Hereditary Hemolytic Anemia Associated With Abnormal Membrane Lipids: Mechanism of Accumulation of Phosphatidyl Choline

By Stephen B. Shohet, Barbara M. Livermore, David G. Nathan, and Ernst R. Jaffé

In order to study the mechanism of the accumulation of phosphatidyl choline (PC) in erythrocytes with abnormal erythrocyte phospholipids from patients with a hereditary hemolytic anemia, the phospholipids of the erythrocytes were labeled radioactively. Labeling of phosphatides was achieved by both passive equilibration with preformed phosphatides, and active “acylase”-mediated incorporation of fatty acid (FA) in the presence of glucose, ATP and coenzyme A. The labeled cells were then reincubated in fresh compatible sera and the catabolism of the labeled erythrocyte phospholipids was followed. In addition, total acylase capacity of erythrocyte stroma was determined under optimal conditions in a system with excess lysophosphatide, FA, ATP, CoA, and Mg++. No differences in passive uptake or release of phosphatides were found between the patients’ erythrocytes and comparable reticulocyte-rich controls. On the other hand, overall active incorporation of FA into PC was abnormally increased in the patients’ erythrocytes, whereas incorporation of FA into phosphatidyl-ethanolamine (PE) was decreased. However, acylase capacity for both lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) was normal in the patients’ cells. This apparent paradox could be explained by the subsequent turnover of actively incorporated PC-FA which was found to be reduced. A brief labeling experiment designed to approximate pulse-chase conditions and to label primarily PC showed a considerable inhibition of the subsequent transfer of PC-FA to PE upon reincubation in fresh serum. This transfer has previously been shown to be responsible for a significant portion of PC-FA catabolism. Reincubation in hyperlipemic sera obtained from patients with liver disease or artificially enriched with PC did not influence the abnormal outflow of phosphatide-FA in actively labeled cells. The findings were consistent with the concept that PC accumulated in these cells because of a defect in the catabolism of actively incorporated PC-FA. This de-
fect appeared to be in the transfer of PC-FA to PE prior to final release from the cell. Passive exchange pathways and the active anabolic acylase pathway were not abnormal in these patients’ erythrocytes.

A familial nonspherocytic hemolytic anemia associated with abnormalities in membrane lipids was described by Jaffé and Gottfried in 1968.¹ They found that the affected patients’ cells had an absolute increase in erythrocyte phosphatidyl choline (PC). There was no evidence for abnormalities in glycolytic enzymes, ATP content, hemoglobin type, or plasma lipids. Osmotic fragility was decreased, Coombs tests were negative, and isologous ⁵¹Cr survival was decreased, while homologous ⁵¹Cr survival was normal.

The authors concluded that the hemolytic anemia was due to an intrinsic cellular defect. While no direct causal relationship between the elevated PC and the hemolytic anemia could be established, the unusual lipid abnormality seemed likely to be related to the hemolysis.

The mature human erythrocyte does not synthesize fatty acids or phospholipids de novo.² However, during circulation the cell continuously loses membrane lipid to the environment.³⁴ As summarized in Fig. 1A, two major pathways for lipid renewal and maintenance are present. Reaction 5 involves the passive equilibration of membrane phospholipids with the same preformed lipids in the plasma.⁵ Reactions 1 and 2 involve the active incorporation of lysophosphatides and plasma free fatty acids (FFA) within the membrane to form complete phosphatides.⁶⁻¹⁰ After an initial passive equilibration of plasma and membrane FFA, the further uptake of fatty acid requires ATP and proceeds through a series of discrete stages into phospholipid.¹¹ In the human erythrocyte, this process is primarily confined to PC and phosphatidyl ethanolamine (PE), with initial fatty acid incorporation rates into PC being two to three times greater than those into PE.⁸¹⁰ The fatty acids of phosphatides incorporated by this active process may be more intimately associated with the structure of the erythrocyte membrane than those of the passively incorporated preformed phosphatides since they are retained in a separate pool of phosphatides.¹³ Their turnover rate is also considerably slower than the turnover rate of passively exchanged phosphatides.¹²¹³

