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DRUG BINDING TO HUMAN ERYTHROCYTES IN THE PROCESS OF IONIC DRUG-INDUCED HEMOLYSIS

FLOW MICROCALORIMETRIC APPROACHES

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Abstract—Erythrocyte hemolysis induced by cationic phenothiazine derivatives and anionic non-steroidal anti-inflammatory drugs was compared, by flow microcalorimetry, with respect to thermodynamic characteristics for drug binding to intact human erythrocytes. Phenothiazines having high hemolytic activities bound strongly to erythrocyte cells, inducing an immediate hemolytic action characterized by an endothermic heat effect prior to saturating available binding sites. The thermodynamic observable ΔH and ΔS fell within the ranges of −119 to −65.1 kJ/mol and −308 to −128 J/mol/K, respectively, for these cationic species. There was a linear relationship between the hemolytic activity and the degree of exothermicity of ΔH which was enhanced significantly by the presence of a halogen atom(s) at the C-2 position of the phenothiazine nucleus in the order of $H < Cl < CF_3$. Anti-inflammatory drugs, however, bound to quite different sites in the erythrocytes with lower affinities and higher capacities than cationic drugs. The latter was characterized by small negative ΔH (−17.3 to −7.1 kJ/mol) and positive ΔS (10 to 41 J/mol/K). In the calorimetric profiles observed during hemolysis by anionic drugs, two stages were seen: the first, an exothermic process, arising from drug binding to the erythrocytes; the second, an endothermic process, corresponding to the heat of dilution of hemoglobin released from erythrocytes. Hemolysis occurred after the binding sites on the erythrocytes were saturated with drugs. Our data suggest that the binding activities of ionic drugs, such as the amounts of the bound drug and their binding energies to erythrocytes, contribute to the hemolysis.

Many kinds of ionic drugs which are distributed and/or bound to human blood cells, as well as plasma proteins, frequently induce erythrocyte hemolysis at high concentrations [1–4]. It has also been observed that anionic and cationic drugs cause different morphological changes of the cells (e.g. membrane externalization and internalization) in the process of the drug-induced hemolysis [5–8] and that they have some different effects on the rate and the extent of hemolysis [9, 10]. Although erythrocyte hemolysis is induced as a result of the interaction between drugs and erythrocytes [10–14], there has been, as yet, no satisfactory elucidation of the relationship between drug binding to intact erythrocytes and drug-induced hemolysis. Furthermore, studies have not resolved what total amount of drug is actually bound to the erythrocytes, which are the probable sites of drug action [15–17].

Recently we reported the usefulness of a differential flow microcalorimetry system for the study of drug interactions in the blood system [18–20]. Since optically clear solutions are not required for the application of this technique, a large concentration range of materials or suspensions can be measured without destruction. In addition, the calorimetric titration curve obtained only at one certain temperature is shown to determine the binding enthalpy change as well as the binding parameters and to evaluate both free energy and entropy changes in the interaction system. In the present paper, we studied both binding and thermodynamic characteristics for the interactions of various ionic drugs with intact human erythrocytes. In addition, the relationship between hemolytic activities and binding actions was investigated to elucidate clearly the mechanism of drug-induced hemolysis.

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MATERIALS AND METHODS

Materials. Fluphenazine dimaleate (FPZ), perphenazine dimaleate (PPZ), trifluoperazine dimaleate (FPRZ), prochloroperazine dimaleate (CPRZ) and perazine dimaleate (PRZ) were obtained from the Yoshitomi Pharmaceuticals Co. Ltd. (Osaka, Japan). Flufenamic acid (FA), mefenamic acid (MA), ibuprofen (IP) and indomethacin (IM) were purchased from the Sigma Chemical Co. (St. Louis, MO); flurbiprofen (FP) was a gift from the Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan). Human erythrocytes donated by the Red Cross Blood Center (Fukuoka, Japan) were washed three times in phosphate-buffered isotonic saline (PBS) (NaCl, 90.0 g; $NaH_2PO_4 \cdot 2H_2O$, 3.43 g; and $Na_2PO_4 \cdot 12H_2O$, 34.425 g/L, pH 7.4) by centrifugation at 1000 *g* for 10 min and then resuspended in the same buffer as a stock solution. Before each use, erythrocytes were washed 2–3 times with PBS until the supernatant was clear and

colorless, to obtain packed cells with a 100% erythrocyte concentration. The number of erythrocytes in the experimental suspension was measured by a Coulter Counter model TA-2 (Hialeah, FL, U.S.A.).

