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Constancy of Cell Volume During Shape Change of Erythrocytes Induced by Increasing ATP Content

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Abstract

Erythrocytes in long-preserved blood are spherical, but when the cells are incubated with inosine and adenine, the resulting increase in ATP content is accompanied by a shape change of the cells to discoidal form via a crenated form. The cells incubated with adenine alone or with no addition remain almost unchanged in shape. When incubated with inosine alone, the elevation in ATP level is less than that with both inosine and adenine, and the cell shape remains unchanged or changes partially into a crenated form. These phenomena occur in the presence of EDTA as well as in the absence of serum protein in the media. The cell volumes are measured as packed cell volume after centrifugation, by means of a Coulter counter (model S), and by determination of the intercellular space by the use of ^{131}I-labeled bovine serum albumin. The results show that no alteration in cell volume occurs during the shape changes. Accordingly, the surface area of the cell must increase with increase in the ATP content. This suggests that both the lipid bimolecular layer and the undermembrane structure are altered during the shape change.

Key Words: Red blood cell; ACD blood; adenine; inosine; ATP; red cell shape; surface area; erythrocyte; undermembrane structure.

Introduction

Nakao and his colleagues pointed out that the shape of red cells changes reversibly from discoidal to smooth spherical via a crenated form and *vice versa* depending on the ATP level in the cells (Nakao *et al.*, 1959; Nakao *et al.*, 1960a; Nakao *et al.*, 1960b; Nakao *et al.*, 1961a). The precise mechanism of this shape change is not known, although the involvement of an undermembrane network structure has been widely accepted (Guidotti, 1972; Sheetz *et*

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al., 1976; Fairbanks *et al.*, 1971; Bennet and Branton, 1977; Bennet, 1978; Nakashima and Beutler, 1978). Further information on this phenomena should be helpful in elucidating the molecular mechanism. Since the shape change apparently resembled mitochondrial swelling (Lehninger, 1959a, b), some change in the cell volume was expected. However, this was not the case, as will be described here.

Materials and Methods

Three different methods were used for determination of the cell volume at room temperature. One was hematocrit determination after centrifugation at 10,000 rpm for 5 min, with correction for the error due to hemolysis (usually less than 10%). Centrifugation for 10 min gave the same values. Another was the use of a Coulter counter (model S), which operates on the principle of determination of the electrical resistance of the cell. The third was to estimate the intercellular space with ^{131}I-labeled serium albumin. A 1-ml sample of plasma obtained from the ACD blood to be used in this experiment was labeled with 15 μCi of ^{131}I essentially according to Roholt and Pressman (1972). The radioactive proteins were purified by column chromatography on Sephadex G-25. Next, 0.3 ml of the labeled plasma was added to 24 ml of the ACD blood from which 4 ml of plasma had been separated to concentrate the cells. Aliquots of 3 ml were each mixed with 0.3 ml of 0.9% NaCl alone, or containing adenine (5 mg), inosine (20 mg), or adenine (5 mg) plus inosine (20 mg), and the mixture was incubated for 0 or 2 hr at 37°C. A 1-ml sample of plasma was counted in a G.M. counter for 5 min. The counting error was within 1%. For the zero time control, samples were preincubated for 5 min at 37°C.

Red cells which had been preserved as ACD blood or ACD packed cells for 6–10 weeks were used. The media used were ACD serum and other isoosmotic media as described in the footnotes to the tables. In some experiments erythrocytes were washed three times with 10 volumes of physiological saline to eliminate the effect of serum proteins. Adenine and inosine were purchased from Ajinomoto Co. (Tokyo). ATP was measured with the ATP assay kit of Boehringer Mannheim.

