Incubation Hemolysis and Red Cell Metabolism in Acanthocytosis *

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Acanthocytosis [1] is a hereditary syndrome characterized clinically by retarded growth, steatorrhea, progressive neurological disease, and retinal degeneration (2). In the blood, half or more of the red cells are acanthocytes ("thorny" cells), and low density (beta) lipoproteins are greatly decreased or completely absent (3-5).

Previous studies have demonstrated abnormalities of red cell membrane lipids in acanthocytosis (6, 7). Lecithins, the major class of phospholipids in normal human red cells, are decreased, both in relative and absolute amount, and sphingomyelins are increased. In addition, the amount of linoleic acid esterified to phospholipid is very low. Striking alterations in plasma lipids are also present, with very low levels of total lipid, phospholipid, and cholesterol, accompanied by an altered distribution of lecithins and sphingomyelins similar to that seen in the red cells of these patients.

In view of these differences in morphology and in the chemical composition of the membrane, it is noteworthy that the red cells in acanthocytosis display altered *in vitro* behavior and, at least in some patients, shortened *in vivo* survival (Table I). The present communication reports red cell metabolic studies and describes a distinctive *in vitro* autohemolytic pattern of these cells useful both in the detection and in the investigation of acanthocytosis.

In the accompanying paper (19) the influence of normal serum and certain of its subfractions on the autohemolytic behavior is described, and the implications of these findings are discussed.

Materials and Methods

Patients. Patient 1 is M. S. in Table I. Most of the current studies were carried out in the summer of 1962 while he was a patient of the U. S. Public Health Service Clinical Research Center at the University of Washington Hospital. The deficiencies in plasma phospholipid, total lipid, and cholesterol and the abnormalities of phospholipid distribution in red cells and plasma previously reported in this patient have been repeatedly demonstrated over a 33-month period of observation (Table II). Patient 2 is R. B. in Table I. Our analyses of his red cell and plasma lipids (Table II) are similar to the values reported by Phillips (7) and to the values found for Patient 1.

Measurement of erythrocyte glycolysis. Approximately 35 ml of the fasting patient's venous blood, defibrinated with glass beads, was centrifuged at 400 g for 10 minutes at room temperature. After removal of the serum, the buffy coat was discarded, and the cells were resuspended in their original serum. The procedure was repeated once, the serum-suspended cells having a final hematocrit of approximately 40%. Sufficient 10% glucose was added to raise the glucose concentration by 50 mg per 100 ml. Hemoglobin, hematocrit, leukocyte, and reticulocyte counts were determined on the fresh-defibrinated, and on the reconstituted blood.

The cell suspension was distributed into sixteen 25-ml Erlenmeyer flasks as shown in the following scheme:

Flask no.	Vol cell suspension	Additions (vol and average, μc)				
		Glucose-1-C¹⁴	Glucose-6-C¹⁴	Glucose-2-C¹⁴	Methylene blue	H₂O
	ml	ml	ml	ml	ml	ml
1, 2, 3, 4	2					0.06
5, 6	1	0.03 (0.04 μc)				
7, 8	1		0.03 (0.4 μc)			
9, 10, 11, 12	2				0.02	0.04
13, 14	1	0.02 (0.004 μc)			0.01	
15, 16	1			0.02 (0.01 μc)	0.01	

TABLE I

Hematologic findings in acanthocytosis

Patient	Sex	Selected references	Year born	Hemoglobin	Reticulocytes	Erythroid marrow	Cr^{51} t$_{\frac{1}{2}}$	Hapto-globin	Acantho-cytes	Osmotic fragility	Mechanical fragility*	Red cell phospholipids
				g/100 ml	%		days		%		%	
R. K.	F	(1, 7)	1932	11.3	Normal to 2.0	Slightly increased				Slightly decreased		Abnormal
L. Z.	M	(7, 8, 9)	1938	12.3	0.7	Normal	26		75	Normal	21	Normal
?	M	(10)	1938	11.7–13.8	0.5	Slightly increased			70–80	Slightly decreased	20	
R. C.	F	(11, 12)	1922	12.9–14.0	3.2–5.0	Slightly increased	18	Absent		Normal	23	
N. S.	M	(13)	1923	14.0		Normal						
G. F.	M	(12, 14)	1953	Slight anemia, later normal				Absent	90	Normal		
C. R.	F	(4, 6, 15, 16)	1947	Mild anemia								Abnormal
A. C.	M	(17, 6)	1933	16.0	3.0	Increased	18			Normal		Abnormal
C. S.	F	(5)	1958	13.1					50	Slightly decreased		
R. B.	M	(7, 18, this paper)	1956	11.9								Abnormal
M. S.	M	(6, this paper)	1954	10.4–11.1	2.4–5.8	Slightly increased	See text	See text	60–70	Normal		Abnormal
R. I.	M	(7, 8)	1953	13.1	5.0					Normal		Abnormal

* Normal mechanical fragility, less than 8% (10).