Drug-induced hemolysis. Concentrated drug solution was added to 5 mL of 2% (v/v) erythrocyte suspension in PBS by microsyringe to make a final drug concentration between 10^{-5} and 10^{-2} M. The mixture was incubated for 90 min at 37° and then centrifuged at 1000 *g* for 10 min. The supernatant was separated and then the absorbance $E^{1\,cm}_{543\,nm}$ of hemoglobin released from the erythrocytes was determined. The percent hemolysis is expressed by comparison with complete hemolysis of the erythrocytes in water.

Calorimetry. Calorimetric experiments were carried out at 37° via a differential flow microcalorimeter [18], which is an adiabatic type and consists of a twin-cell structure with each mixing cell containing a total volume of 60 μL. The instrument was calibrated by the methods of the electric calibration heater and the enthalpy changes of dilution of sucrose solutions. The reaction solutions were introduced at equal flow rates (0.12 mL/min) into the calorimeter through Tygon tubing using a four-channel peristaltic pump (Gilson minipulse 2, Villers-Le-Bel, France). The calorimetric signal was recorded on a chart recorder. The procedures used in the flow experiments have been described elsewhere [18]. The heat of reaction during the binding of drugs to intact erythrocytes was measured as a function of the drug concentration when the concentration of erythrocyte suspension was constant at 4% (v/v). The reaction heat was proportional to the recorded steady-state value. The heat of dilution of erythrocytes in the suspension was instrumentally subtracted by flowing the erythrocyte suspension to the reaction and reference cells. The heat of dilution of drug in the solution was measured separately and subtracted from the reaction heat. The total concentration of the drug in the calorimetric solution was measured by UV absorption.

The heat effect during hemolysis was measured continuously. A baseline was established by flowing erythrocyte suspension and PBS solution, and then the drug solution was added to the suspension by a microsyringe. A recorded signal during erythrocyte hemolysis corresponded to the heat of dilution of hemoglobin released from the erythrocyte cells.

Calculation of binding and thermodynamic parameters. The heat of binding (ΔQ) is proportional to the amount of drug–erythrocyte complex formed with total erythrocyte concentration fixed at E_t as follows:

$$\Delta Q = \Delta H \cdot F_r \cdot D_b \tag{1}$$

where ΔH is the binding enthalpy per mole of a drug, and D_b is the bound concentration of the drug at a constant flow rate of F_r. Assuming that a drug interacts with erythrocytes by stepwise equilibrium, the binding model derived from the law of mass action is:

$$\frac{D_b}{E_t} = \sum_{i=1}^{m} \frac{n_i \cdot K_i \cdot D_f}{1 + K_i \cdot D_f} \tag{2}$$

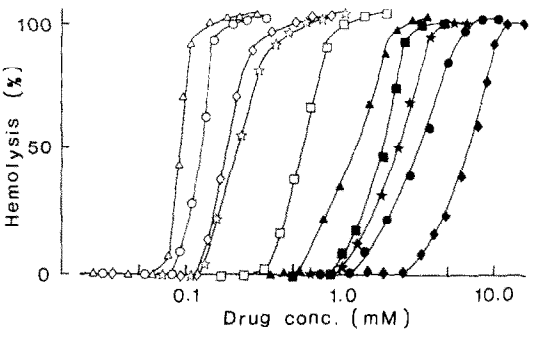

Fig. 1. Hemolytic effects of drugs on human erythrocytes. The concentration of erythrocytes was 2% (v/v) in PBS solution at pH 7.4 and 37°. Key: (Δ) FPZ; (◊) PPZ; (○) FPRZ; (☆) CPRZ; (□) PRZ; (▲) FA; (●) MA; (♦) IP; (★) FP; and (■) IM.

