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THE EFFECTS OF ALL-*TRANS*- AND 11-*CIS*-RETINAL ON THE ERYTHROCYTE MEMBRANE

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SUMMARY

1. When treated with all-*trans*- or 11-*cis*-retinal, rabbit erythrocytes hemolyse above 20° but not below this temperature.

2. All-*trans*-retinal shows much stronger hemolytic potency than 11-*cis*-retinal in the concentration range from $1 \cdot 10^8$ to $2 \cdot 10^8$ molecules per erythrocyte.

3. Erythrocytes treated with hemolytic concentrations of retinal do not hemolyse at 2°, but they do suffer a loss of potassium. All-*trans*-retinal is more potent than 11-*cis*-retinal in causing K^+ release.

4. Measurement of the frequency dependence of impedance for the erythrocyte suspensions treated with all-*trans*-retinal shows that this agent causes destruction of membrane, as indicated by the disappearance of β-dispersion. Similar effects can be observed in the case of treatment with digitonin or sonication.

5. The experimental results are discussed in connection with the mechanism of the permeability change in photoreceptive membranes.

INTRODUCTION

DINGLE and co-workers reported that vitamin A (retinol) causes lysis of erythrocyte[1] and lysosomal[2] membranes as well as swelling of mitochondria[3]. Furthermore, they showed that vitamin A aldehyde (retinal) is effective in lysis of erythrocytes and, as retinol[1], in the non-lytic potassium release from these cells. One may suppose, therefore, that retinol and retinal affect the arrangement of lipid and/or protein molecules in the biomembrane, resulting in some change in the permeability.

On the other hand, retinal is present in the outer segments of visual cells as the chromophore of visual pigment, which is isomerized from the 11-*cis*- to the all-*trans*-form by absorption of light. The problem of how the isomerization of the chromophore leads to the excitation of visual cells is one of key interest in the physiology of vision.

Visual pigment is presumably embedded in the disc membrane composed of lipid and protein. If the chromophore of the visual pigment makes contact with the disc membrane, all-*trans*-retinal, either free or bound, produced by the photoisomerization of the chromophore, may have some effect on the arrangement of lipid

and/or protein molecules in the membrane. Thus, assuming that the all-*trans*-form but not the 11-*cis*-form, can induce some change in the permeability of the membrane concerned with visual excitation, the conversion of the chromophore from the 11-*cis*-form to the all-*trans*-form would cause an increase in the permeability of the membrane, resulting in excitation of the visual cell. This hypothesis is supported by the fact that *cis–trans* isomerization of photochromic compounds (*N-p* phenylazophenyl-*N*-phenylcarbamylcholine chloride and *P*-phenylazophenyl trimethylammonium chloride) can regulate the potential difference across the excitable membrane of the electroplax of Electrophorus[4].

In the present paper, we compared the effects of all-*trans*- and 11-*cis*-retinal on the hemolysis of and potassium leakage from rabbit erythrocytes, whose membrane was regarded as a model of the disc membrane in the outer segments of visual cells.

EXPERIMENTAL METHOD

Preparation of retinal solution

Crystals of all-*trans*- and 11-*cis*-retinal were prepared by the modified method of BROWN AND WALD[5] under the direction of Mr. Y. Maeda. After dissolving about 2 mg of each compound in 2 ml methanol, the exact concentration of retinal was calculated from the absorbance measured spectrophotometrically (Hitachi, EPS-2A). The solution was kept in a deep-freeze (−18°) in the dark and used within 2 weeks.

Preparation of erythrocyte suspension

Two adult male rabbits weighing about 2.5 kg were bled by ear puncture with a heparinized syringe. About 3 ml of blood was transferred to a glass vessel containing 1.5 ml of 0.015 % (w/v) heparin–0.9 % NaCl solution. This suspension was stored at 5° and used within 1 week.

Before each experiment, an erythrocyte suspension was prepared according to the following procedure: erythrocytes were separated from plasma by centrifugation at 5000 rev./min for 10 min; they were washed 3 times by suspending the cells in NaCl–Tris solution (a mixture of 1 l of 0.9 % NaCl and 5 ml of 1 M Tris adjusted to pH 7.4 with 1 M HCl) and by centrifuging at 5000 rev./min for 10 min. After the erythrocytes had been resuspended in NaCl–Tris solution, any clotted blood was removed by filtration. The suspension thus obtained was adjusted to the required concentration by the addition of NaCl-Tris solution, and cooled by immersion in ice water.