As opposed to the passively acquired fatty acid which is released as the complete phosphatide molecule, fatty acid incorporated by the active route is released back to the plasma as free fatty acid (reactions 3B and 4, Fig. 1A). In the process of the return of this phosphatide fatty acid to plasma, a transfer of PC fatty acid to PE fatty acid has been observed (reaction 3, Fig. 1A).¹³ This transfer may account for up to 30% of the accumulation of fatty acid in PE and appears to be a pathway through which a considerable amount of PC fatty acid may proceed before its ultimate release to plasma. The fatty acid is actually transferred between the glycerides; neither demethylation of PC to PE, nor base-exchange of ethanolamine for choline, could be found to speciously simulate this fatty acid transfer in human erythrocytes.¹⁴ Whether this transfer is mediated by a direct PC-LPE transferase or by a PC-phospholipase producing a very discrete intramembrane pool of FFA for LPE acylation is not currently clear. In any event, recent work by Paysant et al.¹⁵ suggests that phospholipases
of either the red cell or the plasma which are necessary for the final return of fatty acid from cell phosphatides to the plasma preferentially attack acidic phosphatides, e.g., PE. Since the neutral phosphatide, PC, is the compound into which free fatty acid is primarily incorporated; and since passive exchange does not serve to release actively incorporated PC fatty acid, this mechanism of fatty acid transfer from PC to acidic phosphatides appears to be important for the cell.

The purpose of this report is to describe experiments designed to investigate these several phosphatide renewal and release pathways in erythrocytes from the patients with hemolytic anemia associated with high erythrocyte PC levels (HPC cells). The data confirm the absolute increase in cell PC levels when compared to comparable reticulocyte-rich samples from patients with other types of hemolytic disorders and hemorrhage. In addition, measurements of the capacity of the stroma of these cells for active incorporation of fatty acid into PC in an optimized system showed no increase over age-matched controls. In plasma reincubation studies of prelabeled cells, the overall release of fatty acid of patients' cells was similar to that of controls. However, the absolute release of fatty acid from PC was considerably reduced while that from PE was unchanged. Finally, specific transfer of PC fatty acid to PE fatty acid was shown to be markedly reduced and delayed in these cells.

Taken together the data suggest that the elevated PC levels in HPC cells are
due to a block in the PC to PE transfer pathway. A fixed, apparently requisite, removal of PE fatty acid continues and, together with the transfer defect, produces a concomitant net reduction in cell PE levels as well.

The resultant imbalance in membrane phosphatide composition may be related to the observed hemolysis.

**MATERIALS AND METHODS**

**Reagents and Radiochemicals**

Linoleic acid, 1-14C, for use in active labeling studies, was obtained from New England Nuclear, Boston, Mass. Thin layer chromatography of this preparation before use showed its radioisotopic purity to be greater than 99%. Preformed 1-2, 14C choline-labeled phosphatidyl choline for passive labeling studies was obtained from Tracerlab, Waltham, Mass., and purified by thin layer chromatography. 1-Acyl-lysophosphatidyl choline (L-PC), coenzyme A, and ATP were obtained from Sigma, St. Louis, Mo. Crystallized human serum albumin was obtained from Dade Division, American Hospital Supply, Miami, Fla. This material was defatted and then refatted with the 14C linoleic acid as previously described. Defatted albumin for the removal of superficial red cell free fatty acid was prepared from human factor V generously supplied by the Massachusetts State Department of Health. This material was defatted by the method of Chen.

Silicic acid for column chromatography, (Biosil-A), was obtained from the Bio-rad Laboratories, Richmond, Calif. All chromatography and extraction solvents were reagent grade and purged with nitrogen prior to use.

**Cell and Serum Collection and Preparation**

Peripheral human blood was simultaneously obtained from the patients and normal donors and defibrinated with glass beads. Both control and patient cells were transported to the laboratory at 4°C (3 hr) after which the serum was removed by centrifugation. The samples were then washed essentially free of white blood cells and platelets as previously described. The separated sera which were used for reincubation studies were immediately centrifuged at 10,000 g for 20 min to remove any contaminating cellular material and then preserved at 4°C prior to use.

