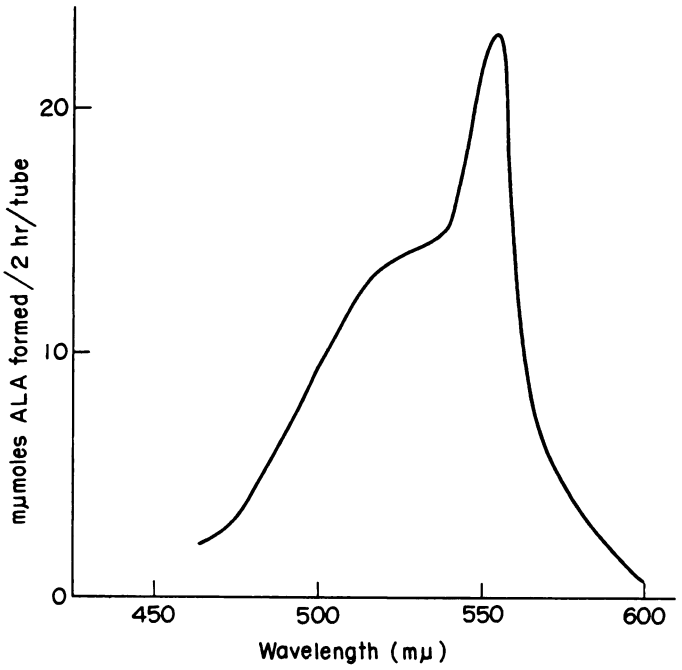

Chart 1. Optical absorption of the Δ-aminolevulinic acid (ALA) pyrrole (2-methyl-3-acetyl-5-propionic acid), showing the absorption of the ALA pyrrole formed with acute leukemia leukocytes. It is identical to that of the ALA pyrrole formed from authentic ALA.

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demonstrate ALA synthetase activity in leukocytes of a patient with chronic myelogenous leukemia (Table 1). Peripheral leukocytes of these 2 cases of acute myelogenous leukemia consisted of 96 and 92 percent leukemic myeloblasts respectively. Table 2 shows ALA dehydrase activity of normal granulocytes, normal lymphocytes, and leukocytes from acute myelogenous, chronic myelogenous, and chronic lymphocytic leukemia. Normal granulocytes and lymphocytes had higher ALA dehydrase activity than leukemic leukocytes. Significance of this finding will be discussed later.

Table 3 demonstrates heme synthetase activity of normal granulocytes, normal lymphocytes, and leukocytes from various leukemias. Leukocytes from acute myelogenous and chronic myelogenous leukemia had lower enzyme activity than normal granulocytes, normal lymphocytes, and chronic lymphocytic leukemia leukocytes.

Assay of the above enzymes pertaining to heme synthesis need large numbers of leukocytes which cannot always be obtained from peripheral blood of leukemic patients. To obtain a general idea of the heme synthesis capacity of normal and leukemic leukocytes, a leukocyte suspension or homogenate was incubated with glycine-2-^{14}C or ALA-4-^{14}C, and the incorporation of these precursors into heme was measured. Chart 2 shows times of incorporation of glycine-2-^{14}C into

Table 1

Name	Disease	WBC per tube	mμmoles ALA formed/2 hr per 10^9 WBC
K. S.	AML	3.40×10^8	5.4
S. K.	AML	1.08×10^8	5.8
K. O.	CML	3.16×10^8	[a]

ALA synthetase activity of leukocytes. ALA, Δ-aminolevulinic acid; WBC, white blood cell; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia.

[a] Not detectable by the present method.

Table 2

Name	Disease	mμmoles PBG/hour/10^8 WBC
S. M.	AML	0.45
T. A.	AML	0.86
E. S.	CML	0.95
K. M.	CML	1.70
S. U.	CML	1.10
F. K.	CLL	2.25
Mature Granulocytes		4.17
		8.96
Lymphocytes		4.42

ALA Dehydrase activity of normal and leukemic leukocytes. ALA, Δ-aminolevulinic acid; PBG, porphobilinogen; WBC, white blood cells; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; CLL, chronic lymphocytic leukemia.