Examination of hemolysis

3 ml of the erythrocyte suspension was injected into a test tube containing rerinal–methanol solution (50 μl) of known concentration. The suspensions were incubated under controlled conditions, and hemolysis was stopped by placing the tubes in ice-cold water for 2 min. The cells were then packed by centrifuging at 5000 rev./min for 10 min at 4°. All procedures up to this point were carried out under orange or red light. The absorbance at 500 nm ($A_{500\ nm}$) of the supernatant was determined with the spectrophotometer. The percentage of hemolysis was calculated from the following formula:

$$\text{Percentage of hemolysis} = \frac{H_r - H_c}{H_t - H_c} \times 100\,(\%) \qquad (1)$$

where H_r and H_c denote $A_{500\,nm}$ of the supernatant from the suspensions with and without retinal, respectively, and H_t denotes the A_{500nm} with excess saponin (100 μg/ml) for complete hemolysis.

Determination of potassium released

The concentration of K^+ in the supernatant obtained above was measured by flame photometry (Hitachi spectrophotometer, EPU-2A, with flame photometer attachment Type H-2). The percentage of potassium release from erythrocytes was calculated from the following formula:

$$\text{Percentage of potassium released} = \frac{K_r - K_c}{K_t - K_c} \times 100\,(\%) \qquad (2)$$

where K_r and K_c represent the respective amounts of potassium released from erythrocytes in the presence and absence of retinal in methanol solution and K_t represents the full amount of potassiam in the case of complete hemolysis caused by addition of distilled water.

Measurement of impedance

The capacity and the conductivity of the erythrocyte suspension hemolyzed by retinal or distilled water were measured in order to estimate the extent of damage to the erythrocyte membrane.

Rabbit blood (10 ml) collected as mentioned above was suspended in 50 ml of 8.7% (w/v) isotonic sucrose solution, and centrifuged at 1000 rev./min for 10 min. From about 4 ml of erythrocytes thus obtained, 1 ml was removed and resuspended in 3 ml of distilled water. The hemolysis obtained after incubation at 37° for 30 min was regarded as complete. Another 2 ml of the erythrocytes were resuspended in 6 ml of isotonic sucrose solution. 3 ml of the suspension thus obtained were injected into a test tube containing 100 μl of all-*trans*-retinal–methanol solution (5 mg/ml), and the rest was used as control. Both suspensions were incubated at 37° for 30 min.

Each suspension (3 ml) was put into a glass cell with cyclic electrodes of platinum black. The capacity and the conductivity were measured in the range of frequencies from $1 \cdot 10$ to $3 \cdot 10^3$ kcycles/sec with an apparatus comprising a dielectric loss bridge (Ando, Electric Co. Ltd., Type TR-IB), a G-C box (Ando Electric Co., Type YS-1) and a decade resistor and capacitor (Yokogawa Co., Model CD-41). The glass cell was immersed in ice water during the measurements.

RESULTS

The time course for the lysis of erythrocytes incubated with retinal at various concentrations is shown in Fig. 1. When the erythrocyte suspension was treated with all-*trans*-retinal at a final concentration of 21.8 μg/ml, almost complete hemolysis was obtained within 30 min. As indicated in Fig. 1, the hemolysis proceeds rapidly during the initial 10 min of incubation and then more slowly until a nearly constant value is reached after 30 min, irrespective of the concentration of all-*trans*-retinal. 11-*cis*-Retinal (10.9 μg/ml) shows a time course similar to that of all-*trans*-retinal

at the same concentration, but the hemolytic action of the former is weaker. Hence the percentage of hemolysis after 30 min incubation was used to compare the hemolytic action of all-*trans*- and 11-*cis*-retinal at various concentrations.