TABLE II

Red cell and plasma lipids* in acanthocytosis

	Total lipid	Lipid phosphorus	Choles-terol	Phospholipid distribution†					Esterified linoleic acid
				Lec	SP	PE	PS	Other	
	mg × 10⁻¹⁰ per cell			*% of total phospholipid‡*					*moles/ 100 ml*
Red cell lipid									
Normal (13) Mean	4.83	0.116	1.18	30.0	24	26	15	6	11.0
SD	0.25	0.006	0.06	1.5	1.7	2.6	1.6	1.7	
Patient 1									
May 1960		0.115	1.18	19.3	34.2	26.2	15.1	5.4	1.8
June 1962	5.23	0.116	1.35	23.6	32.8	22.8	15.7	5.4	2.2
July 1962	5.66	0.124	1.40	23.9	33.0	23.0	15.2	4.6	
January 1963§				22.5	33.4	24.2	16.7	3.0	4.4
Patient 2§		0.111	1.18	21.3	29.3	45		4.4	0.8
	mg/100 ml								
Plasma lipid									
Normal males‖ Mean		8.73	172	69.1	19.0			11.7	
SD		0.88	22						
Patient 1									
May 1960		1.27	25	40.6	37.5			21.8	
June 1962		2.01	37	55.3	29.4			14.9	
August 1962		1.82	34	55.0	29.0			16.0	
January 1963		1.66	41	50.7	38.2			11.1	
Patient 2§		1.14	29	49.5	29.2			21.3	

* The methods of lipid analysis have been described previously (6).
† Lec, lecithin; SP, sphingomyelin; PE, ethanolamine phosphoglycerides; PS, serine phosphoglycerides.
‡ The values are in per cent of total phospholipid recovered from the column (May 1960) or paper (all subsequent) chromatograms.
§ Patient 1 had been receiving 5 to 10 ml safflower oil daily for 7 months, and Patient 2 had been on a fat-free formula for 2 months at the time these assays were carried out.
‖ Phosphorus and cholesterol values from Havel, Eder, and Bragdon (20); phospholipid distribution from Phillips (7).

The flasks were closed with rubber serum stoppers and incubated at 37° C for 60 minutes in a shaking incubator, after which 0.5-ml samples were removed from flasks 1 to 3 and protein-free filtrates (21) prepared for zero time glucose and lactate determinations.

Radioactive glucose in water, methylene blue (MB) (0.2 mg per ml in 0.15 M NaCl, to achieve a final concentration of 6×10^{-6} M), and water were then added to the flasks. Thereafter, each flask was resealed with a serum stopper fitted with a glass center well and incubated an additional 120 minutes at 37° C. At the end of this period, samples for glucose and lactate determinations were collected from flasks 1 to 3 and 9 to 11.

The flasks containing labeled glucose were injected with 0.5 ml of Hyamine hydroxide (22) through the stopper into the center well. Immediately thereafter, metabolism was stopped and the $C^{14}O_2$ released from solution by injecting 0.3 ml of 6 N H_2SO_4 into the reaction mixture, carefully avoiding the center well. The flasks were then shaken at room temperature for 80 minutes to insure complete trapping of CO_2 by the Hyamine. $C^{14}O_2$ was measured as described previously (23).

The contents of flasks 4 and 12 were used to measure pH at the beginning and end of the incubation period. The initial pH was 8.0 ± 0.1 and fell 0.07 to 0.15 U during the 120-minute period.

The added glucose-1-C^{14} and glucose-2-C^{14} accounted for less than 0.5% of the total glucose in each flask. Because of negligible CO_2 production from carbon 6 of glucose, a larger quantity of glucose-6-C^{14} was added, amounting to about 5 to 10% of the total glucose content.

Methemoglobin reduction. Ten ml of fresh venous blood was added to 1.5 ml of a citrate-dextrose solution containing 5.0 g glucose and 2.13 g $Na_3C_6H_5O_7 \cdot 2H_2O$ per 100 ml. Three-ml portions were aerated thoroughly by rotation and then diluted to 20 ml with isotonic, buffered saline-phosphate (10 parts 0.15 M NaCl to 1 part 0.085 M potassium phosphate buffer, pH 7.4). One ml of freshly prepared sodium nitrite (0.3 M in 0.15 M NaCl) was added to give a final nitrite concentration of 0.014 M, and the mixture was incubated at room temperature for 15 minutes. The cells were sedimented (4° C, 10 minutes, 1,500 g) and washed 4 times by resuspension in 20 vol of the buffered saline-phosphate to remove residual nitrite. After the last wash a 10% suspension of these cells, in buffered saline-phosphate containing either 0.01 M glucose, inosine, or no substrate, was prepared, and 5.0-ml portions were incubated at 37° C in a metabolic shaker according to the following scheme:

Suspension	Flask					
	1	2	3	4	5	6
Glucose	x	x				
Inosine			x	x		
Control					x	x
MB		x		x		x

Where indicated, 50 μl of a methylene blue solution (0.2 mg per ml in 0.15 M NaCl) was added to give a final concentration of 6×10^{-6} M.