Results and Discussion

Increase of ATP in the Presence of Adenine Plus Inosine in Various Media

Human red cells which had been preserved at 4°C contain very small amounts of ATP (0.05–0.17 μmol/ml cell) (Table I). Incubation of these cells with adenine plus inosine increased the cellular concentration of ATP rapidly

Table I. ATP Content of Stored Erythrocytes After Incubation with no Addition or with Addition of Adenine, Inosine, or Adenine Plus Inosine[a]

Weeks preserved	Medium	ATP (μmol/ml red cells)				
		0°C−AI	37°C+AI	37°C−A	37°C-I	37°C−AI
5.1	a	0.071	0.930	0.386	0.096	0.072
6.6	a	0.137	1.276	0.612	0.089	0.087
6.7	a	0.050	1.501	0.249	0.075	0.099
6.9	a	0.142	1.300	0.662	0.177	0.145
7.0	a	0.173	1.333	0.558	0.181	0.180
7.4	a	0.145	1.040	0.441	0.128	0.155
8.4	b	—	1.310	0.532	0.150	0.204
10.1	c	0.117	1.068	0	0	0
10.3	c	0.117	1.265	0.240	0.096	0.047
0	b		1.2			
Shape of most cells:		sphere	disc	sphere	sphere	sphere

[a]Medium a: 60 mM sucrose, 123 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 4 mM Na_2HPO_4, 1 mM Na_2EDTA. b: ACD plasma; c: 0.9% NaCl + 0.1 M NaP buffer (10:1); 2 ml of medium containing no addition (−AI) or with 8 mM adenine (−A), 40 mM inosine (−I), or adenine plus inosine (+AI) was added to 1 ml of washed erythrocytes (except in case b) and the suspension was incubated for 3 hr at 37°C.

to 1–1.5 μmol/ml in all media used in this study. The addition of inosine alone partly regenerated ATP, but that of adenine alone was ineffective. The time course showed that the increase of the ATP content was almost saturated at 3 hr. For example, the ATP content was 0.10, 0.84, 1.14, 1.64, or 1.72 μmol/ml cells at 0, 45, 90, 180, or 360 min after the start of incubation, while the number of discocytes or stomatocytes was 0, 10, 50, 70, or 80% respectively. These results are consistent with the positive feedback theory proposed previously (Nakao *et al.*, 1961b).

Shape Change Accompanying the Increase of Intracellular ATP in Various Media

This increase of ATP in red cells accompanied by a change in shape was described previously (Nakao *et al.*, 1960a; Nakao *et al.*, 1960b; Nakao *et al.*, 1961) (Table I, Fig. 1). Most of the erythrocytes changed from spherical to discoidal form via a crenated form as their ATP content increased during incubation with inosine plus adenine, and in cases where the increase in ATP content was smaller, e.g., after incubation of the 8-week-old blood with inosine alone, some erythrocytes changed from spherical to crenated form and some others became discoidal form. As ATP content in some ACD bloods, especially after preservation for more than 8 weeks, did not increase sufficiently when incubated only with inosine, as in Table I, the shape of erythrocytes did not change remarkably. Although the transformation was complete when unwahsed preserved ACD blood was used and stomatocytes,