Fig. 2a shows the percentage of hemolysis for erythrocytes treated with various

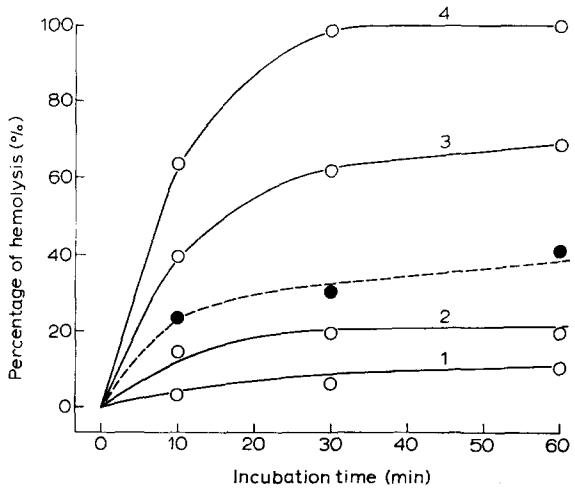

Fig. 1. Time course of hemolysis with all-*trans*- and 11-*cis*-retinal. The erythrocyte suspension (3 ml of $2 \cdot 10^8$ cells/ml, pH 7.4) was mixed with all-*trans*- or 11-*cis*-retinal, and incubated at 37°. The final concentrations of all-*trans*-retinal (○—○) in the suspension are 5.5 μg/ml (Curve 1), 7.3 μg/ml (2), 10.9 μg/ml (3) and 21.8 μg/ml (4). The final concentration of 11-*cis*-retinal (●---●) is 10.9 μg/ml.

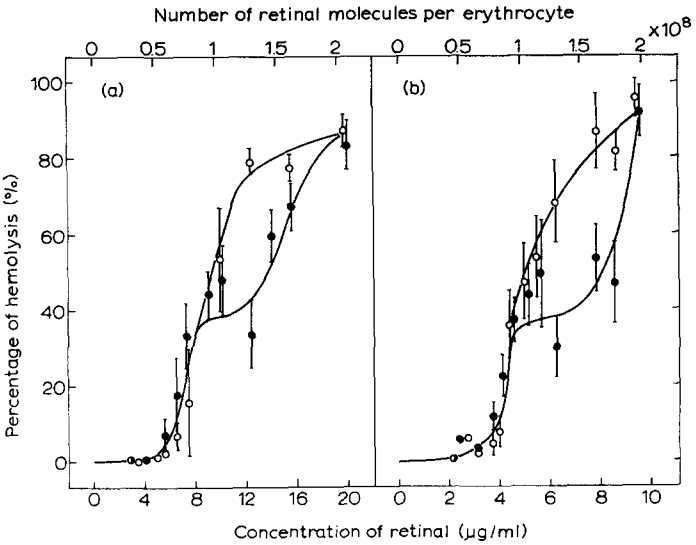

Fig. 2. Comparison of the hemolytic activities of all-*trans*- and 11-*cis*-retinal. The erythrocyte suspensions (3 ml, pH 7.4), in concentrations of $2 \cdot 10^8$ cells/ml (a) and of $1 \cdot 10^8$ cells/ml (b), were treated with various concentrations of retinal. The concentration in the figure is represented by the final retinal concentration. The percentage of hemolysis was determined after incubation for 30 min at 37°. Each plot indicates means from 3 to 10 experiments and the vertical lines show the standard deviations. ○, all-*trans*-retinal; ●, 11-*cis*-retinal.

concentrations of all-*trans*- and 11-*cis*-retinal. It can be seen that all-*trans*-retinal possesses much stronger hemolytic action than 11-*cis*-retinal at concentrations above 10 μg/ml. At lower concentrations the two isomers exhibit no significant difference. It should be noted that the relation between the percentage of hemolysis and the concentration of retinal is represented by a sigmoid curve in the case of all-*trans*-retinal, and by an irregular curve in the case of 11-*cis*-retinal.

In order to examine whether the results mentioned above depend on the total concentration of retinal or on the number of retinal molecules per erythrocyte, the original erythrocyte suspension ($2 \cdot 10^8$ cells/ml) was diluted 2-fold. The results obtained with diluted suspension (Fig. 2b) are almost identical to those with the orginal suspension (Fig. 2a), indicating that the percentge of hemolysis depends on the number of retinal molecules per erythrocyte. Thus, it may be concluded that all-*trans*-retinal possesses much stronger hemolytic action than 11-*cis*-retinal in the range $1 \cdot 10^8$ to $2 \cdot 10^8$ molecules per erythrocyte, assuming that all retinal molecules present in the suspension act on the erythrocytes.