After a 5-minute equilibration period, methemoglobin and total hemoglobin were determined on each sample (24) using 0.5 ml of the incubation mixture. Subsequently methemoglobin was measured at 15-minute intervals for 1 hour on samples containing methylene blue and at hourly intervals for 4 hours on the remaining samples.

Ninety-three to 98% of the hemoglobin was initially converted to methemoglobin by the nitrite. Methemoglobin reduction rates were independent of the total hemoglobin concentration in the range found in the incubation mixtures (1.7 to 2.9 g per 100 ml). In suspensions containing glucose or inosine and methylene blue, the reduction rates were linear for about 45 minutes or until 85 to 90% of the methemoglobin was reduced, except that a slight lag (less than 15 minutes) was observed with inosine plus methylene blue. With glucose alone, rates remained linear for 2 to 3 hours, and with inosine alone, for 4 hours or more. The tabulated values were obtained from the linear portion of the curves.

Autohemolysis. Autohemolysis was measured essentially as described by Young, Izzo, Altman, and Swisher (25) using 2-ml samples of defibrinated blood. When supplemented with energy substrates before the incubation, 0.1 ml of 0.56 M D-glucose in 0.15 M saline or 0.2 ml of 0.09 M inosine in 0.15 M saline was added. These solutions were sterilized by passage through Millipore filters [2] and stored frozen. When heparinized blood was studied, it contained 20 U (0.02 ml) of heparin per ml. Samples collected in disodium EDTA dihydrate (Na$_2$ H$_2$ EDTA) contained 1 to 1.5 mg per ml blood. Samples collected in oxalate contained 1 mg "balanced" oxalate (26) per ml blood. ACD-blood contained 1 part of acid-citrate-dextrose, NIH formula A, to 7 parts of blood. In performing the test, the samples were incubated for 24 hours, then gently rotated by hand to resuspend the cells, and incubated an additional 24 hours. Hemoglobin, both total and supernatant, was measured as cyanmethemoglobin. All determinations were done in duplicate.

GSH content and stability. GSH concentration was determined in blood collected in ACD by a modification of Grunert and Phillips' method (27) as described by Flanagan, Schrier, Carson, and Alving (28). A standard solution was prepared from commercial glutathione, the GSH content of which was verified by iodometric titration (29). Glutathione stability was determined as described by Beutler (30).

Assays for ATP and ADP. One vol of blood was deproteinized with 2 vol of cold 0.6 N perchloric acid, and after centrifugation the supernatant fraction was neutralized with KOH. ATP was measured with the coupled reaction, 3-phosphoglycerate kinase—glyceralde-

hyde-3-phosphate dehydrogenase (31), adapted to the filtrate described above.[3] The final concentrations in the assay system, in a 3-ml vol, were as follows: triethanolamine buffer, pH 7.6, 0.25 M; MgSO$_4$, 3.3×10^{-3} M; D-3-phosphoglycerate, 5×10^{-3} M; DPNH, 6.7×10^{-5} M; glyceraldehyde-3-phosphate dehydrogenase, 0.16 mg; 3-phosphoglycerate kinase, 0.04 mg; and filtrate, 0.5 ml. ADP was measured with the coupled reaction, pyruvic kinase—lactic dehydrogenase (33). The final concentrations in the assay system, in a 3-ml vol were these: glycylglycine buffer, pH 7.6, 5×10^{-2} M; MgSO$_4$, 8×10^{-3} M; KCl, 7.5×10^{-2} M; phosphoenolpyruvate, 1×10^{-3} M; DPNH, 1×10^{-4} M; lactic dehydrogenase, 0.0075 mg; pyruvic kinase, 0.06 mg; and filtrate, 1.2 ml. Assays for ATP and ADP were performed on duplicate extracts of blood on the same day the extracts were prepared.