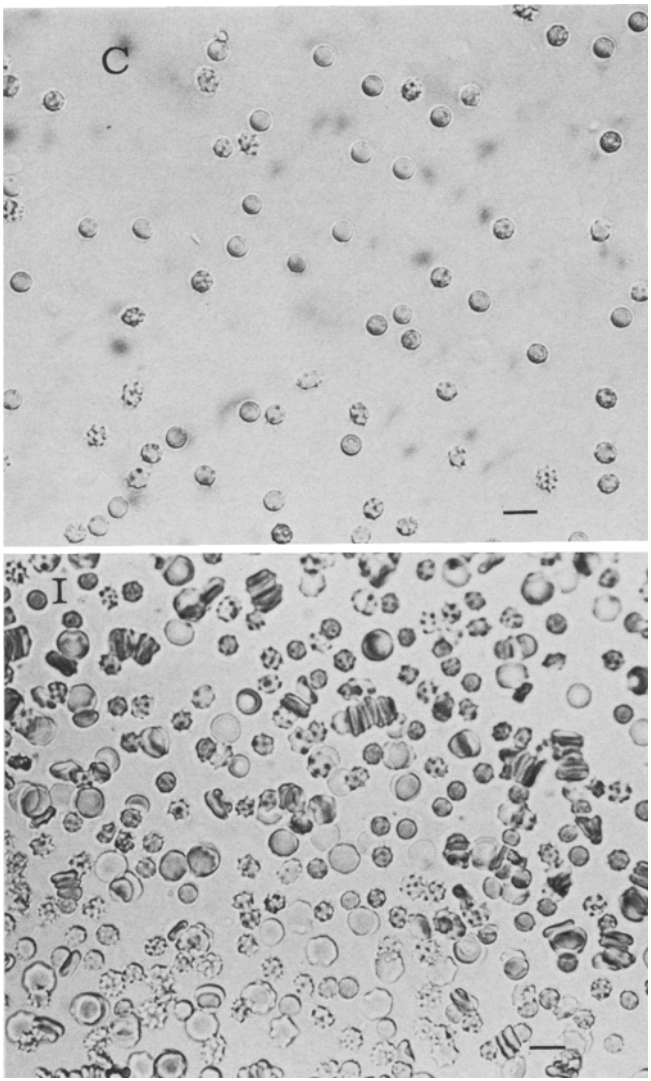

Fig. 1. Shapes of erythrocytes in hanging drops. Two volumes of plasma containing 4 mM adenine, 20 mM inosine or 4 mM adenine plus 20 mM inosine was added to 7-week-old ACD blood, and the suspensions were incubated for 4 hr at 37°C. (C) No addition; (A) plus adenine; (I) plus inosine; (AI) plus adenine and inosine. Scale: 10 μm.

rather than discocytes, increased when ACD packed blood was used, the phenomena were essentially the same when various media and/or well-washed cells were used, as described in Tables II, III, and IV. Several features seem noteworthy in these experiments. First, the presence of calcium

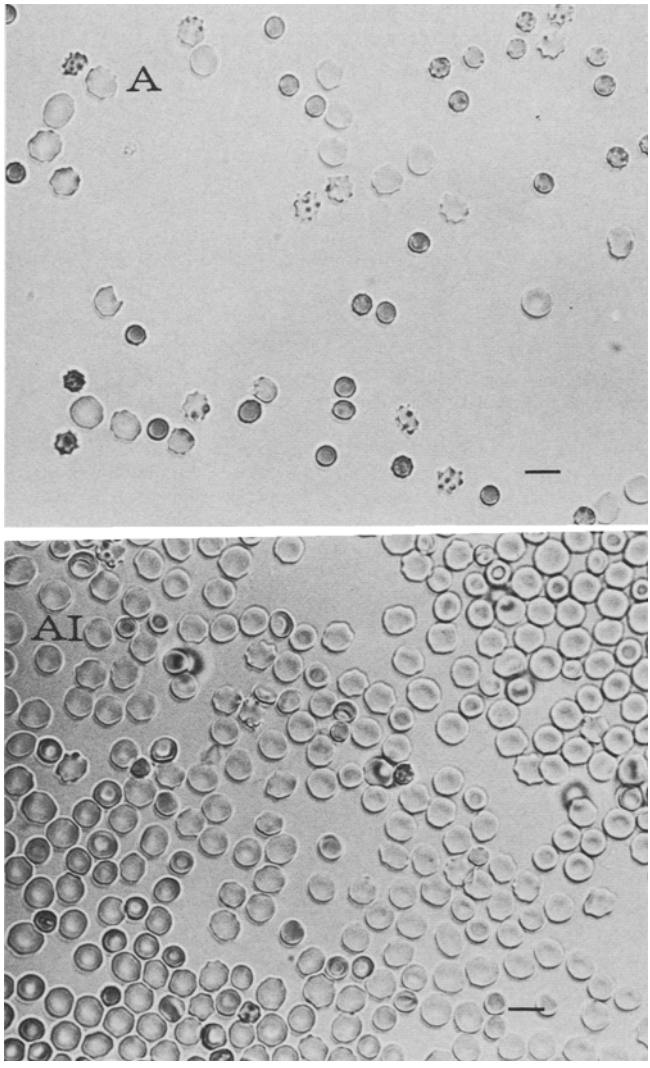

Fig. 1. Continued.