**Acylase Capacity Assay**

Total "acylase capacity" (defined as the net effect of fatty acid thiol-esterification combined with the subsequent action of acyl-CoA: lysophosphatidyl acyl transferase) was measured by modifications of the method of Ferber et al. which will be described in detail elsewhere. In brief, a red blood cell stromal preparation was obtained by lysing erythrocytes prepared as above with 0.001 M EDTA. The stromata were washed with EDTA and a volume equivalent to 0.1 ml of original packed cells was incubated with coenzyme A (0.5 mM), ATP (10mM), magnesium chloride (20 mM), radioactive fatty acid on albumin (0.05 mM–100,000 dpm), and LPC or LPE (0.5 mM) in 20 mM phosphate buffer (pH 7.4), at a final volume of 1.0 ml for 1 hr at 37°C. After incubation, the suspensions were diluted with saline and the stromata were collected by centrifugation and washing at 40,000 g for 20 min. Lipid extracts were prepared from the resuspended washed stromal button and phospholipid was separated from neutral lipid by simple small scale column chromatography on Biosil A or by thin layer chromatography. Under these conditions with added LPC, almost all fatty acid incorporated into phospholipid was found in PC (over 90%); when the assay was performed using LPE as substrate, 76% of incorporated PL-FA was found in PE. These assays were found to be approximately linear for amounts of erythrocyte stroma corresponding to from 0.01 to 0.5 ml of packed red cells.

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Fig. 2.—Uptake and outflow of preformed PC (passive exchange pathway). (A) Washed cells were incubated in serum at 20% Hct with 1 μCi of added preformed PC (1-2 14C choline) and with antibiotics and additives. Aliquots were removed, cells were washed free of media with saline, extracted, and analyzed for counts in PC. (B) Residual cells that had been relabeled as above were washed with saline and reincubated in fresh autologous serum. Points represent averages of duplicate determinations on two different patients (III-28 and IV-30 in reference 1) and two controls. Ranges are indicated by brackets. Squares, patients; circles, controls.

cells. The assays varied considerably with the reticulocyte count or erythrocyte glutamic oxalate transaminase (EGOT) levels of the cells. When assays of patient acylase activity were made, they were compared to controls of similar EGOT in an effort to secure hematologic age-matching.

Experimental Manipulations

The experimental manipulations and subsequent analytic techniques were similar to those previously described, and will not be described in detail here. A brief summary follows:

(1) Active Uptake of Fatty Acid Into Phospholipid: Washed erythrocytes from patients and controls were incubated in buffer with 14C fatty acid bound to human albumin in the presence of glucose, CoA, ATP, penicillin, and streptomycin for 2–4 hr. The media were removed, the cells were washed with saline and defatted albumin and aliquots were taken for lipid extraction and chromatographic analysis.

(2) Subsequent Transfer and Release to Serum of Fatty Acid Incorporated in Cellular Phospholipid: Following active incorporation and washing with saline and defatted albumin, the cells were reequilibrated briefly in fresh autologous serum (5 min at 37°C) to reestablish a normal surface fatty acid pool. These sera were then removed by centrifugation and replaced again with fresh autologous sera, glucose, ATP, CoA, and antibiotics. The cells were then reincubated and sequential aliquots for lipid extraction and chromatographic analysis were removed.

(3) Passive Incorporation and release Studies: Cells similarly prepared as in Sections A and B were incubated in autologous sera containing 14C preformed PC in tracer amounts. Glucose, ATP, CoA, and antibiotics were added to keep the conditions analogous to those in the active incorporation studies. The cells were washed with saline, aliquots were removed for analysis, and the remainder of the cells were reincubated in fresh serum. At various times, lipid extracts were prepared for chromatographic analysis.