where m is the number of classes of independent binding sites, n_i the number of binding sites in the ith class with the binding constant K_i, and D_f is the free drug concentration related to the equation,

$$D_t = D_b + D_f. \tag{3}$$

In the simplest case of drug binding where one case of binding sites is involved ($m = 1$), the value of D_b is obtained from the total concentrations of the drug and the erythrocytes by solving the following equation [19]:

$$D_b = \frac{A - \sqrt{A^2 - 4n \cdot E_t \cdot D_t}}{2} \tag{4}$$

where

$$A = 1/K + n \cdot E_t + D_t. \tag{5}$$

The observed calorimetric titration curve is analyzed by the iterative least squares method of non-linear regression to estimate the best-fit values of the binding and the thermodynamic parameters, K, n, and ΔH. The initial value of ΔH can be calculated from the slope of the initial linear part of the titration curve. The computer program was run on a FACOM M-380R.

RESULTS

Drug-induced hemolysis. The hemolytic effects of drugs on human erythrocytes in PBS solution are shown in Fig. 1. The ionic drugs showed characteristic differences in hemolytic activities; cationic drugs produced a severe lysis, while anionic drugs caused hemolysis at a higher concentration than cationic ones. The concentrations which caused 50% hemolysis (C_{50}) were obtained from the hemolytic curves.

Heat produced by drug-induced hemolysis. Figure 2 shows the hemolytic profiles of the heat effect (upper) and the percent hemolysis (bottom) during incubation of a 4% (v/v) erythrocyte suspension with FPZ, PRZ, FA and IM. As shown in the case of FPZ and PRZ, the erythrocyte hemolysis induced by cationic drugs was rapid, and 100% hemolysis was completed within several minutes. The heat

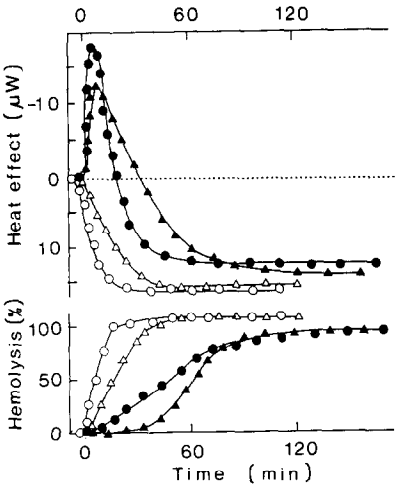

Fig. 2. Hemolytic profiles of heat effect (upper) and percent hemolysis (bottom) at 37°. Human erythrocytes in 4% (v/v) suspension were incubated with 0.15 mM FPZ (○), 1.0 mM PRZ (△), 2.0 mM FA (●), and 3.0 mM IM (▲). The percentage of hemolysis was expressed as the ratio of the absorbance $E^{1\,cm}_{543\,nm}$ in the final calorimetric solution to the absorbance after the complete hemolysis in water.

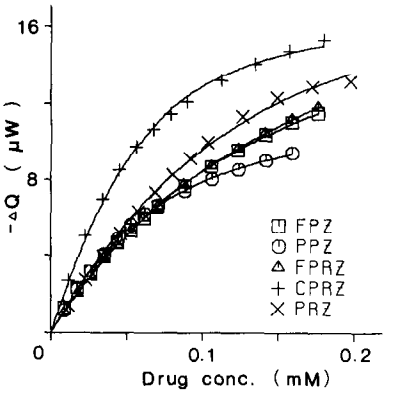

Fig. 3. Calorimetric titration curves for binding reactions of phenothiazines with human intact erythrocytes at pH 7.4 in PBS solution and 37°. Human erythrocytes were suspended in PBS solution to make an initial hematocrit value of 4% (v/v). Each point is the mean value of three measurements. Solid lines represent computer-generated best-fit curves assuming a one-class binding model with binding parameters given in Table 1.