outside the cells was necessary for this shape change (Table II, experiment 4). The shape of the cells which had been preserved in ACD medium for several weeks changed. Second, the shape change occurred even in the absence of serum protein. This also eliminated the possibility that a detergent molecule or lipid might be released from or bind to cell membranes and induce the transformation, because such a molecule could not be solubilized into the media in the absence of plasma proteins. Third, various concentrations of sucrose did not affect the shape change, although the absolute cell volume

Table II. Cell Volumes Determined by the Hematocrit Method[a]

Experiment number	Weeks preserved	Number of experiments	Relative hematocrit (%) 37°C+AI	37°C−A	37°C−I	37°C−AI	pH
1	10.6	2	100	101.1 ± 2.1	104.7 ± 3.2	101.2 ± 2.5	6.5
2	6.7–7.3	5	100	98.0 ± 4.2	98.3 ± 4.2	100.7 ± 1.0	7.0
3	8.3	1	100	103.8	103.3	94.8	6.5
4	8.4–10.0	4	100	99.9 ± 1.4	105.6 ± 1.2	103.7 ± 1.0	6.9
5	9.9–10.4	3	100	99.5 ± 2.5	101.1 ± 2.0	102.6 ± 4.7	7.7
6	10.1–10.3	2	100	100.3 ± 1.5	99.5 ± 4.1	102.6 ± 2.2	6.8
Shape of most cells:			Disc	sphere and crenated	sphere	sphere	

[a]Medium for experiment No. 1: 50 mM sucrose, 100 mM NaCl, 5 mM NaH_2PO_4, 1 mM Na_4EDTA; 2: 60 mM sucrose, 123 mM NaCl, 3 mM KCl, 1 mM Na_4EDTA, 5 mM P buffer; 3: 60 mM sucrose, 110 mM NaCl, 10 mM Na_3 citrate, 2 mM $MgCl_2$, 1 mM EGTA, P buffer; 4: ACD plasma, 5 mM P buffer; 5: 50 mM sucrose, 5 mM P buffer; 6: 0.9% NaCl, P buffer; 2 ml of medium containing no addition or with 8 mM adenine (−I), 40 mM inosine (−A), or adenine plus inosine (+AI) was added to 1 ml of washed erythrocytes (except in experiment No. 4) and the suspension was incubated for 3 hr at 37°C. Control experiments with different cell volumes were performed as follows. To 5-week-old ACD packed cells (A) 5 volumes of 0.25 M sucrose was added. After 3 hr at 37°C (B), the cells were washed and incubated in 0.75% NaCl for a further 3 hr (C). Hematocrit % (by centrifugation) per hemoglobin % was (A) 4.84, (B) 2.77, and (C) 3.55. Osmotic fragility according to Parpart's method (Parpart *et al.*, 1947) was (A) 0.49% NaCl, (B) 0.29% NaCl, and (C) 0.43% NaCl, and these figures corresponded with the hematocrit values.

was changed when the cells were incubated in media containing various percentages of isoosmotic sucrose. For example, ACD blood (8 weeks old) was incubated with 0.8 volume of 0.25 M sucrose containing 10 mM Na citrate and 4 mM Na_2HPO_4 for 24 and 48 hr at 37°C. The shape of cells was both 10% sphere and 90% crenated sphere, but the cell volumes measured with the Coulter counter were 93 and 84.5 μ^3 respectively. On the other hand, when the cells were incubated in the same medium with 8 mM adenine and 40 mM inosine, the shape of the cells was discoidal or stomatocytic both at 24 and 48 hr incubation although the cell volumes were 95.5 and 82.5 μ^3 respectively. Similar results were also obtained with the aid of the centrifugation hematocrit method. These findings indicated that the shape change under consideration did not involve any effect of sucrose or osmotic pressure.

