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VARIOUS TOPOCHEMICAL ARRANGEMENTS OF SIALIC ACIDS ON HUMAN ERYTHROCYTES AS DETECTED BY PARTITION IN AQUEOUS TWO-POLYMER PHASE SYSTEMS

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Summary

Human and rabbit red blood cells were subjected to partition in an aqueous, buffered Ficoll-Dextran two-phase system. The effect of neuraminidase treatment on the cell partition behaviour was examined and compared with the amount of sialic acids released from the cell surface and with the change in the electrophoretic mobility of the cells. The data obtained in the study indicate that there are two main groups of sialic acids differing in their topochemical arrangement on the human erythrocyte surface, and their relative hydrophobicity was evaluated. The results obtained in the case of rabbit red cells seem to indicate that sialic acids present on the cell surface are not the only major ionogenic surface components as is the case for human red cells.

The data obtained support the assumption that the membrane surface charge is the determinant of cell partition only as a factor affecting the relative hydrophobicity of the cell surface.

Introduction

The partition of cells in aqueous two-phase polymeric systems [1] is known to be an extremely useful method for the separation and subfractionation of cells as well as for gaining certain information relating to the surface properties of the cells examined [2,3]. It has been shown recently [4] that the partition technique can be used for semiquantitative characterization of the relative hydrophobicity of the surface of the cells subjected to distribution in an aqueous two-polymer phase system. It is obscure at present what are the contri-

butions of different cell membrane constituents to the cell surface properties as measured by the partition technique.

One of the main surface charge components, particularly of various erythrocytes, is known to be sialic acids [5]. The negative charge on the red cell is due mainly to the presence of charged sialic acid groups, which can be selectively removed through treatment with neuraminidase [6]. A correlation between the partition of cells in the Dextran-poly(ethylene glycol) phase systems and their relative electrophoretic mobilities was found [7,8], the effect of neuraminidase treatment of red cells on their partition in the above phase systems was studied [3,9—11] and it was concluded that the membrane charge is the main determinant of cell partition. Some results [3,10—12], however, indicate that the partition measures surface properties other than charge at the plane of shear and it has been suggested by Walter et al. [3] that the membrane charge either does not determine or is not the main determinant of cell partition.

It was suggested by us recently [13] that the membrane surface charge dependent on the presence of ionogenic groups at the periphery of a given cell is the determinant of cell partition only as a factor affecting the relative hydrophobicity of the cell surface. The present study describes the effect of neuraminidase treatment of human and rabbit red cells on their partition in the isotonic Ficoll-Dextran phase system as a function of the ionic strength (or the ionic composition) of the system.

Materials and Methods

Materials. Dextran-40 with molecular weight about 40 000 was obtained from Ferak, Berlin, Germany; Ficoll-400 was obtained from Pharmacia Fine Chemicals, Sweden. Neuraminidase (from Vibrio cholerae) was purchased from Behringwerke, Marburg-Lahn; *N*-acetylneuraminic acid was obtained from Koch-Licht, U.K. Salts were of analytical reagent grade.

Erythrocytes. Fresh blood was collected in 3.8% sodium citrate solution. Rabbit blood was obtained from three animals by heart puncture, human blood was obtained by venipuncture from four different donors. Erythrocytes were used in the experiments to be described within 48 h.

The cells were washed three times with sodium phosphate-buffered isotonic saline (pH 7.4) before the experiments.

Treatment of erythrocytes with neuraminidase. The cells were washed three times with 310 mosM sodium phosphate buffer (pH 5.95). After that, 1 vol. of packed cells was resuspended in 2 vols. of the same buffer and 1 ml of the neuraminidase solution (500 U/ml) was added to 30 ml of the cell suspension. The mixture was incubated at 37°C for 3 h. At appropriate times an aliquot, usually 2—3 ml, was removed and immediately cooled to 0°C in order to inhibit enzyme activity. The aliquot was centrifuged and the supernatant was decanted for sialic acid determination. The cells from the aliquot were further washed three times with 0.15 M NaCl in an 0.01 M sodium phosphate buffer (pH 7.4), resuspended in this buffer and used for electrophoresis and in the partition experiments. The control sample of erythrocytes was treated in an identical manner but without neuraminidase in the incubation mixture.