(4) Control Studies: Parallel studies of active incorporation and reincubation were
Fig. 3—Uptake of FA into PC and PE (active incorporation pathway). Cells were incubated in Krebs-Henseleit buffer at 20% Hct with \(^{14}\)C linoleic acid on albumin (1 \(\mu\)mole FA acid 1.2 \(\mu\)Ci \(^{14}\)C/cc) in the presence of glucose and additives. At various times aliquots were removed. Lipid extracts were prepared and lipid classes were separated by thin layer chromatography. Points represent averages of duplicate determinations on two patients and two controls as in Fig. 2. Squares, patients; circles, controls.

Conducted using compatible sera obtained from normal controls and from two patients with markedly abnormal red cell lipids on the basis of serious liver disease. One of these patients had advanced to the stage of so-called “spur cell anemia,” while the other had severe obstructive biliary disease. The authors are grateful to Dr. Richard Cooper of the Thorndike Memorial Laboratory and Harvard Medical School for supplying these cells and sera. In one experiment, actively prelabeled patients’ cells were reincubated in sera from these patients with liver disease and the transfer and release of fatty acid was followed as in 2, above. In another control experiment, actively prelabeled patients’ cells were reincubated in normal sera fortified with the addition of 10 mM PC.

RESULTS

Lipid Analyses

Lipid analyses of total erythrocyte lipids are presented in Table 1. The data are presented as \(\mu\)g phosphorous per ml of cells and as \(\mu\)g per cell. The normal reticulocyte-rich cells (hereafter referred to as control cells) were obtained from a heterogeneous group of patients with nonhemolytic reticulocytosis (patients recovering from hemorrhage or megaloblastic anemias). In comparison to the controls, the cells from the high PC patients demonstrated a slight increase in total phospholipid and a marked increase in the absolute amount of PC, together with a less striking but significant decrease in the absolute amount of PE. These changes were present whether the calculations were made on a per cell or on a volume basis.

Other data not presented in detail here confirmed earlier observations that the patients’ sera were unremarkable with a mean total phospholipid of 81 ± 6 \(\mu\)m/ml (vs. control 83 ± 8 \(\mu\)m/ml) a mean PC of 56 ± 4 \(\mu\)m/ml (vs. control 54
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**Lipid Analysis of RBC Extracts**

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<tr>
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<th>Patients</th>
<th>Controls</th>
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<tbody>
<tr>
<td></td>
<td>µg/Cell x 10^-10</td>
<td>µg/cc RBC</td>
</tr>
<tr>
<td>Total PL</td>
<td>152 (± 11)</td>
<td>145 (± 10)</td>
</tr>
<tr>
<td>PC</td>
<td>55 (± 4)</td>
<td>53 (± 4)</td>
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<tr>
<td>PE</td>
<td>31 ± 3</td>
<td>30 ± 3</td>
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</tbody>
</table>

* Cells were washed free of plasma and extracted with isopropanol and chloroform by Rose's method.22 Average M.C.V. patients 105 cu µ; controls 99 cu µ.

† Controls: pernicious anemia responding to vitamin B₁₂, glucose-6-phosphate dehydrogenase deficiency, mild pyruvate kinase deficiency, recent acute hemorrhage, chronic hemorrhage on iron therapy.

I Standard deviation is shown in parentheses. By Student's t test differences between patients (N = 5) and controls (N = 6) are significant for PC (p < 0.001) and PE (p < 0.01). Differences in total lipid are not significant.
Fig. 5.—Per cent fractional turnover of actively labeled PC-FA and PE-FA. (A) Turnover of counts in PC; (B) turnover of counts in PE. Cells were preincubated for 4 hr with $^{14}$C linoleic acid and glucose to actively label PC-FA and PE-FA. They were washed free of media and surface FA with saline and DFA, respectively. They were then reincubated in fresh serum and aliquots were taken for lipid extraction and TLC analysis at various times. The counts present a 0 time in each fraction are arbitrarily set at 100%. Points represent averages of duplicate determinations on two patients and two controls as in Fig. 2. Squares, patients; circles, controls.

