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STUDIES ON RED CELL MEMBRANE PROTEINS

I. ELECTROPHORETIC PATTERNS OF VARIOUS MEMBRANE PROTEIN EXTRACTS ON POLYACRYLAMIDE DISC GEL ELECTROPHORESIS

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Abstract

Electrophoretic patterns of membrane proteins in mature human red cells were investigated on the SDS-polyacrylamide disc gel electrophoresis. The best resolution of these membrane proteins was obtained on the 11 % gel system with discontinuous buffers. Some of peripheral proteins of red cell membranes were lost either by lysing red cells wish 5 mM PO_4 buffer (pH 8.0) or by washing red cell ghosts more than three times even with the 20 mOsm Na/K PO_4-buffered saline (pH 7.4). Contrary to commonly held belief, on the 11 % gel system, a major glycoprotein of red cell membrane proteins (PAS-1) appears to be different from the band III stained by coomassie blue.

The electrophoretic patterns consisted mainly of bands I and II in peripheral protein extracts, and of bands of the lower molecular weight including the band III in integral protein extracts, respectively.

The SDS polyacrylamide disc gel electrophoresis would be suitable as a preparatory procedure for extraction and purification of specific protein fractions of red cell membrane proteins.

INTRODUCTION

Membrane proteins of human red cells have recently been extensively investigated[1-6]. This fact will be attributed chiefly to the great success in solubilization[7-10] of red cell membrane proteins especially with sodium dodecyl sulfate (SDS). The SDS polyacrylamide disc gel electrophoresis[11-14] has been widely utilized for the fractionation of red cell membrane proteins. The fractions of red cell membrane proteins are nominated by Fairbanks *et al.*[13], such as bands I, II, etc., and the phy-

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sicochemical characteristics[1,13,15,16] of these membrane protein fractions have been clarified, even partially. Recently, the two-dimensional slab gel electrophoresis[17] has been introduced to investigate human red cell membrane proteins.

On the other hand, various procedures for the extraction of red cell membrane proteins from whole ghosts[18,19] have been described, such as peripheral proteins[20,21], integral proteins[22,23], lipid-extracted proteins[24], and glycophorin[25,26]. These extracted membrane proteins are extremely useful to investigate a role of the membrane proteins on the structure and functions of human red cells.

Thus, in this communication, electrophoretic characteristics of these various membrane protein preparations of human red cells were investigated on the SDS polyacrylamide disc gel electrophoresis. In addition, several factors affecting the electrophoretic pattern of the membrane proteins were also described.

MATERIALS AND METHODS

(I) Preparation of membrane proteins from mature human red cells.

1) Preparation of red cell ghosts.

Venous blood was drawn from normal individuals with heparin as an anticoagulant, and plasma and buffy coat were eliminated from the blood by centrifugation at 4°C at 10,000×g for 20 minutes. One volume of red cells was washed three times with 10 volumes of isotonic sodium/potassium phosphate-buffered saline (310 mOsm, pH 7.4) according to the method of Dodge *et al.*[18]. After washing the red cells, 28 volumes of the sodium/potassium phosphate-buffered saline (20 mOsm, pH 7.4) were added to 2 volumes of the packed red cells, and the mixtures were kept at 4°C for 10 minutes. After the hypotonic lysis of red cells, the mixtures were centrifuged at 300,000×g for 40 minutes on the Sorvall refrigerated centrifuge, and the supernatant was eliminated. The red cell ghosts as pellets were further washed three times with 20 mOsm lysing buffer, and so-called nuclear proteins at the bottom were taken out. The red cell ghosts were resuspended in the buffer with 0.1 N HCl, and kept at 4°C overnight. The final preparations of red cell ghosts were obtained after centrifugation at 300,000×g for 40 minutes.

In some experiments, red cells were washed with isotonic saline solution instead of isotonic Na/K phosphate-buffered saline, and lysed with the 20 mOsm buffer (1 : 30 v/v). The final preparations of red cell ghosts were obtained without the addition of 0.1 N KOH.