To get the stromal sialic acid content ghost erythrocytes prepared as in Ref.

14 were submitted to hydrolysis in 0.05 M H_2SO_4 for 1 h at 80°C. Sialic acids were assayed by the resorcinol method [15] using *N*-acetylneuraminic acid as a standard. All the samples were assayed in duplicate with an accuracy of 2%.

The electrophoretic mobilities of the red blood cells were measured in 0.01 M sodium phosphate buffer (pH 7.4) in isotonic saline with a Zeiss cytopherometer as described previously [13]. The rates of migration of more than 10 individual cells were determined with reversal of the polarity of the electrical field after each estimation. The mobilities of the cells were calculated in μm/s per V per cm and the standard error of the mean values amounts to 0.05 μm/s per V per cm.

Phase systems. Buffered, Ficoll-Dextran aqueous two-phase systems were prepared as previously described [4,13]. All of the phase systems used in the present experiments had the same polymer concentrations, 14% (w/w) Ficoll and 10% (w/w) Dextran, but differed in salt composition as indicated in Table I.

Partition experiments. The phase systems were poured into calibrated tubes and were thoroughly mixed. A known aliquot (from 0.1 to 1.0 ml) of red blood cell suspension was added to a tube containing the system to obtain the required cell concentration, namely about 10^5—10^8 cells per total 5 g of the system, depending on the cells examined. The test tubes were inverted for mixing numerous times and the phases were then allowed to settle at room temperature for 21—24 h. At the end of this time the phases were separated and aliquot was withdrawn by pipette from the top or the bottom phase of the system (depending on the cell partition behavior) and diluted with a suitable quantity of sodium dodecyl sulphate solution [13]. After that the concentration of cells in the sample was determined by measuring the absorbance of the cell lysate at 410 or 540 nm as previously described [13].

Presentation of data. The cell partition is characterized by the partition coefficient, K, calculated as the ratio of the quantity of cells in the top phase to the quantity of cells attached to the interface or as the ratio of the quantity of cells at the interface to that of cells in the bottom phase, depending on the cells' partition behaviour in each system. The quantity of cells at the interface was calculated as the difference between the total quantity of cells added and the quantity of cells in the top or in the bottom phase. The partition experiments were performed more than four times with each blood sample and the quantity of cells in the phases was determined with the deviation from the mean value not exceeding 3% for all the samples.

Results and Discussion

Fig. 1 shows the relationship between the logarithm of the partition coefficient and the ionic strength of the phase system for rabbit (A) and human (B) red blood cells treated and neuraminidase for 3 h or not treated. The prolonged treatment was used to provide the greatest possible reduction in the electrophoretic mobility and in the sialic acid content of the cells examined. It must be emphasized here that the term 'ionic strength' used throughout this paper should be considered as the parameter representing the ionic composition of the medium containing NaCl and sodium phosphate buffer. The salts con-

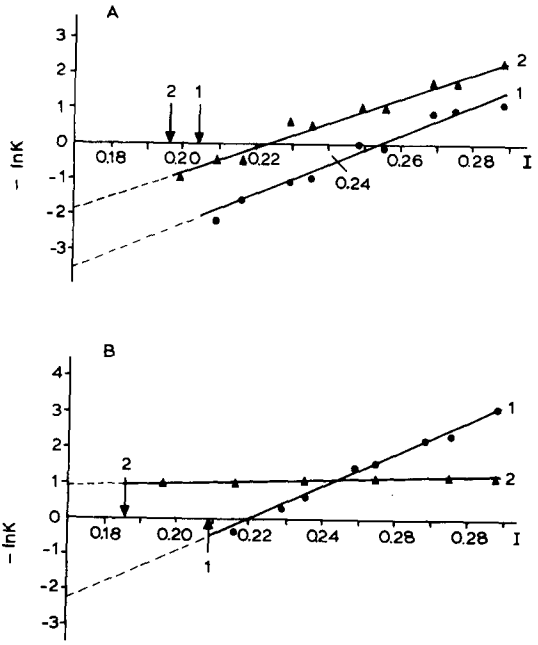

Fig. 1. Partition of neuraminidase-treated and intact rabbit (A) and human (B) erythrocytes in Ficoll-Dextran phase systems. Logarithm of the partition coefficient as a function of the ionic strength of the system containing 14% (w/w) Ficoll-400, 10% (w/w) Dextran-40 and different amounts of NaCl and sodium-phosphate buffer, pH 7.4, indicated in Table I. 1, intact red blood cells; 2, red cells treated with neuraminidase for 3 h.

centrations used and the respective ionic strength values are given in Table I.