G-6-PD and 6-PGD determinations. Erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) and a 6-phosphogluconate dehydrogenase (6-PGD) were determined by the method of Glock and McLean (34) as adapted to hemolysates by Zinkham, Lenhard, and Childs (35, 36), with minor modifications. Blood was collected in acid-citrate-dextrose (ACD); the plasma and buffy layer were discarded and the cells washed once in 8 vol of 0.15 M NaCl, resuspended in 3 vol of 0.15 M NaCl, and hemolyzed by freeze-thawing once. The final concentrations in the assay system were the following: MgCl$_2$, 0.01 M; Tris, pH 8.0, 0.065 M; TPN, 2×10^{-4} M; 10 μl of hemolysate; and either disodium G-6-P, 6.6×10^{-4} M, or disodium 6-PG, 6.6×10^{-4} M, both, or neither (blank), in a final vol of 3 ml. The reaction was started by adding the hemolysate, and TPNH formation was followed at 340 mμ in a Beckman DU spectrophotometer with the temperature controlled at 25° C. The rate of TPNH formation in the cuvette containing 6-PG alone was taken as a measure of 6-PGD activity, whereas the difference in rates between this cuvette and the one containing both substrates represented G-6-PD activity (34). As defined by Zinkham and associates (35), 1 U of enzyme activity catalyzes the formation of 1 μmole of TPNH per minute at 25° C.

Centrifugal fractionation. Whole blood, collected in EDTA, was distributed in 4-ml Sanford McGath tubes, allowed to stand vertically at room temperature for 30 to 60 minutes, and then centrifuged at 1,000 *g* for 30 minutes. After removal of the plasma and most of the buffy layer, the top $\frac{1}{10}$ of the cell column from each tube was aspirated with a capillary pipette and pooled. The intervening $\frac{8}{10}$ of the cell column was then aspirated, and, finally, the bottom $\frac{1}{10}$ was collected and pooled. Each cell pool was resuspended in 0.9% NaCl after removing a portion for morphologic studies.

Standard methods. Morphologic examinations were

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[2] Millipore Filter Corp., Bedford, Mass.

[3] We found that a 1:1 perchloric acid filtrate, prepared as described in the directions, led to incomplete extraction of the ATP. A detailed evaluation of this method is in preparation (32).

TABLE III

Centrifugal separation

	Top	Whole blood	Bottom
Fe^{59}, cpm/mg Hgb	105	35	6
Reticulocytes, %	28.5	10	0.5
MCHC*	28.6	34.7	38.3
Morphology			
% Normal cells	46	40	2
% Crenated microspherocytes	2	20	46

* MCHC = mean corpuscular hemoglobin concentration.

performed on smears stained with Wright's stain and on wet preparations suspended in a solution of 1% formalin in 3% trisodium citrate (26). Hemoglobin was measured as cyanmethemoglobin; hematocrit was determined by the microcapillary method, after centrifugation for 4 minutes at 12,000 g. Red cell counts were performed on a Coulter counter. Reticulocytes were enumerated on dried smears of cells stained supravitally with new methylene blue (37), counting 80 reticulocytes. Osmotic fragility was measured by adding 50 μl of fresh, fasting, oxygenated heparinized blood to tubes containing 5 ml of hypotonic saline buffered to pH 7.4, allowing 1 hour at room temperature for lysis to be complete (26). Glucose was determined with glucose oxidase (38) and lactate with lactic dehydrogenase (39). Measurements of pH were performed at room temperature with a Beckman model G pH meter and standard glass electrodes.

The metabolic, autohemolytic, and fragility experiments were run concurrently with a sample of normal blood and except where indicated were performed on blood from Patient 1.

Materials included lactic dehydrogenase,[4] pyruvic kinase,[4] 3-phosphoglyceraldehyde dehydrogenase,[4] 3-phosphoglycerate kinase,[4] glucose oxidase,[5] glutathione,[6] glucose-1-C^{14} [7] (SA, 3.2 to 8.3 μc per mg), glucose-2-C^{14} [8] (SA, 2.95 μc per mg), glucose-6-C^{14} [8] (SA, 2.33 to 3.25 μc per mg), and hydroxide of Hyamine 10X.[9]

Results and Discussion

Morphologic studies. The patient's average red cell was of normal size and normochromic (Table III), but 60 to 70% of the cells were distorted. The abnormalities in shape varied from cells with one or more bleb-like protuberances to