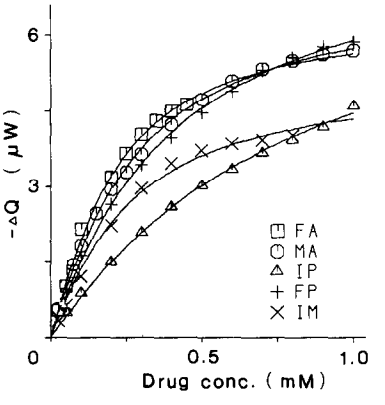

Fig. 4. Calorimetric titration curves for the binding reaction of anti-inflammatory drugs with intact erythrocytes at pH 7.4 and 37°. The experimental conditions are identical to those given in the legend of Fig. 3.

effect increased endothermically while increasing the degree of hemolysis. On the other hand, the percentage of hemolysis induced by anionic drugs such as FA and IM increased gradually following a lag phase for 10–20 min and complete hemolysis required more than 60 min (Fig. 2). The calorimetric profiles were characterized by two stages: the heat effect was produced exothermic within the lag time and then reversed to an endothermic process as hemolysis was caused by the drugs. In all the cases of hemolytic profiles, there was a significant correlation between the heat effect and the degree of hemolysis, indicating that the endothermic heat was proportional to the quantity of free hemoglobin released from the erythrocyte cells.

Calorimetric titrations of intact erythrocytes with drugs. The results from the heat of binding of phenothiazines and of anti-inflammatory drugs to intact erythrocytes are shown in Figs. 3 and 4 respectively. The concentration of erythrocyte suspension was 4% (v/v). The total concentrations of the drugs in the calorimetric solutions were in the range of 0.01 to 0.2 mM for phenothiazines and 0.05 to 1.0 mM for anti-inflammatory drugs, where no hemolysis was caused by the drugs. Each data point is an average of three measurements and solid lines represent the best-fit curves obtained by the curve-fitting procedure assuming a one-class binding model. The data fitted well to the curves with the correlation coefficients exceeding $r = 0.99$.

The estimated values of the binding and thermodynamic parameters are summarized in Table 1; the free energy (ΔG) and entropy changes (ΔS) are also listed in this table. The values of ΔG were calculated from the tabulation of equilibrium constants, according to the familiar thermodynamic relation $\Delta G = -RT \ln K$, where R is the gas constant and T is the temperature in Kelvin. The ΔS values were then calculated from ΔG and ΔH using the relationship: $\Delta S = (\Delta H - \Delta G)/T$.

The heat of binding proceeded exothermically with increasing concentration of the drugs. In the binding of phenothiazines, both values of ΔH and ΔS were considerably negative and varied from −119 to −65 kJ/mol and from −308 to −128 J/mol/K respectively. The absolute values of ΔH and ΔS decreased along the series FPZ > FPRZ > PPZ > CPRZ > PRZ, indicating that the interactions between phenothiazines and erythrocytes were influenced much more by the variation of a substituted halogen atom(s) at position C-2 than for the alkyl chain length of the side chain at N-10 in the phenothiazine nucleus. However, ΔG values calculated from the equilibrium binding constants were relatively constant (i.e. −23.3 to −27.5 kJ/mol). Anti-inflammatory drugs bound with a lower

Table 1. Binding and thermodynamic parameters for the binding of ionic drugs to human intact erythrocytes assuming a one-class binding model at pH 7.4 and 37°

Drug	K (10^4 M^{-1})	n (fmol/cells)	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (J/mol/K)
Fluphenazine	0.941	0.286	−119.1	−23.9	−308
Perphenazine	1.992	0.207	−99.5	−25.5	−239
Trifluoperazine	0.852	0.331	−101.8	−23.3	−253
Prochlorperazine	4.241	0.303	−93.0	−27.5	−211
Perazine	2.004	0.435	−65.1	−25.5	−128
Flufenamic acid	0.282	0.580	−17.3	−20.5	10
Mefenamic acid	0.177	0.809	−13.9	−19.3	18
Ibuprofen	0.209	1.503	−7.1	−19.7	41
Flurbiprofen	0.256	0.744	−11.9	−20.2	27
Indomethacin	0.275	0.699	−16.5	−20.4	13

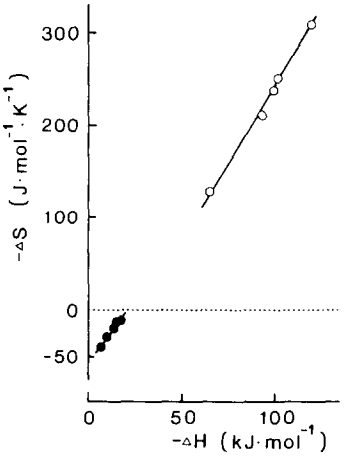

Fig. 5. Enthalpy and entropy compensation for the binding system between ionic drugs and intact human erythrocytes. Key: (●) anionic anti-inflammatory drugs; and (○) cationic phenothiazines.