Constancy of Cell Volume During Shape Changes

The cell volume was compared after incubation with no addition, or with addition of inosine alone, adenine alone, or inosine plus adenine. The results of measurement by the three different methods are summarized in Tables II, III, and IV.

The cells incubated with adenine plus inosine mostly became discoidal, with some spherical cells. Some of the erythrocytes incubated with inosine alone changed to a crenated form or discoidal form when the cellular ATP

Table III. Red Cell Volumes Determined by Measurement with a Coulter Counter[a]

Experiment number	Weeks preserved	Medium	MCV (μ^3) 37°+AI	37°−A	37°−I	37°−AI	pH
1	10.8	a	92.0	93.5	92.3	92.0	6.6
2	6.0	a	92.0	94.5	95.0	95.0	6.5
3	6.7	b	87.8	89.7	92.3	91.5	7.1
4	7.3	b	85.0	87.5	90.0	88.8	7.0
5	7.3	b	86.0	90.0	92.0	91.5	7.1
6	8.3	c	97.0	98.5	96.3	95.0	6.5
7	10.3	d	96.1	96.2	93.8	94.5	—
8	10.3	d	98.4	98.2	95.7	95.2	—
Shape of most cells:			disc	disc and sphere	sphere	sphere	

[a]Medium a: 50 mM sucrose, 100 mM NaCl, 5 mM NaH_2PO_4, 1 mM Na_4EDTA; b: 60 mM sucrose, 123 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 4 mM Na_2HPO_4, 1 mM Na_4 EDTA; c: 60 mM sucrose, 110 mM NaCl, 3 mM KH_2PO_4, 2 mM K_2HPO_4, 10 mM Na_3 citrate, 2 mM $MgCl_2$, 1 mM EGTA; d: 30 mM sucrose, 139 mM NaCl, 1 mM KH_2PO_4, 3 mM K_2HPO_4. Experimental details are the same as described in the footnote to Table II. Control experiments with different cell volumes were performed as follows: To 8-week-old ACD packed cells (A) 0.7 volume of 0.25 M sucrose containing 10 mM Na citrate, 4 mM NaH_2PO_4, 8 mM adenine, 40 mM inosine, and 15 mM glucose was added and incubated for 3 hr at 37°C (B). MCV was 99 μ^3 (A) and 87 μ^3 (B). Mean corpuscle hemoglobin was 32.0 pg (A) and 31.6 pg (B).

content increased a little. In other cases, the cells remained spherical. Nevertheless, in the cases of all four different additions, the cell volume did not change or changed only very slightly during each series of incubations in various media. It is generally accepted that any method for measuring the cell volume is not perfect and small differences are usually observed between values obtained by different methods using different principles. Therefore, the coincidence of the results obtained by the three different techniques in this experiment should be stressed. In Table III MCVs of the cells in medium b (Nos. 3–5) incubated with adenine plus inosine were smaller than those without adenine plus inosine by about 5%. However, the relative hematocrit values in the same medium in Table II (No. 2) show no difference for cells

Table IV. Erythrocyte Volume Estimated from the Intercellular Space Determined by the Use of ^{131}I-Labeled Serum Albumin[a]

Additon	Cell volume (% of cell suspension) Before	After	Shape of most cells
None	46.4%	45.0%	Sphere
Adenine	46.4	47.0	Sphere
Inosine	48.0	48.0	Sphere and crenated
Adenine plus inosine	47.1	45.6	Disc

[a]Experimental details are as described in Materials and Methods. Data are averages of duplicate samples.

incubated with and without addition of adenine plus inosine. The cause of this discrepancy is unclear at present. The volume change of 5% did not affect our discussion on cell surface area.

Unchanged Distribution of Cell Volume in Differently Shaped Cells

The distribution of cell size was examined with the Coulter counter, model S-plus, in order to compare the cell volume distributions of the cells incubated with no addition and with addition of adenine alone, with inosine alone, and with adenine plus inosine. All the distribution profiles were symmetrical and essentially identical, even though the cells with and without adenine plus inosine were spherical and discoidal, respectively. The data for no addition, addition of inosine alone, and addition of adenine plus inosine are shown as an example in Fig. 2.