In some experiments, whole blood (25 ml) was obtained with 1 ml of ethylenediaminetetraacetic acid (EDTA), and the supernatants were eliminated after centrifugation of the whole blood with 26 ml of the mixture of 5 mM sodium phosphate buffer and 0.15 M saline (pH 8.0) at 4°C at 10,000×g according to the method of Steck *et al.*[19]. The red cells were further washed three times with the mixture, and the packed red cells were lysed (1 : 30 v/v) with the 5 mM sodium phosphate buffer (pH 8.0) at 4°C. The final preparation of red cell ghosts were obtained after the supernatants and the nuclear proteins were eliminated by the centrifugation at 300,000×g.

2) Preparation of peripheral proteins.

The peripheral proteins were prepared by dialysing 1 ml of the white ghosts obtained by the method[18,19] described above against 100 ml of 1 mM tris HCl (pH 8.0) at 4°C for 36 hours. The soluble fractions of the dialysed membrane proteins were obtained by the centrifugation at 9,000,000×g for 30 minutes, and the final concentration was adjusted to 3 mg/ml (w/v).

3) Triton X-100 extractable proteins.

The red cell ghosts (540 μl) prepared by the method of Dodge *et al.*[18] were incubated with 60 μl of 20 % Triton X-100 at room temperature for 5 minutes. The Triton X-100 extractable membrane proteins were obtained as supernatants after the centrifugation at 12,000,000×g for 30 minutes.

4) Lipid-extracted proteins[24].

After plasma and the buffy coat were eliminated, red cells were washed three times with 310 mOsm tris HCl buffer (pH 7.5), and red cell ghosts were prepared by the method of Dodge *et al.*[18]. To one volume of the red cell ghosts, 15 volumes of the mixture of ethanol and ether (3 : 1 v/v) were added and kept at -15°C for 2 hours. The precipitates were washed three times by the centrifugation with the buffer at 13,000 ×g for 30 minutes, and the lipid-extracted proteins were obtained as sediments after the filtration with Whatman filter paper # 50.

(II) Polyacrylamide disc gel electrophoresis.

Two different procedures were utilized for the electrophoresis.

1) The 5.6 % polyacrylamide disc gel method[13].

The following stock solutions were prepared.

(A) Acrylamide-bisacrylamide concentrates;
40 g of acrylamide (Eastman # 5521)

1.5 g of N,N'-methylenebisacrylamide (Eastman # 8383)
made 100 ml by adding bidistilled water.

(B) 20 % sodium nodecyl sulfate (SDS) solution,

(C) 1.5 % (w/v) ammonium persulfate solution,

(D) 0.5 % (w/v) N,N,N',N'-tetramethylethylenediamine (TEMED; Eastman # 8178) solution,

(E) A ten-fold concentrated buffer solution (pH 7.4);
40 ml of 1.0 M tris
10 ml of 2.0 M sodium acetate
10 ml of 0.2 M EDTA
pH to 7.4 with acetate acid
made 100 ml by adding bidistilled water

The composition of the buffer for the electrophoresis consisted of;
100 ml of the 10 fold concentrated buffer solution (E)
50 ml of 20 % SDS solution (B)
850 ml bidistilled water.

Polyacrylamide gels were prepared as follows;
1.4 ml of acrylamide-bisacrylamide concentrates (A)
1.0 ml of the 10 fold concentrated buffer solution (E)
0.5 ml of 20 % SDS solution (B)
5.6 ml bidistilled water.

To polymerize the gel mixture, 1.0 ml of 1.5 % ammonium persulfate (C) and 0.5 ml of 0.5 % TEMED (D) were added sequentially, and 2.5 ml of the mixtures were transferred into the glass columns (150 mm×6 mm). The final concentrations of acrylamide was 5.6 %.

After the completion of polymerization, solubilized membrane proteins (150~200 μg membrane proteins per column) were applied on the gels, and the electrophoresis was carried out at 2 mA per column for 4 to 6 hours. Pyronin Y was added as a marker of the electrophoresis.

Membrane proteins were solubilized for the electrophoresis:
25 μl of whole ghosts
10 μl of 20 % SDS
10 μl of β-mercaptoethanol
55 μl of the buffer for electrophoresis.

After electrophoresis was carried out, membrane proteins in gels were fixed and stained by incubation overnight with the mixture of 25 % isopropyl alcohol solution and 10 % acetic acid solution containing 0.05 % coomassie blue solution (50 ml per gel). The excessive dyes in gels were eliminated by the mixture of 10 % isopropyl alcohol and 10 % acetic acid, and by 10 % acetic acid alone, sequentially.