It can be seen from the curves in Fig. 1 that the enzyme treatment affects the relationships indicated for rabbit and human erythrocytes.

It was shown earlier [13] that the observed relations can be expressed as follows:

$$-\ln K = A + B \cdot I \tag{1}$$

where I is the ionic strength, A and B are the constants, which can be expressed as:

$$A = E \cdot n \tag{2}$$

where E represents the free energy of transfer of CH_2 group between the system phases, ΔG^{CH_2}, according to equation $\Delta G^{CH_2} = RT \cdot E$ as considered earlier in Ref. 16; n is the total number of CH_2 groups required to represent the overall relative hydrophobicity of all the cell surface groups at the zero ionic strength of the medium.

The parameter B can be expressed as:

$$B = \sum_i (D_i \cdot c_i) \tag{3}$$

where D_i refelects the effect of the ionic strength (or the ionic composition) on

TABLE I

PARTITION OF HUMAN ERYTHROCYTES IN FICOLL-DEXTRAN PHASE SYSTEMS AS A FUNCTION OF THE AMOUNT OF SIALIC ACIDS PRESENT ON THE SURFACE OF THE CELLS

(Polymer composition: 14% (w/w) Ficoll-400, 10% (w/w) Dextran. Salt composition: NaCl and sodium phosphate buffer, pH 7.4. The NaCl concentration C_{NaCl} in the system at a given ionic strength I can be calculated from the equation $C_{NaCl} = (0.288-I)/0.75$; the sodium phosphate buffer concentration C_{NaPB} at the same ionic strength can be calculated from the equation $C_{NaPB} = 0.11-0.67 \cdot C_{NaCl}$. Partition is expressed as quantity of cells (percent of total cells added) found in the top phase (T), at the interface (TB) and in the bottom phase (B).

Amount of sialic acids present on the cell surface * (%)	Time of neuramidase treatment (min)	Partition at the indicated ionic strength (M/kg)																		
		0.176			0.196			0.216			0.235			0.255			0.275			0.288	
		T	TB	B	T	TB	B	T	TB	B	T	TB	B	T	TB	B	T	TB	B	T	TB	B
100	0	91	9	—	55	45	—	—	57	43	—	34	66	—	18	92	—	8	92	—	4	96
57.6	5	64	36	—	—	57	43	—	42	58	—	30	70	—	21	79	—	12	88	—	10	90
45.1	10	44	56	—	—	50	50	—	40	60	—	31	69	—	23	77	—	19	81	—	13	87
37.8	20	39	61	—	—	41	59	—	37	63	—	38	62	—	37	63	—	35	65	—	35	65
34.4	30	37	63	—	—	40	60	—	38	62	—	37	63	—	35	65	—	33	67	—	32	68
22.6	60	29	71	—	—	39	61	—	37	63	—	35	65	—	33	67	—	33	67	—	31	69
6.2	180	10	78	12	—	27	73	—	26	74	—	25	75	—	24	76	—	22	78	—	22	78

* The initial stromal sialic acids content of the cell membrane is 115 $\mu g/10^{10}$ cells.

the free energy of transfer of the ith type of ionogenic cell surface group between the system phases; c_i is the amount of the ith groups on the surface of the cell being partitioned in the phase system.

It seems evident that parameter B reflects the overall effect of the ionic strength of the system with a given ionic composition on all ionogenic groups present on the surface of the cells distributed in the phase system. Parameter A reflects the relative hydrophobicity of the surface of the cell under study provided the ionic strength of the medium is zero.

Since sialic acid is the main surface charge component of various erythrocytes [5,6] the observed changes in both A and B values for human and rabbit red cells seem to be in line with the above physical meaning of the parameters. It should be noted that the change in the A and B values produced by neuraminidase treatment of human erythrocytes (see Table II) exceeds significantly the changes observed in the case of rabbit red cells (in the case of untreated cells $A = -10.8$, $B = 42.4$ kg/mol and in the case of neuraminidase treated cells $A = -7.49$, $B = 33.92$ kg/mol with the deviations from the given values not exceeding 5%). For a better interpretation of these changes it seemed necessary to establish a relationship between the release of sialic acids from cells treated with the enzyme and the partition behaviour of these cells.