Table 2. Comparison between drug concentration (C_{50}) and the amounts of bound drug (r_{50}) to the erythrocytes which cause 50% hemolysis

Drug	C_{50}* (mM)	r_{50} (10^{-16} mol/cells)	r_{50}/n† (%)
Fluphenazine	0.09	1.12	39.2
Perphenazine	0.19	1.58	76.3
Tifluoperazine	0.13	1.55	46.8
Prochlorperazine	0.22	2.68	88.4
Perazine	0.59	3.97	91.3
Flufenamic acid	1.45	4.60	79.3
Mefenamic acid	3.40	6.90	85.3
Ibuprofen	7.20	14.07	93.6
Flurbiprofen	2.20	5.96	85.3
Indomethacin	1.95	6.14	82.5

* These data were obtained from Fig. 1.
† Degree of saturation at binding sites of erythrocytes.

affinity (i.e. $K = 10^3\,M^{-1}$) and a higher capacity (i.e. $n = 0.58$ to 1.5 fmol/cell) than phenothiazines. The thermodynamic parameters were characterized by slightly negative ΔH values (−17.3 to −7.1 kJ/mol), positive ΔS values (10 to 41 J/mol/K), and almost constant ΔG values (i.e. −19.3 to −20.5 kJ/mol).

In all the drug–erythrocyte binding systems, enthalpy–entropy compensation analysis was examined by plotting ΔS against ΔH (Fig. 5). These plots are expressed by Equations 6 and 7 with an excellent correlation coefficient ($r = 0.997$ and 0.995). Phenothiazines:

$$\Delta H\,(\text{J/mol}) = 296\,\Delta S\,(\text{J/mol/K}) + 28.1 \times 10^3 \quad (6)$$

Anti-inflammatory drugs:

$$\Delta H\,(\text{J/mol}) = 325\,\Delta S\,(\text{J/mol/K}) + 20.4 \times 10^3 \quad (7)$$

The existence of ΔH–ΔS compensation shows that a single mechanism predominates in the binding process as stated by Breslauer *et al.* [21]. Thus, it is tempting to rationalize the linearities on the basis of the nature of the common binding sites of drugs on the erythrocyte cells.

Relationship between hemolysis and drug binding to erythrocytes. To examine a quantitative relationship between drug binding and erythrocyte hemolysis, the drug concentration (C_{50}) which caused 50% hemolysis was obtained from the hemolytic curve in Fig. 1, and the amounts of drug bound to an erythrocyte cell (r_{50}) at C_{50} were compared (Table 2). The value of r_{50} was calculated from the calorimetric titration curve by using Equation 2. The ratio of r_{50} to n (e.g. r_{50}/n), which indicates the degree of saturation at binding sites, was obtained. In phenothiazines, r_{50} and the ratio of r_{50} to n were increased with increasing C_{50}. However, the r_{50} values of the anti-inflammatory drugs were almost equal except for IP which induced hemolysis at a very high concentration; the values of r_{50}/n were in a range of 79.3 to 93.6%. Thus, when hemolysis by these drugs occurred, the binding sites of erythrocytes were already saturated by them.

Figure 6 shows linear relationships between ΔH of the drug binding to the erythrocytes and the logarithms of $1/C_{50}$ which represents apparent hemolytic activity. Evidently, drugs having high

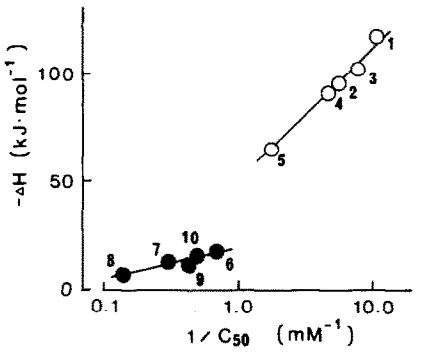

Fig. 6. Relationship between hemolytic activity ($1/C_{50}$) and ΔH for the binding of ionic drugs. Symbol key is the same as for Fig. 5, and numbers are (1) FPZ; (2) PPZ; (3) FPRZ; (4) CPRZ; (5) PRZ; (6) FA; (7) MA; (8) IP; (9) FP; and (10) IM.

hemolytic activities are bound to the erythrocytes with a high exothermic enthalpy. These thermodynamic observables are represented by two independent linear relations for the groups of anionic and cationic drugs, indicating that the differences in the hemolytic activities of the two classes of drugs may be directly related to the thermodynamic characteristics for drug binding to erythrocytes.