4 Obtained from C. F. Boehringer and Sons through the California Corp. for Biochemical Research, Los Angeles, Calif.
5 Worthington Biochemical Corp., Freehold, N. J.
6 Schwartz BioResearch, Inc., Mt. Vernon, N. Y.
7 Volk Radiochemical Co., Chicago, Ill.
8 National Bureau of Standards, Washington, D. C.
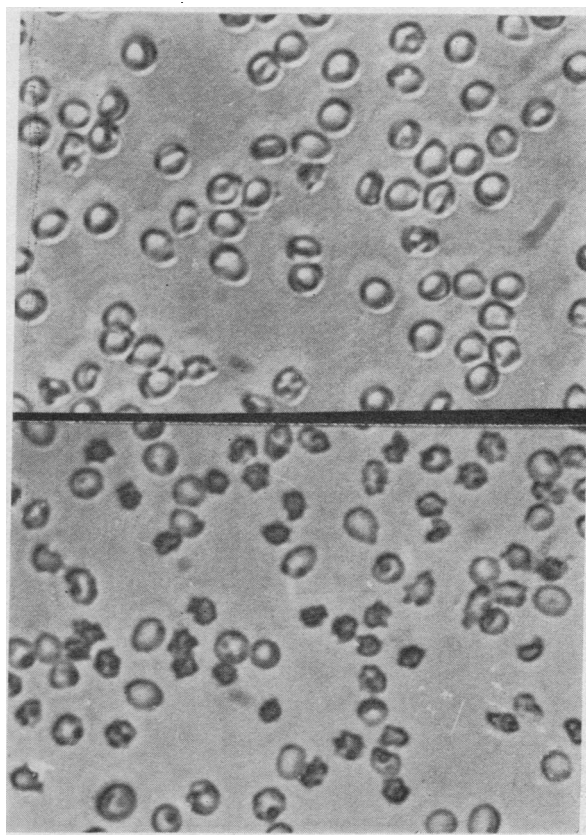9 Packard Instrument Co., La Grange, Ill.

FIG. 1. CENTRIFUGAL SEPARATION OF RED CELLS. The cells from Patient 1 were separated and wet preparations prepared as described (Methods). Upper—top layer; lower—bottom layer.

densely staining microspherocytes with a few spine-like processes. Intermediate forms included crenated spheres and cells that appeared normal in size but had several spiny excrescences. These morphologic variants were better seen in wet preparations, fixed in formalin (see Methods).

Centrifugal separation of the patient's blood, after a 2-week period of extensive experimental blood loss and 4 days after the injection of transferrin-bound Fe^{59}, produced a top layer enriched with younger cells as shown by the high concentration of reticulocytes and specific radioactivity (Table IV). Morphologically, the top fraction contained few crenated cells; the severely distorted acanthocytes accumulated at the bottom (Figure 1). The mean corpuscular hemoglobin concentration (MCHC) was significantly different in each of the layers (highest at the bottom and lowest at the top), which apparently resulted in a

Red cell metabolism and osmotic fragility

		Normal	± 2 SD	Patient 1	
				$\mu moles/g$ hemoglobin/hour	
				6/4/62	8/13/62
Glucose catabolism	(14)*				
Glucose disappearance		7.0	0.8	8.6	9.2
Lactate formation		11.6	0.9	11.6	15.1
C^1O_2 formation		0.31	0.05	0.42	0.28
C^6O_2 formation		0.015	0.009	0.012	0.012
With methylene blue (MB)					
Glucose disappearance		8.6	1.0	9.6	9.6
Lactate formation		12.7	1.5	15.1	16.8
C^1O_2 formation		4.7	1.0	3.1	5.1
C^2O_2 formation		1.4	0.5	0.5	1.6
				8/8/62	
Methemoglobin reduction	(8)				
Glucose		1.1	0.46	0.9	
Inosine		1.3	0.46	1.0	
Glucose with MB		13.2	2.2	18.8	
Inosine with MB		15.3	3.7	20.0	
				$\mu moles/g$ hemoglobin	
Glutathione	(17)	7.5	2.6	6.1†	
Glutathione "stability"	(17)	91%	12.8%	89%†	
ATP (heparinized blood)	(40)	4.04	1.26	5.2†	
ADP (heparinized blood)	(19)	0.57	0.32	0.56†	
				U/g hemoglobin	
Enzymes	(8)				
G-6-PD		4.87	0.24	8.2	
6-PGD		2.97	0.22	4.1	
Osmotic fragility	(9)				
Hemolysis, 1%		0.49	0.03	0.48†	
Hemolysis, 50%		0.41	0.03	0.40†	

* Parentheses indicate number of normal specimens tested.
† Average values, two or more determinations.

sufficiently large specific gravity gradient to account for the separation achieved (40). These differences in MCHC, in combination with the quantitative morphology, specific radioactivity, and reticulocyte counts, suggest but do not prove that the older cells of the blood were concentrated in the bottom layer. Thus, in this patient, there was minimal distortion of the younger circulating erythrocytes (a concept also supported by the absence of deformed red cell precursors in the marrow), with an increase in deformity as the cells matured or aged or both. In a previously studied patient, the acanthocytes were also predominately segregated in the "older" cell population (cited in 7), but this was apparently not observed in the patient reported by Schwartz and his associates (8).