Table 3

Name	Disease	mμmoles Fe incorporated per 10^9 cells
T. A.	AML	0.86
S. A.	CML	1.23
Y. T.	CML	0.59
E. S.	CML	0.10
K. M.	CML	0.16
F. K.	CLL	2.34
Mature Granulocytes		2.05
Lymphocytes		1.65

Heme synthetase activity of normal and leukemic leukocytes. AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; CLL, chronic lymphocytic leukemia.

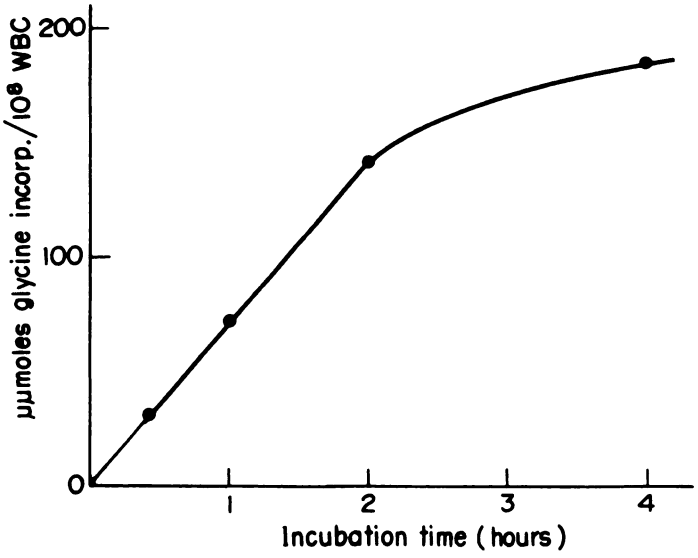

Chart 2. Incorporation of glycine-2-^{14}C into heme in leukocytes of an acute leukemia case (case Y. K.).

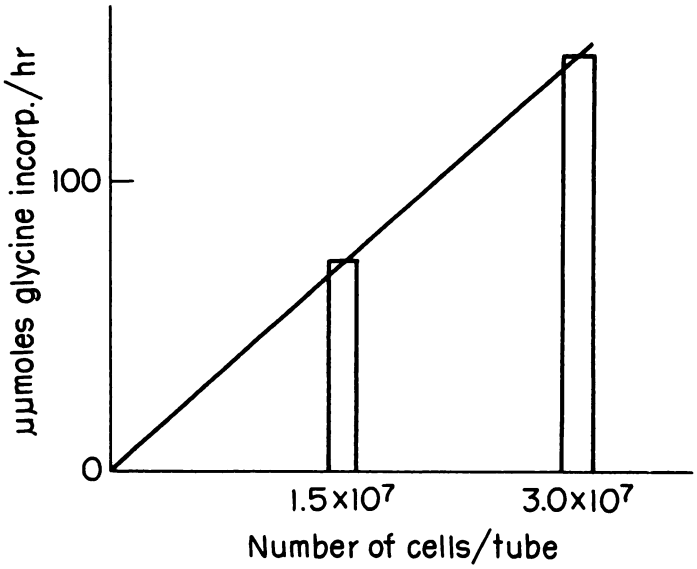

Chart 3. Cell counts incubated and amounts of glycine-2-^{14}C incorporated into heme. Leukocytes were obtained from the acute leukemia case demonstrated in Chart 2.

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heme in leukocytes from a patient with acute leukemia. Although Chart 2 demonstrates a typical curve, this increase in incorporation was not observed in leukocytes from all leukemic patients. In some cases of acute leukemia, a plateau was reached at the second hour of incubation. Chart 3 shows the relationship between the cell counts incubated and the amounts of glycine-2-^{14}C incorporated into heme in the leukocytes of the acute leukemia case shown in Chart 2. As shown in Table 4, incorporation of glycine-2-^{14}C into heme was observed in leukocytes from all cases of acute myelogenous leukemia and all cases of chronic myelogenous leukemia except one. Leukocytes from a patient with chronic lymphocytic leukemia could not incorporate glycine-2-^{14}C into heme. Similar inability to utilize glycine-2-^{14}C for heme synthesis was observed in normal granulocytes and normal lymphocytes (Table 5).