DINGLE AND LUCY[1] reported that the hemolysis induced by all-*trans*-retinol is greatly affected by temperature. Fig. 3 shows the effect of temperature on hemolysis induced by all-*trans*- and 11-*cis*-retinal after incubation for 30 min. Neither isomer possesses hemolytic activity at 2°. Above 20° the hemolytic action of both isomers increases markedly, all-*trans*-retinal being more potent than 11-*cis*-retinal. The temperature dependence of the effect of all-*trans*-retinal is almost identical to that obtained with all-*trans*-retinol reported by DINGLE AND LUCY[1].

In the above experiments (Figs. 2a, 2b), it was demonstrated that no hemolysis

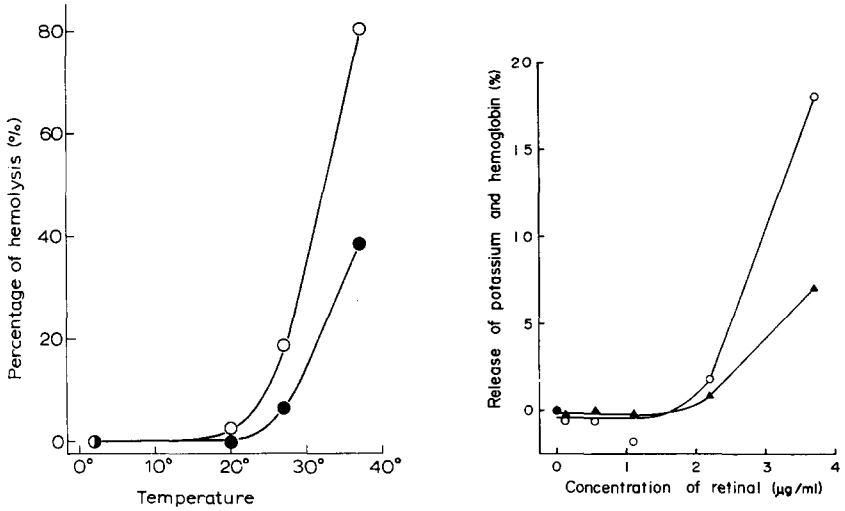

Fig. 3. Effect of temperature on hemolysis by all-*trans*- and 11-*cis*-retinal. The erythrocyte suspension (3 ml of $2 \cdot 10^8$ cells/ml, pH 7.4) was treated with all-*trans*- and 11-*cis*-retinal (final concentration, 12 μg/ml) and the percentage of hemolysis was determined after 30 min incubation. ○, all-*trans*-retinal; ●, 11-*cis*-retinal.

Fig. 4. Release of potassium and hemoglobin from erythrocytes treated with lower concentrations of all-*trans*-retinal. The erythrocyte suspension (3 ml of $1 \cdot 10^8$ cells/ml, pH 7.4) was mixed with various concentrations of all-*trans*-retinal dissolved in methanol (50 μl). The concentration in the figure represents the final retinal concentration. The percentage of hemolysis was determined after incubation for 30 min at 37°. ○, potassium; ●, hemoglobin.

occurred at concentrations of retinal below $5 \cdot 10^7$ molecules per erythrocyte. However, we may assume that even at subhemolytic concentrations retinal might affect the erythrocyte membrane. DAVSON AND DANIELLI[6] reported that treatment of rabbit erythrocytes with subhemolytic concentrations of pentanol or dihydric phenol causes release of potassium from the cells. Accordingly, we investigated the release of potassium from erythrocytes treated with subhemolytic concentrations of all-*trans*-retinal. Results in Fig. 4 demonstrate that, at concentrations of all-*trans*-retinal below 2 μg/ml, release of hemoglobin and potassium from erythrocytes is negligible. At a concentration of 3.7 μg/ml, 18 % of the K^+ was released, but only 7 % of hemoglobin. PONDER[7] mentioned that the loss of hemoglobin was substantially an all-or-none phenomenon for individual erythrocytes, but the potassium loss might be either a loss of all the potassium from some of the cells, a loss of some of the potassium from all of the cells, or any combination of these two extreme possibilities. If his view is correct, our experimental results suggest that a part of the potassium released comes from erythrocytes which have not yet been hemolysed by treatment with all-*trans*-retinal. To check this possibility, we measured the release of potassium from the erythrocytes treated with all-*trans*- and 11-*cis*-retinal at 2°, the temperature at which no hemolysis occurs with retinal (see Fig. 3). This experiment also served to examine whether potassium release was affected differently by the two retinal isomers.