Cell Surface Area

The shape change from sphere to disc was carefully re-examined. When the cells were fixed with a cross-linking reagent, such as glutaraldehyde or osmium tetroxide at various concentrations and at various temperatures, small changes were induced, especially in the form and number of spikes. Therefore, samples suspended in the media described above were directly observed under a microscope (Zeiss photomicroscope) with or without differential interference optics. The diameter of each cell was determined with a scale in an ocular lens. Since there was no difference between the volume of the sphere and that of the disc obtained after incubation with adenine plus inosine, the surface areas must be different. The average diameter of erythrocytes incubated for 3 hr with no addition in ACD plasma was $5.3 \pm 0.19\ \mu$ ($n = 50$). On the assumption that the spherocytes are complete spheres, the volume was calculated to be $77.9\ \mu^3$ and the surface area, $88.2\ \mu^2$. When the stored erythrocytes were incubated in ACD plasma in the presence of adenine plus inosine, about 35% of the cells were apparently discocytes and the rest were stomatocytes. The diameter of these discocytes was determined to be $8.19 \pm 0.55\ \mu$. As it is difficult to calculate the volume and surface area of the stomatocytes from the diameter, they were calculated on the assumption that these stomatocytes were of the same volume and surface area as the discocytes in the sample. If all the erythrocytes in this experiment have the same ratios of diameter:volume:surface area as fresh erythrocytes [diameter $8.5\ \mu$, cell volume $87\ \mu^3$, surface area $163\ \mu^2$ according to the literature (Ponder 1971)] the volume and surface area should therefore be $78.2\ \mu^3$ and $151.8\ \mu^2$ respectively. As the cell volumes of spherocytes ($77.9\ \mu^3$) and discocytes ($78.2\ \mu^3$) thus calculated are in good agreement, this result is consistent with that of direct measurement of the cell volumes described

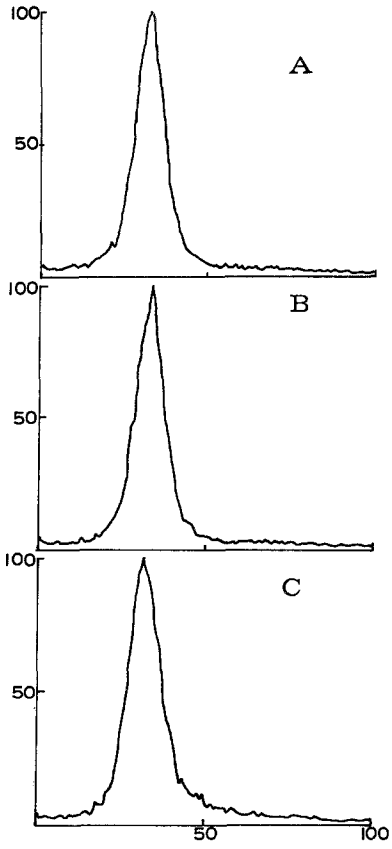

Fig. 2. Distribution of cell volumes. Erythrocytes incubated with no addition (A), with addition of inosine (B), and inosine plus adenine (C) (experiment I in Table II) were analyzed with a Coulter counter model S plus. MCV: (A) 94.5 μ^3; (B) 961. μ^3; (C) 96.2 μ^3. Abscissa, relative cell volume; ordinate, frequency.

above. Accordingly, the difference in the calculated surface area (88.2 μ^2 for spherocytes and 152 μ^2 for discocytes) seems to be well established. As the structure of human erythrocytes consists of a lipid bilayer and an undermembrane network (spectrin, actin, 4.1, 4.9 structure), both structures can presumably expand reversibly depending on the ATP level in the cell (Nakao *et al.*, 1961a). The expansion of the lipid bimolecular layer might passively follow the expansion of the undermembrane network since the latter shape change is presumably a direct response to the increase in the ATP level in the cell.

Acknowledgments

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