As shown in Table 6, when the ALA-4-^{14}C was incubated with leukocyte homogenate, incorporation of ALA-4-^{14}C into heme was observed in all leukocytes studied (normal granulocytes, normal lymphocytes, and leukocytes from acute myelogenous, chronic myelogenous, and chronic lymphocytic leukemia).

Table 4

Name	Disease	WBC per tube	Glycine-2-^{14}C per ml (μc)	Incubation (hr)	$\mu\mu$moles glycine-2-^{14}C incorporated/ 10^8 WBC
S. K.	AML	6.24×10^7	1	4	54.9
K. S.	AML	6.48×10^8	1	4	37.6
T. A.	AML	2.26×10^7	1	1	99.6
T. A.	AML	2.26×10^7	1	2	113.2
Y. K.	AML	4.30×10^7	2	1	73.5
Y. K.	AML	4.30×10^7	2	2	142.0
Y. K.	AML	4.30×10^7	2	4	185.0
I. Y.	A.mono.L.	0.96×10^7	2	1	182.0
I. Y.	A.mono.L.	0.96×10^7	2	2	348.0
K. O.	CML	6.75×10^7	1	3	54.4
S. A.	CML	2.45×10^8	1	3	48.6
K. M.	CML	3.00×10^7	1	3	341.0
S. T.	CML	4.10×10^7	5	2	309.0
K. S.	CML	2.68×10^8	2	4	67.5
M. F.	CML	2.67×10^8	1	2	[a]
F. K.	CLL	2.00×10^7	1	2	[a]
F. K.	CLL	1.80×10^7	4	2	[a]

Incorporation of glycine-2-^{14}C into heme (leukemic leukocytes). WBC, white blood cells; AML, acute myelogenous leukemia; A.mono.L., acute monocytic leukemia; CML, chronic myelogenous leukemia; CLL, chronic lymphocytic leukemia.

[a] No incorporation of glycine-2-^{14}C into heme.

Table 5

WBC per tube	Glycine-2-^{14}C per ml (μc)	Incubation (hr)	$\mu\mu$moles glycine-2-^{14}C incorporated/ 10^8 cells
Granulocytes			
2.36×10^7	1	2.0	NI
7.00×10^7	1	2.0	NI
2.80×10^7	2	4.0	NI
1.84×10^7	2	4.0	NI
2.22×10^7	4	2.0	NI
3.95×10^7	4	2.5	NI
Lymphocytes			
1.83×10^7	1	2.0	NI
2.80×10^7	4	2.5	NI

Incorporation of glycine-2-^{14}C into heme (mature leukocytes). WBC, white blood cells; NI, no incorporation of glycine-2-^{14}C into heme.

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Table 6

Name	Disease	WBC per tube	ALA-4-^{14}C per ml (μc)	Incubation (hr)	$\mu\mu$moles ALA incorporated/ 10^8 WBC
T. A.	AML	4.00×10^7	1.0	3.0	147.2
T. A.	AML	3.00×10^7	0.5	2.0	544.7
K. M.	CML	4.00×10^7	1.0	3.0	126.6
S. T.	CML	4.10×10^7	0.5	2.0	85.3
M. F.	CML	1.76×10^8	1.0	2.0	125.0
F. K.	CLL	2.00×10^7	0.5	1.0	54.6
		Mature granulocytes			
		1.66×10^7	0.5	2.0	164.2
		2.22×10^7	0.5	2.0	340.0
		2.25×10^7	1.0	1.0	478.2
		2.25×10^7	1.0	2.5	497.0
		7.00×10^6	0.8	2.0	550.0
		7.00×10^6	0.8	4.0	1020.0
		Lymphocytes			
		6.70×10^6	0.8	2.0	281.7
		6.70×10^6	0.8	4.0	1155.0

Incorporation of ALA-4-^{14}C into heme (normal and leukemic leukocytes). ALA, Δ-aminolevulinic acid; WBC, white blood cells; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; CLL, chronic lymphocytic leukemia.