Fig. 5a clearly demonstrates that, at a concentration of 8.3 μg/ml, all-*trans*-retinal causes a greater release of potassium from the erythrocytes than does 11-*cis*-retinal, while at 1.7 μg/ml there is no difference in the extent of potassium liberation between the isomers. On the other hand, hardly any hemoglobin is released from

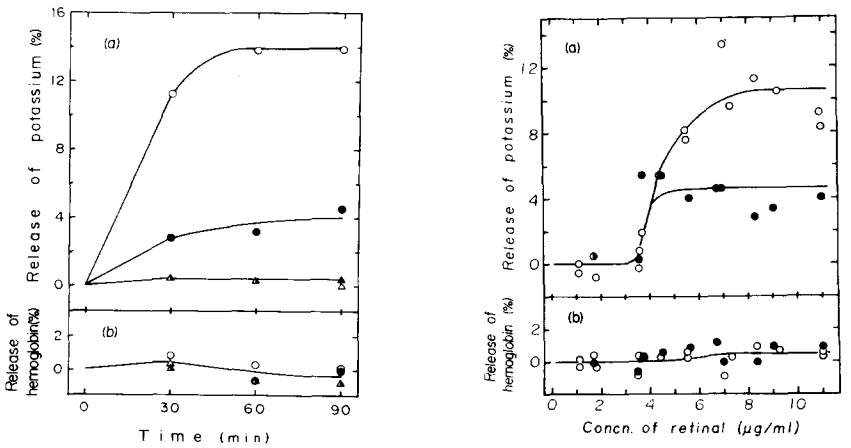

Fig. 5. Time course of the potassium release from erythrocytes treated with all-*trans*- and 11-*cis*-retinal. The erythrocyte suspension (3 ml of $1 \cdot 10^8$ cells/ml, pH 7.4) was treated with various concentrations of all-*trans*- or 11-*cis*-retinal at 2°. The concentration represents the final retinal concentration. The release of potassium (a) and hemoglobin (b) was measured. ○, all-*trans*-retinal, 8.3 μg/ml; △, all-*trans*-retinal, 1.7 μg/ml; ●, 11-*cis*-retinal, 8.3 μg/ml; ▲, 11-*cis*-retinal, 1.7 μg/ml.

Fig. 6. Release of potassium and hemoglobin caused by all-*trans*- and 11-*cis*-retinal. The erythrocyte suspension (3 ml of $1 \cdot 10^8$ cells/ml, pH 7.4) was treated with various concentrations of all-*trans*- or 11-*cis*-retinal at 2°. The concentration represents the final retinal concentration. The release of potassium (a) and hemoglobin (b) from the erythrocytes was determined after incubation for 30 min. ○, all-*trans*-retinal; ●, 11-*cis*-retinal.

the cells under these conditions (Fig. 5b). Results concerning the release of potassium and hemoglobin from erythrocytes treated with various concentrations of all-*trans*- and 11-*cis*-retinal are summarized in Fig. 6. Release of potassium becomes noticeable at a concentration of retinal of 3 to 4 μg/ml, increases rapidly between 4 and 6 μg/ml, and reaches a constant value above this concentration; above 4 μg/ml, all-*trans*-retinal is more effective than 11-*cis*-retinal.

The results described above indicate that at low temperature (2°) the penetration of retinal into the erythrocyte membrane may cause a slight change of membrane structure leading to the leakage of potassium, whereas above 20° it may cause a severe change resulting in the release of hemoglobin. It was of interest to determine whether the hemolysis caused by retinal is due to a mere increase in the permeability of the erythrocyte membrane for a molecule as large as hemoglobin, or to a more drastic structural change in the membrane.

In order to elucidate this point, the impedance of an erythrocyte suspension treated with all-*trans*-retinal was measured. As shown in Fig. 7, suspensions of normal erythrocytes as well as of erythrocytes hemolyzed with distilled water show β-dispersion in the frequencies from $1 \cdot 10^2$ to $3 \cdot 10^3$ kcycles/sec, whereas the suspension of erythrocytes hemolyzed by retinal does not.