Red cell metabolic studies. Several *in vitro* studies were carried out to determine whether the

defects in membrane composition and plasma environment were associated with abnormalities in red cell metabolic function (Table IV). Measurements of glycolysis (glucose disappearance, lactate formation), of phosphogluconate oxidation ($C^{14}O_2$ production from glucose-1-C^{14}, G-6-PD, and 6-PGD activity), and of pentose phosphate metabolism ($C^{14}O_2$ from glucose-2-C^{14}) were characteristic of a population slightly enriched with young red cells but were otherwise unremarkable. As with normal red cells, no significant amount of $C^{14}O_2$ was evolved from glucose-6-C^{14}.

These conclusions are based on the experiment of August 13, 1962. In the original study (June 4, 1962), $C^{14}O_2$ production from glucose-1-C^{14} was elevated, whereas in the presence of methylene blue, $C^{14}O_2$ evolution was depressed both from glucose-1-C^{14} and from glucose-2-C^{14}. Because

these results were inconsistent with the other metabolic data to be presented, the glycolytic study was repeated, and essentially normal results were obtained.

We can offer no explanation for the discrepancy between these two studies. The samples of normal blood studied concomitantly in both experiments gave values within the normal range.

The capacity of acanthocytes to utilize these pathways of energy production for certain red cell metabolic functions was also investigated. Methemoglobin reduction, with either glucose or inosine as substrate, with or without methylene blue, was normal or increased, indicating that the reduction of methemoglobin could be coupled normally both to the Embden-Meyerhof sequence and to the phosphogluconate oxidative pathway. Likewise, the normal level of reduced glutathione and its adequate maintenance after incubation with acetylphenylhydrazine and glucose indicate that glutathione reduction, which is linked to the phosphogluconate oxidative pathway, was not impaired. Finally, the ATP level was slightly elevated (also probably a reflection of a population slightly enriched with young cells), indicating adequate coupling of red cell glycolysis to production of high energy compounds *in vivo*.

These studies indicate that acanthocytes are normal with respect to the pathways of red cell intermediary carbohydrate metabolism considered of major importance to red cell integrity. They do not, however, exclude a possible defect in the linkage between energy production and processes necessary for maintenance of membrane structure or function.

Physical properties (Table I). In previous studies, acanthocytes were reported to have increased mechanical fragility (8, 10, 11) and increased lysolecithin fragility (10), but their permeability to Na+ and K+, based on measurements of influx and outflux of both Na+ and K+, was normal (41). Osmotic fragility has also been normal or slightly decreased (2, 5, 7, 8, 10, 11, 17), and such measurements were normal in this study (Table IV). In view of the presence of crenated and smooth microspherocytes in morphologic preparations, the initial osmotic fragility might have been expected to be increased. That it was not suggests that the membrane of acan-

thocytes is normal in surface area but folded upon itself, or that it is thicker than normal and can spread. Also, the normal mean osmotic fragility of these cells might not have been predicted on the basis of recent findings (42) indicating increased osmotic fragility when normal red cells are incubated *in vitro* in plasma having a low concentration of free cholesterol—a situation analogous to that of acanthocytes *in vivo*.

Autohemolytic behavior. In an average of ten experiments on blood from Patient 1, 40% of the red cells lysed when defibrinated blood was incubated at 37° C for 48 hours, as compared with 3.5% of control cells (Table V).[10] Curiously, autohemolysis of defibrinated blood stored at 4° C for 48 hours was also markedly elevated, averaging 28%, yet at room temperature the values were only slightly above normal. An identical pattern was present in Patient 2.

At 37° C, autohemolysis after the shorter incubation period of 24 hours was not abnormal (Patient 1, 0.6%; Control, 0.3%); at 4° C, however, the 24-hour value was distinctly elevated (Patient 1, 3.6%; Control, < 0.1%). Even here, the major increment in hemolysis occurred during the second 24-hour period. Autohemolysis of defibrinated blood after 24 hours at 37° C in a patient with acanthocytosis has previously been reported by Druez (11), whose value of 3% is elevated compared to the normal range of 0 to 0.5% given by Dacie (26).

Glucose supplementation before the incubation markedly inhibited the lysis but did not reduce it to normal (Table V). Inosine, in contrast to its action in hereditary spherocytosis, had much less effect even when its initial concentration was doubled.