In some experiments, glycoproteins of red cell membranes were stained by the method[13] with periodic acid Schiff (PAS) after membrane proteins were fixed with 25 % isopropyl alcohol and 10 % acetic acid. The gels were treated sequentially with (1) 0.5 % periodic acid (H_5IO_6) solution for 2 hours, (2) the mixture of 0.5 % sodium arsenite and 5 % acetic acid for 60 minutes, (3) the mixture of 0.1 % sodium arsenite and 5 % acetic acid twice for 20 minutes, and finally (4) 5 % acetic acid for 20 minutes. Each gel was further incubated overnight with 10 ml of the Schiff's solution, and then destained by the mixture of 0.1 % sodium metabisufite ($Na_2S_2O_5$) and 0.01 N HCl for 5 hours, until the addition of formaldehyde did not cause the red color in these gels any more.

2) The 11 % polyacrylamide disc gel method.

To obtain a better resolution of membrane proteins, the procedures described above were modified by utilizing the higher concentration of polyacrylamide gel (11 %) and for the different discontinuous buffer system.

The reagents were;

(A) 20 % (w/v) SDS solution

(B) Cathodal buffer (pH 8.64);
0.04 M boric acid
0.041 M tris
0.1 % SDS

(C) Anodal doubly-concentrated buffer (pH 9.2);
0.0612 N HCl
0.8688 M tris

(D) Tris-sulfate buffer (pH 6.1)
0.053 N H_2SO_4
0.108 M tris

Gels were prepared by;
11.0 g of acrylamide
0.1 g of N,N'-methylenebisacrylamide
50 ml of the anodal boudly-concentrated buffer (C)
made 100 ml with bidistilled water.

Gels were polymerized by the addition of 50 mg of ammonium persulfate and 150 μl of TEMED, sequentially. Two and a half milliliter of the polymerized gels were poured into glass columns (6 mm × 150 mm).

In this method, the preparatory stacking gels were overridden onto the running gels for electrophoresis. The stacking gels consisted of;

3.0 g of acrylamide
0.2 g of N,N'-methylenebisacrylamide
50 ml of tris-sulfate buffer (pH 6.1)

made 100 ml with the bidistilled water, and the gels were polymerized by 50 mg of ammonium persulfate and 150 μg of TEMED, as mentioned above. A half milliliter of the stacking gels was overriden onto the polymerized running gels, and then the membrane proteins (150~250 μg per column) were applied on the gels. The electrophoresis was carried out at 3 mA per column for 2 hours, with pyronin Y as a marker. The fixation and staining of membrane proteins in gels were exactly the same as described above.

RESULTS

(I) Various factors affecting the electrophoretic patterns of red cell membrane proteins.

1) Effect of washing red cell ghosts.

Red cell ghosts were prepared from washed red cells by hypotonic lysis according to the method of Dodge *et al.*[18]. Effect of washing red cell ghosts on the electrophoretic patterns of their membrane proteins was examined on the polyacrylamide disc gel electrophoresis. Results are shown in Figure 1. Some of red cell ghosts were lysed only once with 20 mOsm Na/K phosphate buffered saline (pH 7.4), and then packed by the centrifugation at 300,000×g for 40 minutes. In addition to the ghost preparations described above ("No wash"; the upper portion of Fig. 1), most of red cell ghosts were washed 3 times with the 20 mOsm

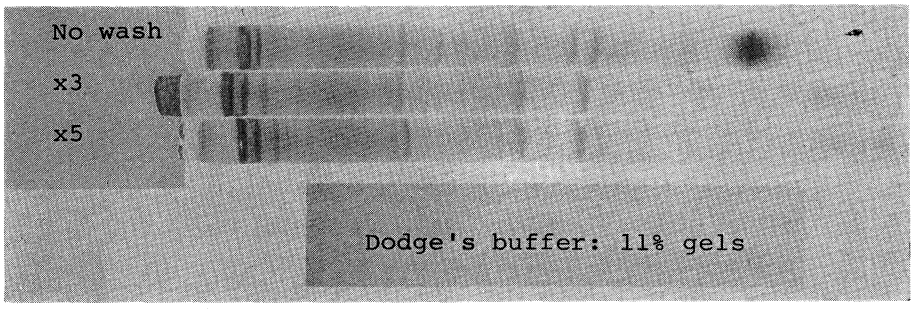

Fig. 1. Effect of washings on the pattern of red cell membrane proteins. Red cell membrane ghosts were lysed against the hypotonic Dodge's buffer (NO WASH), and further washed three times ("×3") or five ("×5") against the buffer. Electrophoresis was carried out on 11 % gels for 2 hours.

buffer ("×3"); the middle portion of Fig. 1, or 5 times with the buffer ("×5"); the lower of Fig. 1.