**Acylase Capacity Studies**

Since it seemed that the changes in cellular PC which had been detected were independent of the passive exchange pathway, (Fig. 2) and dependent upon the active incorporation pathway (Fig. 3), assays of the total PC and PE acylase capacities of the patient and control cells were made following the methods outlined in Section C. The results of these assays are shown in Fig. 4. In spite of the substantial overall increase in PC in the patients' intact cells in comparison to control cells (Table 1), no significant differences in PC or PE acylase capacities were detected. This observation prompted the following studies.

**Turnover of Actively Labeled Phosphatides in Autologous and Homologous Sera**

Actively prelabeled patient and control cells were reincubated in various sera and the percentage turnover of $^{14}$C fatty acid labeled PC and PE was followed (Fig. 5).

The percentage turnover in PC was markedly slowed in patients’ cells in comparison to controls, whereas the percentage turnover in PE was increased. Measurements of the total counts of all labeled fatty acid released to the sera were similar for both control and patient cells.

As shown in Fig. 6, the nature of the reincubation sera did not influence the rate of PC and PE turnover in these actively prelabeled cells. In contrast, in similar experiments not shown here performed with passively prelabeled normal...
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Fig. 6.—Per cent fractional turnover of actively labeled PC and PE in patient cells reincubated in hyperlipemic liver disease sera or in normal sera fortified with 10 mM preformed PC. (A) Turnover of counts in PC; (B) Turnover of counts in PE. Patient cells were prepared and prelabeled as in Fig. 5. They were then reincubated in various hyperlipemic sera and analyzed as in Fig. 5. Point represents averages of duplicate determinations on one patient (III-26 in reference 1). Circles, patient cells in spur cell serum; squares, patient cells in obstructive jaundice serum; X, patient cells in normal serum with 10 mM PC added; dashed line, control cells in normal serum (from Fig. 5).

and patient cells, a marked increase in the release rate of passively prelabeled cell PC was induced by the lipemic and PC fortified sera.

PC to PE Transfer in Briefly Prelabeled Cells

Patient and control cells were incubated with high specific activity 14C linoleic acid for 3 hr to label primarily PC. The radioactive surface pool of fatty acid was removed with defatted albumin and replaced with nonradioactive

### Table 2.—PC to PE Transfer in Briefly Prelabeled Cells

<table>
<thead>
<tr>
<th>Reincubation Time (hr)</th>
<th>Net Rise in PE Counts as Percentage of Initial PC Counts</th>
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<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
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<td>8</td>
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<td>16</td>
<td>16</td>
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<tr>
<td>24</td>
<td>8</td>
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Cells were prelabeled with high specific activity FA for 3 hr, then washed with saline and defatted albumin to remove media and surface FA, respectively. They were then reincubated in fresh serum and sequential aliquots were removed for extraction and analysis. The cumulative increases in PE counts were calculated as per cent original PC counts and represent the net effect of continued incorporation of PC counts into PE less the release of PE counts into the serum.

The initial distribution of radioactivity at 0 time was as follows: control cells: 58% PC, 24% PE, 13% NL, 5% other; patient cells: 72% PC, 14% PE, 9% NL, 5% other; total radioactivity at 0 time was $1.2 \times 10^6$ CPM/ml cells in controls and $1.65 \times 10^6$ CPM/ml cells in patients.
fatty acid from autologous sera. At various times the cells were analyzed for counts in PC and PE. As shown in Table 2 with the control cells, counts rose in PE before eventual fall and discharge to the incubating media. With the patients' cells the rise in PE counts was less extensive and considerably delayed.

**DISCUSSION**

Abnormalities in red cell lipids in hemolytic conditions have been described in the past. Most of these abnormalities have reflected primary defects in the serum. These have included hyperlipemia secondary to liver disease (spur cell anemia), hypercholesterolemia secondary to hereditary defects in plasma cholesterol phosphatidyl choline transferase, or hypercholesterolemia secondary to the lack of a lipid carrying protein (abetalipoproteinemia). Some of the red blood cell defects observed in these disorders were partially corrected by incubation in normal serum.

In contrast, the cells of the patients described here are apparently inherently defective, as originally suggested by cross-transfusion data. We, therefore, became interested in exploring the assumed defect in cellular lipid metabolism in these cells.