Anticoagulants also influenced the autohemolysis of the patients' cells. In heparin, autohemolysis was normal or only slightly elevated when measured either at 37° C or at 4° C. In Na_2H_2 EDTA, lysis was markedly enhanced at 37° C and at room temperature; the value at 4° C was also very high although less than that of defibrinated

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[10] This patient's autohemolysis at 37° C after 48 hours was previously determined by Dr. Clare N. Shumway, Buffalo, N. Y., in April 1958, when the patient was 4 years old. A normal value of 2.2% was obtained. The basis for this apparent change in autohemolytic behavior is not evident.

TABLE V

Autohemolysis and ATP maintenance

	Autohemolysis after 48 hours				ATP after 24 hours*			
	Normal	± 2 SD	Patient 1	Patient 2†	Normal	± 2 SD	Patient 1	pH‡
	%	%	%	%	%	%	%	
37°								
Defibrinated	3.5	2.7	40	45	6	5.1	0†	7.55
Glucose	0.66	0.36	1.5	2.1	70	19	53	7.10
Inosine	0.66	0.38	21	16	16	10	10†	7.25
Inosine × 2			33†		35		24†	
Normal serum			5	8			0†	
Heparin	3.2	1.1	4		7	4		7.55
Na₂H₂ EDTA	3.8	2.8	87		8			7.39
21–23°								
Defibrinated	0.31	0.24	0.9	1.0	51		33	7.67
Na₂H₂ EDTA	0.34	0.30	14	18	70			7.33
Oxalate	0.2		0.5		13			7.48
4°								
Defibrinated	0.24	0.20	28		21	12	16	7.84
Glucose			24†		25†			7.78
Normal serum			0.9†					
Heparin	0.23	0.22	1.0		29	7	41	7.66
Na₂H₂ EDTA	0.21	0.24	11		58	14	67	7.59
ACD§	0.05	0.20	0.3		84	13	91†	7.11

* % of fresh heparinized blood value remaining.
† Single experiment; all other values represent averages of two or more experiments.
‡ Average value in normal subjects after 24 hours.
§ ACD = acid-citrate-dextrose.

blood. Lysis in ACD at 4° C was insignificant. This could not be attributed to the presence of glucose, since supplementing defibrinated blood with glucose at this temperature did not significantly affect the autohemolytic rate.

Normal serum added to the patients' blood also inhibited the lysis. If as little as 7% of the serum in the incubation mixture was normal, the lysis was almost reduced to that seen in control cells, both at 37° and at 4° C. [This phenomenon is the subject of the accompanying paper (19).]

In an attempt to find a common basis for this striking hemolytic behavior, the level of intracellular ATP was determined under the various conditions of incubation (Table V). In defibrinated blood, the decrease in ATP at the various temperatures correlated well with the degree of autohemolysis, yet the patient's cells behaved like normal cells with respect to ATP maintenance in all instances studied.

Although these observations suggest that maintenance of intracellular ATP may be important in inhibiting abnormal autohemolysis in acanthocytes, certain observations indicate that other mecha-

nisms operate as well. First, although small amounts of normal serum are effective in inhibiting the abnormal autohemolysis, they have no influence on the fall in ATP. Second, Na₂H₂ EDTA enhanced, whereas heparin inhibited, the abnormal lysis without apparent relation to ATP maintenance.

It is interesting that Na₂H₂ EDTA augmented the autohemolysis of acanthocytes but oxalate did not. Since Na₂H₂ EDTA binds both calcium and magnesium whereas oxalate precipitates only calcium, the deleterious effect of Na₂H₂ EDTA on these abnormal cells may relate to its chelation of Mg⁺⁺, which has been postulated to act as an internal cohesive of the red cell membrane controlling monovalent cation permeability (41). The escape of hemoglobin would, in this concept, be secondary to the primary change in cation permeability induced by Na₂H₂ EDTA.

To date, acanthocytosis and paroxysmal nocturnal hemoglobinuria are the only conditions in which we have observed an inhibitory effect of heparin on hemoglobin permeability (43, 44); the mechanism of this inhibition has not been studied.

Thus, under various *in vitro* conditions, acanthocytes become permeable to hemoglobin much more rapidly than do normal cells. This process may be inhibited by better preservation of intracellular ATP, by heparin, and by some factor in normal serum not present in serum of patients with acanthocytosis. Accentuation of hemoglobin permeability may occur as a result of chelating Mg^{++} either extracellularly or within the membrane. Whether a common mechanism underlies these observations remains to be determined.

Erythrokinetic studies. To assess the capacity of acanthocytes to maintain viability *in vivo,* we determined several indexes of red cell production and destruction (45). The studies were carried out over a period of $2\frac{1}{2}$ months, during which there was experimental blood loss of sufficient magnitude to require a doubling of the normal rate of red cell production to maintain hematologic equilibrium.