As shown in Figure 1, several bands of membrane proteins in red cell ghosts were lost by washing the ghosts 3 times ("×3") or 5 times ("×5"), compared to the results without washing red cell ghosts (NO WASH). The membrane proteins lost were identified as some of peripheral proteins as bands V, VI, and others nominated by Fairbanks *et al.*[13]. As shown in Fig. 1, the contents of hemoglobin contaminated in the red cell ghosts, as shown at the far right on each gel, were substantially diminished in sample ("×3") or ("×5"), compared to the results without washing ("NO WASH"). There could be observed no significant differences of the membrane protein patterns between in red cells washed three times ("×3") and in red cells washed five times ("×5").

2) Effect of various buffers on the patterns of membrane proteins of red cell ghosts.

Red cell ghosts were prepared by hypotonic lysis with the 20 mOsm phosphate buffer (pH 7.4) or with the 5 mM phosphate buffer (pH 8.0). The ghosts were washed three times with either buffer. The electrophoresis was carried out on the 11 % gel system with polyacrylamide disc gels.

Results are shown in Figure 2. Although the conditions for the preparation of red cell ghosts are exactly identical between the two except for the different buffers, several bands of membrane proteins

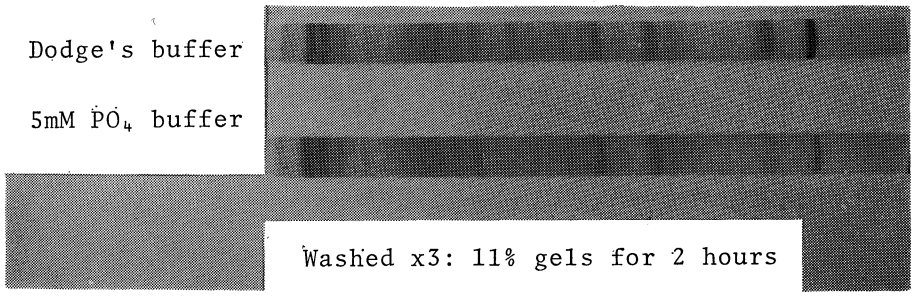

Fig. 2. Effect of the Dodge's buffer or of 5 mM phosphate buffer on the pattern of red cell membrane proteins.
Red cell ghosts were lysed, either against the Dodge's buffer or the 5 mM PO_4 buffer, and washed three times with the Dodge's buffer or the 5 mM PO_4 buffer, respectively. Electrophoresis was carried out on 11 % gels for 2 hours.

were lost in the cases with the 5 mM PO_4 buffer (pH 8.0; the upper portion of Fig. 2), compared to the results with 20 mOsm PO_4 buffer (pH 7.4; the lower portion of Fig. 2). These bands lost appear to be some of peripheral proteins of red cell membranes such as bands V, VI, and others.

3) Comparison of the electrophoretic patterns of red cell membrane proteins between on the 5.6 % gel system and on the 11 % gel system.

The major differences of those two procedures are summarized in Table 1. On the 11 % gel system, β-mercaptoethanol was added to solubilize ghost membrane proteins, and the concentration of acrylamide gels is much higher (11 %) than that on the 5.6 % gel system. Furthermore, on the 11 % gel system, different buffers are utilized at the cathode and the anode for electrophoresis, and only shorter period is required for the electrophoresis.

TABLE 1.