DISCUSSION

The results of this study suggest that drug binding to erythrocytes plays an important role in hemolysis and that the different hemolytic actions between anionic and cationic drugs may be related to the binding mechanism and the nature of the binding sites on the erythrocyte cells.

The cationic phenothiazines exhibited high hemolytic activities and bound strongly to erythrocyte cells which was characterized by large negative values of ΔH and ΔS. A quantitative relationship between the apparent hemolytic activities and the amounts of drug bound to intact erythrocytes was observed. Both binding and hemolytic activities were influenced by the halogen atom(s) at position C-2 on the phenothiazine nucleus, resulting in a significant increase in the order of PRZ < CPRZ < PPZ < FPRZ < FPZ (refer to Fig. 6). A similar tendency is shown in the binding affinity of phenothiazines to the cell membranes obtained by Kanaho *et al.* [13] and in the surface activity of drugs reported by Zografi and Munshi [12], reflecting hydrophobic characteristics in a decreasing order of $CF_3 > Cl > H$.

Since the thermodynamic parameters for drug binding to intact erythrocytes result in contributions from the overall reactions with the erythrocyte cells containing hemoglobin, the large negative ΔH and ΔS values indicate that either the drugs may be inserted into erythrocyte cells instead of remaining on the membrane surface or the drugs are reacting directly with the intra- or intercellular components of erythrocytes. As the drug enters the potential surface of the erythrocyte binding sites, the more strongly it is bound (the more negative the ΔH of binding) the more its rotational and translational freedom will be restricted (the more negative the ΔS). Ogiso *et al.* [22] studied the relationship between drug penetration and hemolysis and reported that drugs which penetrate into the membrane probably exist in the non-polar portions of lipid molecules and at the interfaces between lipid and protein molecules. According to the reports by Lieber *et al.* [14], the erythrocyte membranes are expanded by chlorpromazine and, therefore, hemolysis could result from an intermittent opening of weak seams at phase boundaries. A significant correlation between hemolytic activities and the large negative values of ΔH (refer to Fig. 6) suggests that drugs with high hemolytic activity produce a large amount of energy for the binding to intact erythrocytes. Thus, cationic phenothiazines preferentially bind to the inside face of the erythrocyte cells, inducing immediate disruption of the membrane structure and the formation of pores, through which hemoglobin is released from the cells.

Comparison of the two linear ΔH–ΔS compensation patterns depicted in Fig. 5 suggests that anionic drugs bind to quite different sites in the erythrocyte cells than their cationic counterparts. The binding of anti-inflammatory drugs is characterized by small negative ΔH and positive ΔS values, indicating a contribution from not only hydrophobic interactions but also from electrostatic forces [23] in the binding process. The class of binding sites observed here appears to be similar to the low-affinity class of sites observed by Cousin and Motais [24]. These investigators suggest that the binding sites exhibit a positively charged group(s) close to the hydrophobic surface with electron-donor groups on the erythrocyte membrane. Thus, upon binding of anionic drugs, the negative charge of the carboxylate anion of the drug is neutralized by a cationic locus on the protein; the complexed anion then behaves as a neutral molecule with respect to hydrophobic interactions. Hemolysis may be induced by the drugs as a result of a gradual translocation into the inner membrane to disturb the arrangement of the asymmetric distribution in the cell membrane upon incubating following the saturation of the binding sites.

These results support the bilayer couple hypothesis for drug-induced morphological change by Sheetz and Singer [5]; cationic drugs intercalate mainly into the inner monolayer, thus inducing invagination (membrane internalization) and anionic drugs interact with the outer monolayer and cause the cell to crenate (membrane externalization). However, it will be necessary to investigate further the thermodynamic characteristics for the binding interactions between drugs and erythrocyte membranes.

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