In Fig. 2 the percentage changes in the amount of sialic acids released and the corresponding changes in electrophoretic mobility of human red cells after neuraminidase treatment for different time periods are given. It can be seen from the data in Fig. 2 that the percentage change in mobility of the cells after neuraminidase treatment for 30 min is complete and amounts to 80% which is in agreement with the literature data [6,17]. The corresponding change in the quantity of sialic acids released amounts to about 70%. This finding is in line with the observation [5,6,17—19] that not all of the sialic acid residues released by neuraminidase make a contribution to the membrane surface charge. We could not find in the literature available any data on the correlation

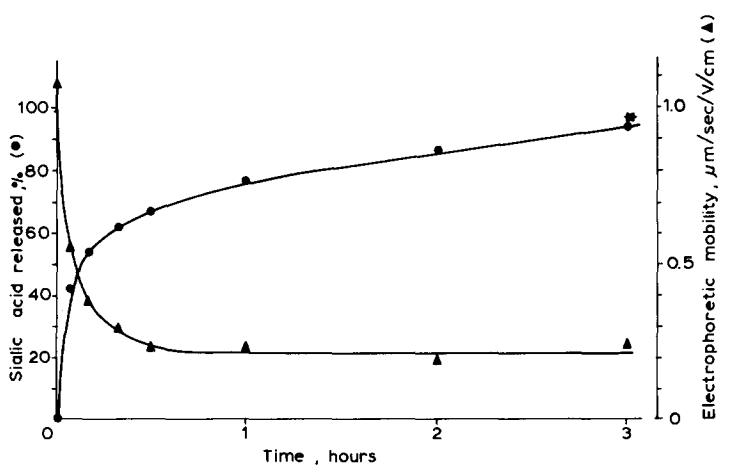

Fig. 2. Percentage of sialic acids released from the surface of human red cells under treatment with neuraminidase and electrophoretic mobility of the cells as a function of the enzyme treatment time.

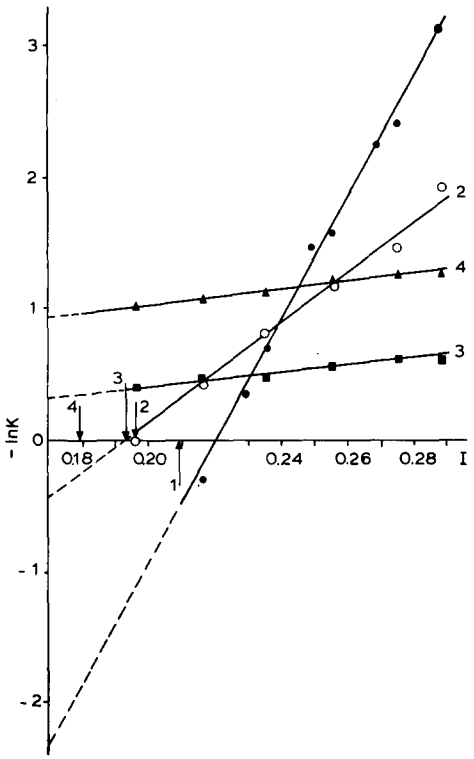

Fig. 3. Logarithm of the partition coefficient of human red cells as a function of the ionic strength in Ficoll-Dextran phase systems. (Composition of the systems as indicated in the caption to Fig. 1.) 1, intact red cells; 2, red cells treated with neuraminidase for 10 min; 3, red cells treated with the enzyme for 20 min; 4, red cells treated with the enzyme for 180 min.

between the decrease of the electrophoretic mobility and the amount of sialic acids releasing during the neuraminidase treatment. Usually the enzyme treatment is completed in 30—40 min [6,18,20], but it is not possible to compare directly any of the results, since they have been obtained under different experimental conditions.