Characteristics of polyacrylamide disc gel electrophoresis by the Fairbanks' method and by the modified method with 11 % gels

	POLYACRYLAMIDE DISC GEL ELECTROPHORESIS	
	FAIRBANKS' METHOD (BIOCHEM. 10:2606, 1971)	MODIFIED METHOD
SOLUBILIZATION OF GHOST PROTEINS BY	1 % SDS, 1 mM EDTA, 40 mM DTT	1 % SDS, 40 mM DTT, 1 % B-MERCAPTOETHANOL
GEL COMPOSITION	5.6 % POLYACRYLAMIDE	11 % POLYACRYLAMIDE
BUFFERS: ANODAL:	0.4 M TRIS 0.2 M SOD. ACETATE 0.02 M EDTA (pH 7.4)	0.0306 N HCl 0.0434 M TRIS (pH 9.2)
CATHODAL:	SAME AS ABOVE	0.205 M TRIS 0.04 M BORIC ACID (pH 8.64)
ELECTROPHORESIS	3 mA/COLUMN FOR 3 HRS	3 mA/COLUMN FOR 2 HRS

As shown in Figure 3, the better resolution of membrane proteins was obtained on the 11 % gel system (the lower part of Fig. 3), compared to that on the 5.6 % system (the upper part). Due to the higher concentration of acrylamide, each band of membrane proteins is sharply

isolated as thinner bands, with the additional advantage of better handling, especially for slicing gels, such as in tracer experiments.

4) PAS-positive bands of membrane proteins.

Red cell ghosts were prepared by washing red cells three times with the 20 mOsm PO_4 buffer (pH 7.4). Polyacrylamide disc gel electrophoresis was carried out on the 11 % gel system. Some gels were stained by coomassie blue, and others by the PAS staining, respectively.

Results are shown in Figure 4. Three PAS-positive bands (the lower part of Fig. 4) were observed on electrophoretic patterns of red cell membrane proteins. The major PAS-positive band, PAS-1, was located at the position slightly toward to the lower molecular weight side from the band III visualized by coomassie blue, which is shown at the upper

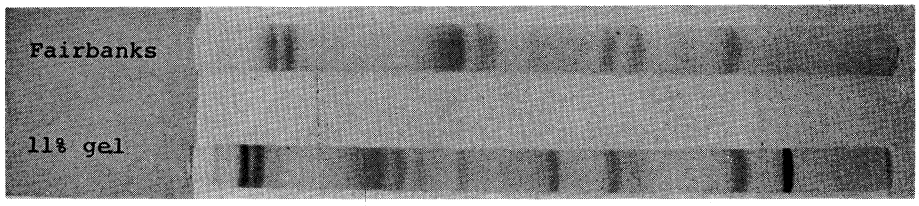

Fig. 3. A better resolution of red cell membrane proteins on 11 % gels
Red cell membrane ghosts were lysed against the Dodge's buffer and washed three times. Electrophoresis was carried out either by the Fairbanks' method or 11 % of gel method.

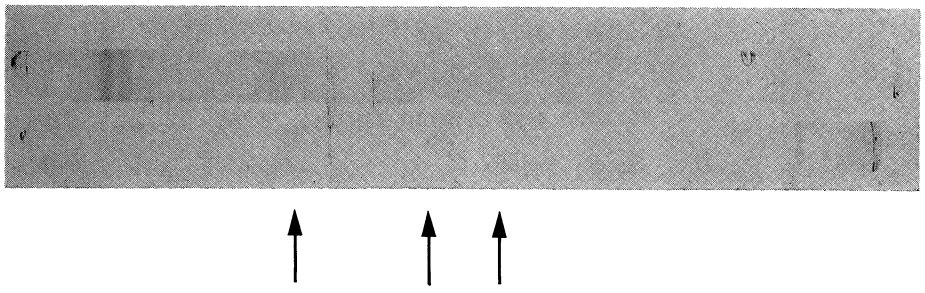

Fig. 4. Polyacrylamide disc gels stained by coomassie blue or by PAS
Red cell membrane ghosts were prepared with the Dodge's buffer, and electrophoresis was performed on 11 % gels. An upper gel was stained by coomassie blue for membrane protein fractionation, and a lower gel was stained by PAS for glycoproteins.

gel of Fig. 4. Two other bands, PAS-2, and PAS-3, are located at the high molecular weight side of the band V, and at the low molecular weight side of the band VI, respectively.

(II) Electrophoretic pattern of red cell membrane protein extract on the SDS polyacrylamide disc gel electrophoresis.