DISCUSSION

Previous reports indicated an inability to demonstrate activity of ALA synthetase or utilization of glycine for heme synthesis in leukemic leukocytes. Our present results (Chart 1; Table 1) indicate an existence of ALA synthetase activity in leukocytes of patients with acute myelogenous leukemia, more than 90% of which were myeloblasts. The existence of ALA synthetase in leukocytes of acute myelogenous leukemia was further substantiated by demonstrating the utilization of labeled glycine for heme synthesis in these cells. Similar utilization of labeled glycine for heme synthesis was also observed in leukocytes from chronic myelogenous leukemia patients. Numbers of leukocytes incubated or amounts of labeled precursors added per tube differed in each incubation. Moreover, the incorporation of glycine-2-^{14}C into heme was not always linear even up to 2 hours of incubation. Therefore, it was not feasible for us to compare the capacity of incubated leukocytes from each patient to utilize glycine for heme synthesis. However, the results in Tables 3 and 4 show that only the leukocytes from acute and chronic myelogenous leukemia could utilize glycine for heme synthesis. The possibility of the above utilization of glycine for heme synthesis by contaminated reticulocytes rather than by leukemic leukocytes has to be considered, despite our attempt to eliminate red cells by differential centrifugation. However, the inability of glycine utilization in normal granulocytes and lymphocytes, which were separated from larger amounts of blood than leukemic leukocytes, seemed to exclude the above possibility.

The contamination of red cells should be considered seriously in measurement of ALA dehydrase activity of the leukocytes. However, since ALA dehydrase is an enzyme found in various kind of cells not synthesizing heme, and since in our experiments the leukocytes were exposed to differential hemolysis and several washings for elimination of red cells, it is probable that leukocytes contain ALA dehydrase activity. The ability of leukemic leukocytes to convert ALA to porphyrins has already been reported by Vannotti and Jeunet (12). The difference in ALA dehydrase activity between normal granulocytes or lymphocytes in acute or chronic myelogenous leukemia observed in our study could be due to contaminated red blood cells. Demonstration of ALA dehydrase and heme synthetase activities in all normal and leukemic leukocytes coincides well with the presently reported incorporation of ALA-4-^{14}C into heme in these cells. As in the case of glycine-2-^{14}C incorporation, the number of cells incubated and amount of ALA-4-^{14}C added per tube were not uniform in all incubations. Linear increase in the incorporation of ALA was not found consistently. Therefore, comparison of the amounts of utilized ALA in each case was not feasible. However, the present results show that ALA could be utilized for heme synthesis in normal as well as in leukemic leukocytes.

Normal granulocytes, normal lymphocytes, and leukocytes from patients with chronic lymphocytic leukemia could not utilize glycine-2-^{14}C for heme synthesis. These findings show that these cells cannot synthesize heme from glycine because of the absence of ALA synthetase activity. The present results also suggest that, in leukocytes, the ALA synthetase is a regulatory enzyme in heme synthesis (also suggested by Granick (5) in hemoglobin synthesis in erythroid cells). Existence of whole enzymes necessary for heme synthesis was demonstrated only in the leukemic cells. However, considering an existence of heme proteins in mature granulocytes, it is most probable that this existence of enzymes is due to an immaturity of cells rather than to leukemic changes in these cells. In support of this speculation, the chronic myelogenous leukemia case (Case H. F. in Table 4), whose peripheral leukemic cells could not

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incorporate labeled glycine into heme, had a low percentage of peripheral immature granulocytes. As in the case of erythroid cells, leukocytes would lose enzymes for heme synthesis in the process of cellular differentiation, the ALA synthetase being the first enzyme lost. The difference in heme-synthesizing enzymes between mature erythrocytes and granulocytes is that the latter still possess heme synthetase which the former has lost.

Because of the rarity of the incidence of chronic lymphocytic leukemia in Japan, we have not yet studied enough numbers of the case to conclude that the inability of these peripheral leukocytes to synthesize heme from glycine is universal. It is speculated that this inability could be due to the maturity of lymphocytes in the peripheral blood of chronic lymphocytic leukemia. It would be of interest to know if peripheral blast cells in acute lymphocytic leukemia can utilize glycine for heme synthesis.

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