Turning to the data of Table I and Fig. 3 on the partition behaviour of human red cells after treatment with neuraminidase it is noteworthy that all the main characteristics of the partition behaviour [13], namely I_0, A and B, appear to be affected by the quantity of sialic acids present on the surface of the cells under study. It should be noted that while the meaning of the parameters A and B was indicated above (see Eqns. 1—3), I_0 as defined previously [13] is the ionic strength at which the largest portion of the total quantity of cells is concentrated at the interface. It should be indicated also that an increase in the sodium phosphate buffer concentration with a concomitant decrease in the NaCl concentration results in a gradual transfer of the cells distributed in the Ficoll-Dextran phase system [4,13,21] from the top Dextran-rich phase to the interface and then into the bottom Ficoll-rich phase of the system. The ionic strength thereby increases from 0.176 M to 0.288 M. When the ionic strength is varied from 0.176 M to I_0, a given cell population distri-

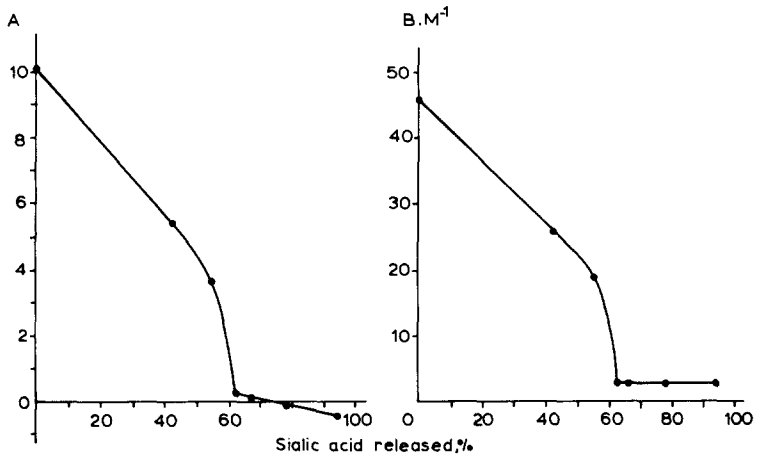

Fig. 4. Main characteristics of the human red cell partition — A and B — as a function of the percentage of sialic acids released from the surface of the cells under treatment with neuraminidase. (For details and explanation see text.)

butes between the top phase and the interface, and when the ionic strength is varied from I_0 to 0.288 M the cells distribute between the interface and the bottom Ficoll-rich phase of the system. It was shown earlier [13] that the I_0 value is species-specific but the significance of the parameter I_0 remains obscure at present. It should be noted, however, that the I_0 value appears to depend on the quantity of sialic acids released from human erythrocytes and reduces from 0.209 M to 0.176 M with the decrease of the sialic acids content on the surface of the cells. It still remains to clarify which factors influence the I_0-value: the cell surface charge, the surface relative hydrophobicity, the cell membrane structure, etc.

One thing, however, is clear: that the other main characteristics of the cell partition behaviour as a function of the ionic strength (or the ionic composition) of the system, A and B, should be dependable on the quantity of the ionogenic groups present on the cell surface. Hence, the relationships between these parameters and the percentage change in the amount of the released sialic acids are plotted in Fig. 4 and listed in Table II.

It should be noted, first, that both relationships given in Fig. 4 appear to involve two main ranges with respect to the percentage change in the quantity of sialic acids released from the cell surface. It seems that the removal of the surface acids up to about 62% is accompanied by significant changes in both A and B values, but the further removal of sialic acids does not affect the B value and is accompanied by just a slight change in the A value. The data in Table II and Fig. 2 indicate that the removal of about 67% of the surface sialic acids is followed by the complete reduction in the erythrocyte electrophoretic mobility. Hence, it seems that there are two main differing groups of sialic acid residues present on the surface of human red cells with respect to the electrophoretic mobility as well as to the cell partition behaviour. The second range of the relationship between parameter B and the percentage change in the amount of sialic acids released indicates that after about 62% of the surface sialic acids

TABLE II

PERCENTAGE CHANGE IN THE AMOUNT OF SIALIC ACIDS RELEASED FROM THE SURFACE OF HUMAN ERYTHROCYTES AND THE CORRESPONDING CHANGES IN ELECTROPHORETIC MOBILITY AND IN THE CHARACTERISTICS OF THE PARTITION OF THE CELLS IN FICOLL-DEXTRAN PHASE SYSTEMS AS A FUNCTION OF THE NEURAMINIDASE TREATMENT TIME

For experimental details see text. n.d., not detected.