Various membrane protein extracts of human red cells have been described, such as peripheral proteins by Mazia *et al.*[20], spectrins by Marchesi *et al.*[16], integral proteins by Bhakdi *et al.*[22,23], and lipid-extracted proteins by Rosenberg *et al.*[24]. The physico-chemical characteristics of the last preparation of membrane protein extracts have been known to be close to those of whole ghosts. The extraction procedures for red cell membrane proteins are summarized in Table 2.

TABLE 2.

Various extraction procedures for red cell membrane proteins

PREPARATION OF MEMBRANE PROTEINS OF HUMAN RED CELL GHOSTS
1. WHOLE GHOSTS:
WASHED RED CELLS x3 WITH SALINE.AND
LYSED AND WASHED (1:23 v/v) BY 20 mOsm
DODGE'S BUFFER (pH 7.4).
2. LIPID EXTRACTED PROTEINS:
TREATED GHOSTS WITH ETHANOL-ETHER
MIXTURES.
SOLUBILIZED BY TRITON X-100.
3. PERIPHERAL PROTEINS:
DIALYSED GHOSTS FOR 36 HRS WITH 1 mM
TRIS-HCl BUFFER (1:20 v/v, pH 8.0).
4. TRITON X-100 EXTRACTABLE PROTEINS:
SOLUBILIZED GHOSTS WITH 2% TRITON X-100.

In this communication, the electrophoretic pattern was examined on (1) whole ghosts, (2) peripheral proteins and (3) integral proteins. Ele-

ctrophoresis was carried out by the 11 % gel system of SDS-polyacrylamide disc gel electrophoresis.

The electrophoretic patterns of whole ghosts (the upper portion), peripheral proteins (the middle), and integral proteins (the lower) are shown in Figure 5.

The major constituents of peripheral proteins are the bands I, II, V, and VI, and spectrin fraction (the bands I + II) are especially dominating. On the other hand, the integral proteins consisted mainly of membrane proteins of lower molecular weight, and spectrin fraction were not observed in the preparation.

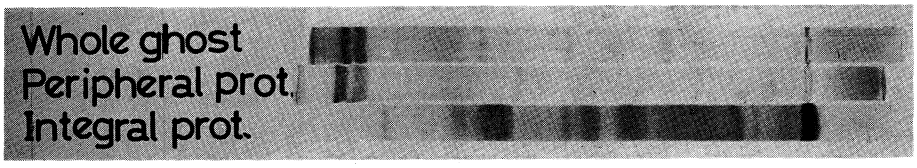

Fig. 5. Patterns of membrane proteins on various membrane preparations. Various preparations of red cell membrane proteins were examined on 11 % gels. Details are described in text.

DISCUSSION

The SDS polyacrylamide disc gel electrophoresis has been widely utilized to investigate red cell membrane proteins. In spite of the fact, standardized procedures of the electrophoresis have not been established and, by the reason, there are some difficulties to compare results of the pattern of red cell membrane proteins studied by various procedures. In addition, several factors affecting the pattern of red cell membrane proteins has been known, such as the conditions to prepare red cell ghosts, the conditions of the electrophoresis, etc.

Thus, in this communication, the effect of these factors on the electrophoretic pattern of red cell membrane proteins is extensively investigated.

Conditions for preparation of red cell ghosts should be set based on the purposes of the research projects. To keep peripheral proteins including spectrins intact, washings of red cell ghosts should be minimized to prevent from losing these proteins, and the Dodge's buffer (pH 7.4) would be preferable for the purpose. In stead of that, the 5 mM PO_4 buffer and/or the extensive washings would be suitable for investigation of integral proteins with the least contamination of hemoglobin.

The 11 % gels with the discontinuous buffer system appear to be

superior to the conventional gel electrophoresis with the same buffers for the cathodal and the anodal. The separation of red cell membrane proteins on the 11 % gels is much clear, and the minimal time for the electrophoresis is required.

On the 11 % gel system, the electrophoretic patterns of red cell membrane protein preparations were studied. Peripheral proteins consisted of bands I, II, V, Vl and others, integral proteins band III and others, respectively, compared to membrane proteins of red cell whole ghosts. These protein preparations seem to be suitable for investigation of some specific membrane proteins.

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