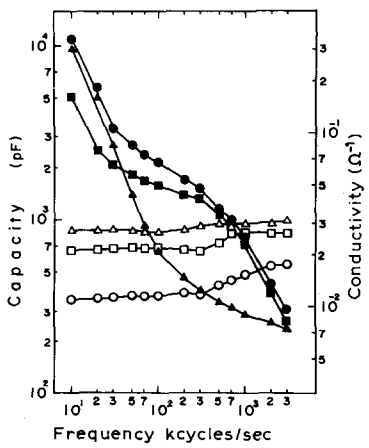

Fig. 7. Frequency dependence of the impedance of suspensions of erythrocytes hemolyzed by treatment with distilled water and with all-*trans*-retinal. Open and closed symbols indicate conductivity and capacity, respectively. Circles, normal erythrocytes; squares, treated with distilled water; triangles, treated with all-*trans*-retinal (see text).

In another series of experiments, we observed disappearance of the β-dispersion when erythrocytes were hemolyzed with digitonin–methanol solution (20 mg/ml, 100 μl), and also when erythrocytes hemolyzed with distilled water were treated with all-*trans*-retinal–methanol solution (5 mg/ml, 100 μl), digitonin or sonication (10 kcycles/sec for 5 sec). SCHWAN[8] interpreted the experimental results of FRICKE AND CURTIS[9] as follows: in the case of hemolysis with saponin or digitonin, these lysins destroy the plasma membrane of erythrocytes, resulting in the disappearance of β-dispersion, while in the case of hemolysis with distilled water, β-dispersion is still observed, implying that hypotonic treatment does not destroy the membrane

but merely increases the permeability of the membrane to hemoglobin. If his interpretation is correct, our results, in accordance with those of FRICKE AND CURTIS[9], indicate that retinal destroys the membrane.

DISCUSSION

The experimental results described above show that penetration of retinal across the erythrocyte membrane not only increases the permeability of the membrane to potassium, but also destroys the membrane structure. DINGLE AND LUCY[10] reported that the process of hemolysis caused by retinol consists of two steps: The initial step is the penetration of retinol into the membrane which occurs both in the cold and at physiological temperature; the second step is the destruction of the membrane which can be observed above 20°. Since the temperature dependence of hemolysis by retinol reported by them is very similar to our results with all-*trans*- and 11-*cis*-retinal (Fig. 3), the process of hemolysis by retinal seems to be similar to that of retinol. However, it is not yet clear whether retinal acts on the same site on the erythrocyte membrane as retinol. How does retinal attack erythrocyte membranes? DINGLE AND LUCY[1] demonstrated that the ability to cause lysis of and potassium release from erythrocytes is connected not only with the end group of the vitamin A molecule, but also with the length of its polyene chain. The present experiments indicate that the all-*trans*-form of retinal causes greater lysis of and potassium release from erythrocytes than the 11-*cis*-form, which means that the steric configuration of retinal may also be connected with its ability to cause a permeability change in the erythrocyte membrane. Therefore, retinal may affect not only the polar site of the erythrocyte membrane but also the hydrophobic region, which interacts with the hydrocarbon chain of retinal.

On the other hand, DAEMEN AND BONTING[11] reported that retinal increases potassium release from the outer segments of the rods. This fact may permit us to extend the effects of retinal on the erythrocyte membrane to those on the disc membrane of the visual cell. In the disc membrane, 11-*cis*-retinal existing as the chromophore of the visual pigment is fixed tightly to a lipoprotein moiety, opsin, so that its ability to cause the permeability change of the disc membrane to cations may be suppressed. The photoisomerization of the chromophore of visual pigment induces a rearrangement of the opsin structure, resulting in the appearance of all-*trans*-retinal, which is free or loosely bound to opsin, so that the all-*trans*-retinal can presumably effect an increase in the permeability of the disc membrane. Therefore, it is reasonable to suppose that the conversion of the chromophore of the visual pigment from the 11-*cis*- to the all-*trans*-form causes a more marked change in the cation permeability of the disc membrane than expected from our results on the effect of all-*trans*- and 11-*cis*-retinal on potassium release from erythrocytes.

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