The marrow revealed mild erythroid hyperplasia and reticuloendothelial iron in normal or increased amounts. The plasma iron concentration was 66 mg per 100 ml, and the total iron binding capacity, 324 to 383 mg per 100 ml. The plasma iron turnover was 1.28 mg of iron per 100 ml whole blood per day, indicating that the activity of the erythroid marrow was approximately two to three times normal (45). The destruction rate of circulating acanthocytes was determined in the patient's own circulation and attempted in a normal compatible recipient by Cr^{51} labeling. The survival curve of the patient's cells in his own circulation was within the normal range when corrected for elution and blood withdrawn during the survival study (46). Since removal of blood in the weeks preceding the Cr^{51} labeling had altered the normal age distribution of the patient's red cell population toward enrichment with young cells (47), the "normal" Cr^{51} survival curve obtained is not inconsistent with some degree of accelerated destruction. Nevertheless, when taken together with the plasma iron turnover data, the patient's red cells appear to survive at least 60 days and possibly longer.

Evaluation of the Cr^{51} survival data obtained in the recipient was unsatisfactory due to an abrupt loss of labeled cells 2 weeks after the infusion. This is frequently encountered when homotransfusions with small volumes of cells are carried out (48). Antibody hemolysis is a suspected etiology but has rarely been demonstrated. Attempts to demonstrate such an antibody to the patient's cells in the recipient were unsuccessful.

Certain other erythrokinetic measurements indicated that in this patient accelerated red cell destruction *in vivo* may have been intermittent in nature. In the summer of 1962, reticulocyte counts were between 2.4 and 4.5% (before any significant experimental blood loss), and haptoglobin was repeatedly absent or barely detectable on starch electrophoresis (49). One year later (May 1963) reticulocyte counts were between 1.6 and 2.1%, and haptoglobin (type 2-1) was easily visible. In June 1963, haptoglobin was again just barely visible on starch electrophoresis; serum haptoglobin at this time was about 15 mg per 100 ml by the peroxidase activation method (50).

Red cell survival in acanthocytosis, as measured by Cr^{51}, has been shortened in two of three previously reported cases (8, 11, 17), and anhaptoglobinemia has been described in an additional patient (Table I). Accelerated *in vivo* destruction, perhaps intermittent, has not been excluded in other cases.

Family studies. Attempts to demonstrate abnormalities in the parents and brother of Patient 1 were unsuccessful. Red cell morphology and autohemolysis after 48 hours and even after 72 hours, as well as the erythrocyte and plasma lipids, were all within normal limits (6).

Detection of acanthocytosis. The property of red cells in acanthocytosis to lyse after 48 hours in Na_2H_2 EDTA at room temperature should serve as a convenient screening test for this exceedingly uncommon disorder. To date, gross hemolysis under these conditions has been observed only in acanthocytosis and in two families with unclassified congenital hemolytic anemia, although minor degrees of lysis (0.8 to 3.9%) occur in hereditary spherocytosis (44). To test for false positive results, a series of 690 routine blood samples collected in Na_2H_2 EDTA at the University Hospital was screened as follows: The blood samples were kept at room temperature for 48 to 72 hours and then mixed several times by inversion, after which the cells were left to resediment. After an additional 24 to 48 hours, the supernatant plasma was examined for gross hemolysis. A single positive result in an infant with

hemolytic disease of the newborn due to a Coombs positive ABO incompatibility was obtained.

Summary

1. Red cells in acanthocytosis can be segregated on the basis of their age by centrifugal fractionation. The younger cells show minimal or no morphologic distortion; the degree of- cell deformity increases with cell maturation or aging or both.

2. Glycolysis, phosphogluconate oxidation, and pentose phosphate metabolism in acanthocytes are characteristic of a population slightly enriched with young cells. The capacity of acanthocytes to utilize these pathways for reduced glutathione and adenosine triphosphate maintenance and for methemoglobin reduction is normal.

3. Red cells in acanthocytosis become permeable to hemoglobin more rapidly than normal cells during 48 hours incubation at 37° C and at 4° C. Inhibition of the lysis can be produced a) by certain conditions of incubation leading to better preservation of intracellular adenosine triphosphate, b) by the addition of normal serum, and c) by heparin. Accentuation of hemoglobin permeability occurs in ethylenediamine tetraacetate, possibly due to chelation of Mg^{++}. The extensive lysis of acanthocytosis blood, collected into ethylenediamine tetraacetate and kept at room temperature for 48 to 72 hours, may serve as a convenient screening test for this uncommon disorder.

4. The *in vivo* life span of the red cells is probably slightly shortened, but this accelerated destruction may be intermittent.

5. No abnormality in morphology or autohemolytic behavior was found in the red cells from the parents and brother of one patient.

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