Lipid analyses confirmed the previous observation that erythrocyte PC was elevated in these patients. Because of the reticulocytosis, the cells were somewhat increased in total volume (MCV 105 cu μ). Therefore, the data were calculated on both a single cell and a packed cell basis. When these data were compared to reticulocytes of similar age and size, it became apparent that in addition to the increase in PC, there was a concomitant decrease in the absolute amount of PE in patients' cells versus controls. This change in the concentration of PE was sufficient to explain the only slight increase of total lipids in the face of a major increase of PC in these cells and indicated a major imbalance in the PC/PE ratio in these cells.

The studies on the passive uptake and release of preformed serum PC (Fig. 2) supported the concept that the increased PC in these patients' cells was not derived from preformed serum phospholipids since there were no differences in the passive exchange of cellular and plasma PC between patient and control cells. However, important differences were noted in the active incorporation pathway. More fatty acid radioactivity was incorporated into PC in the patients than in control cells. There was also a moderate reduction in fatty acid incorporation into PE. These data suggested two possibilities: (1) PC acylase activity could be especially high in the patients' cells. This could increase total PC synthesized and reduce PE synthesized by competition for a common substrate, e.g., free fatty acid on the cell surface. (2) PC acylase activity could be normal or even decreased if outflow transfer pathways from PC were blocked. This too could increase net counts in PC by reduction of catabolism. In addition, if the transfer pathway to PE was the site of the block, the reduced counts in PE would be explained.

PC acylase activity was normal for cell age, which excluded the first of these two alternatives. The data in Fig. 5 revealed that actively labeled PC in these patients' cells did not turn over as fast as that of normal controls. Calculations of the absolute extent of the defect in turnover showed that it was not merely
due to an increased total pool size (which alone could explain about 35% of the turnover defect) but rather that it was the result of an absolute decrease in the flow of fatty acid out of the PC compartment (from 18 nmoles/hr to 5 nmoles/hr/ml cells). On the other hand, the percentage turnover of PE was increased, consistent with a continuous fixed removal of PE fatty acid (13 nmoles/h/ml cells) from a pool of reduced size. This combination accounted for the finding of approximately normal overall fatty acid return to the serum in these reincubation studies.

The brief prelabeling study (Table 2) was undertaken to try to demonstrate a precise defect in the transfer of fatty acid from PC to PE. By using a brief period of preincubation, the majority of the incorporated counts were contained in PC. Hence, changes in the disposition of label occurring during reincubation in fresh serum were more representative of PC catabolism than in the previous prolonged preincubation studies. The considerable reduction and delay of PC to PE transfer despite higher initial PC labeling in the patients' cells vs. controls provided strong evidence for a defect in this pathway in these cells.

Taken as a whole these data suggest a mechanism for the increased PC and decreased PE levels in the cells of these patients which is diagramatically summarized in Fig. 1B. The passive exchange pathway between the cell and the serum is normal (reaction 5). Overall LPC and LPE acylation activity likewise is normal (reactions 2 and 2b). However, the transfer of PC fatty acid to other acceptors, notably PE, is abnormally low (reaction 3). Reaction 3b is probably not involved since total FA released to the plasma is normal and since a reduction in this pathway could not explain the reduced PE levels. As a consequence of this block combined with the ongoing synthesis of PC from plasma LPC, PC levels rise. Also because of this block, there is a lack of PC fatty acid to supply PE synthesis; this, combined with an apparently fixed requisite loss of PE fatty acid (reaction 4), establishes a new level of PE which is now solely dependent upon direct acylation (reaction 2B).

The resultant imbalance in two major structural phospholipids, PC and PE, may alter the permeability of the membranes of erythrocytes. Recently, we have detected a major abnormality of cation permeability in these cells which is probably responsible for the clinically observed hemolytic anemia. The studies of the permeability defect will be reported subsequently.

References


14. —: The apparent transfer of fatty acid from phosphatidyl choline to phosphatidyl ethanolamine in human erythrocytes. J. Lipid Res. 12:139, 1971.


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