Time of neuraminidase treatment (min)	Percentage change in the amount of sialic acids released (%)	Electrophoretic mobility (μm/s/V/cm)	Percentage change	*A*	*B* kg/mol
0	0	1.08	0	−10.1	45.8
5	42.4	0.56	48.2	−5.4	26.3
10	54.9	0.39	64.0	−3.7	19.1
20	62.2	0.30	72.0	−0.23	3.08
30	66.6	0.24	78.2	−0.16	3.08
60	77.4	0.23	78.7	0.10	3.08
120	86.6	0.24	78.2	n.d.	n.d.
180	93.8	0.24	78.2	0.40	3.08

are removed any further change in their quantity does not produce any change in the B value. From Eqn. [3] parameter B can be expressed as follows:

$$B = D_{\mathrm{NANA}} \cdot c_{\mathrm{NANA}} + \sum_j (D_j \cdot c_j) \qquad (4)$$

where D_j and c_j are as defined above when considering Eqn. 3, provided sialic acids are not included in the jth type of the ionogenic cell surface groups, D_{NANA} and c_{NANA} are related to the surface sialic acids.

It is clear from the data in Fig. 4 that after about 62% of the surface sialic acids are removed, the D_{NANA} value is to be zero, which means that the ionic strength (or the ionic composition) does not affect the relative hydrophobicity of the remaining groups. It seems possible to explain this fact in terms of various topochemical arrangements of sialic acid residues on the cell surface. As the cell periphery is a three-dimensional structure, sialic acids may be located in it at different depths. The further away is an ionized group from the elctrokinetic surface of the cell, the less will be the dependence of its properties on the ionic strength (or ionic composition) of the medium. This point of view is generally adopted to explain the discrepancy observed between estimates based on the release of free sialic acids from cells treated with neuraminidase and estimates on changes in electrophoretic mobilities after incubation of cells with the enzyme [5,6,17—19]. From the data in Fig. 4 and Table II it appears that electrokinetically effective sialic acids located at the electrokinetic surface of human erythrocytes make contributions to the A and B values differing from those characteristic for sialic acids buried deep in the membrane structure.

It should be noted that Walter et al. [9] in the study on the partition behaviour of neuraminidase treated human red cells in the Dextran-500-poly-(ethylene glycol)-6000 phase system containing 0.11 M sodium phosphate buffer (pH 6.8) have found that the cells' partition did not change after 62% of sialic acids were removed from the cell surface.

From the data in Table II and Fig. 4 it is possible to estimate the relative hydrophobicity of both surface-located and buried types of sialic acid residue. Sialic acid residue of the buried type seems to be represented by the $-1.8 \cdot 10^{-6}$ CH_2 groups and the surface-located residue seems to be represented by the $-1.0 \cdot 10^{-5}$ CH_2 groups, provided all these residues of a given type are identical. It is noteworthy, however, that the complicated character of the first range of both relationships plotted in Fig. 4 appears to indicate that in reality the residues located nearby the electrokinetic surface of human red cells differ from each other with respect to their properties displayed in the cell partition behaviour. It seems also noteworthy that the B value reduces by 93% as a result of neuraminidase treatment which seems to support the general view [5,6,19] that sialic acid is the major ionogenic surface constituent of the human erythrocyte membrane.

Turning now to the results presented in Fig. 1 it seems possible to give a better interpretation of the observed changes in the partition behavior of rabbit neuraminidase-treated red cells. In the case of rabbit cells the B value changes just by 20%. This fact can be taken as an indication that sialic acids present on the rabbit red cell surface are not the only major ionogenic surface components as in the case of human red cells. To study this cell membrane surface constituents more closely is beyond the scope of this work.

The results obtained in this study seem to indicate that the membrane charge is not the main factor determining the cell partition behaviour. The data obtained support the suggestion [13] that the membrane surface charge is the determinant of cell partition only as a factor affecting the relative hydrophobicity of the cell surface. The results also seem to support the earlier proposed way [4,13] of interpreting quantitatively the data on cell partition in two-phase systems. The sensitivity of the technique to the topochemical arrangements of the cell membrane constituents demonstrated here underscores the potential value of the method in the analytical study of cellular membranes and other biological structures.

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