The Blood Bag Plasticizer Di-2-Ethylhexylphthalate Causes Red Blood Cells to Form Stomatocytes, Possibly by Inducing Lipid Flip-Flop

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Summary

Background: During storage of red blood cell (RBC) concentrates, the plasticizer di-2-ethylhexylphthalate (DEHP) that keeps the blood bags soft leaches out and can be taken up by the RBCs. DEHP is known to be beneficial for the RBC storage quality, but the molecular mechanisms of the action are unknown. **Methods:** Aqueous suspensions of DEHP were added to RBCs in buffer. The morphological effects were observed on RBCs from 5 donors. Flow cytometry with annexin A5 binding was used to measure the exposed phosphatidylserine. **Results:** DEHP induced the formation of stomatocytes at concentrations as low as ng/ml, provided that the cell suspension was also sufficiently dilute. Some spherocytes, which were susceptible to lysis, were also formed; after lysis, RBC ghosts were seen to continue the transition to the cup-shaped stomatocyte form. Incubation with DEHP increased the exposed phosphatidylserine, an effect that was also observed in the presence of vanadate, which inhibits the ATP-dependent translocases that maintain the membrane's lipid asymmetry. **Conclusions:** DEHP can have an active effect on RBC shape, instead of just preventing the storage-related shape changes. The effect appears to be mediated by increased flip-flop of lipids between the leaflets of the RBC membrane.

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Introduction

Red blood cells (RBCs) that are stored for transfusions are typically kept in bags made of polyvinyl chloride (PVC) that has 30–40 wt% di-2-ethylhexylphthalate (DEHP) added in order to make the bags soft [1]. This DEHP is not extracted from the bag by aqueous solutions of salts, but by solutions that contain albumin [2]. Red cell concentrates (RCCs) that are stored for transfusions do contain albumin, and can extract DEHP from the bag; by day 28 of the storage period, the total DEHP concentration for RCCs stored in PVC/DEHP blood bags has been measured at 80 µg/ml [3].

In general, the toxicity of phthalates is low, and adults are typically not considered to be at risk from normal exposure levels [4]. Exposure levels in the general population will frequently be greater than zero due to the ubiquity of phthalates: people with no work-related exposure have been shown to have a wide range of concentrations of phthalate metabolites in their urine [5]. Oral administration of DEHP to rats at dosage levels in the range of 400–750 mg/kg/day does cause fetal development problems in male rats [6, 7], leading to concerns that DEHP could affect neonatal human males.

Storage materials with alternative plasticizers have been investigated for RCCs, due to the potential health effects of DEHP [8–12]. One challenge associated with the replacement of DEHP is that it has beneficial effects on the RBCs, extending the shelf life of the stored RCCs [8–10]. The DEHP reduces RBC vesiculation and loss of hemoglobin [9] and also reduces the extent of morphological changes that occur during storage [10].

As the cells age during storage, the smooth biconcave shape of the discocytes found in vivo first develops bumps around the rim, and then over the top and bottom surfaces; the overall disc shape is

Fig. 1. The initial discocyte form of RBCs changes to a spiculated echinocyte form during storage, in association with an increase in the area of the outer membrane leaflet relative to the area of the inner leaflet. The echinocyte spicules become more pronounced as the area difference increases.

lost as the cells become round; the spicules become more prominent and then become finer. The formation of these spiculated forms, known as echinocytes, is associated with a decrease in ATP levels [13]; this affects the ATP-dependent systems that maintain the lipid asymmetry of the membrane [14, 15] and can also lead to the degradation of lipids incorporated into the bilayer [16]. These factors affect the relative areas of the inner and outer leaflets of the membrane bilayer, affecting the RBC shape [14–17]. The convex bumps of the echinocytes are associated with an increase in the area of the outer leaflet relative to that of the inner leaflet [17] as shown in figure 1, with the cytoskeleton acting to stabilize the spiculated forms, preventing the formation of vesicles [18, 19]. The effects of DEHP on lipid asymmetry were investigated here by measuring the exposure of phosphatidylserine (PS), a lipid which is normally predominantly in the inner leaflet [20]. These experiments were then repeated in the presence of orthovanadate, which inhibits ATP-dependent enzymes [14], in order to determine if DEHP was acting directly on the membrane lipids or indirectly by affecting the ATP-dependent systems that maintain lipid asymmetry.

The goal of the work presented here is to investigate the RBC shape-changing effects that can be attributed specifically to DEHP. Many factors can affect cell shape [21]; in order to simplify the analysis, it was desirable to introduce DEHP to the cells using a method that does not cause any such background changes. The approach taken was to add the DEHP directly to the aqueous medium used to suspend the cells; this resulted in a low DEHP concentration so that low numbers of RBCs collected in a DEHP-free environment were suspended in this mixture in order to maintain a relatively high ratio of DEHP to membrane lipids. The effects of DEHP could therefore be observed while minimizing other factors affecting RBC morphology.

Material and Methods

Preparation of the DEHP Mixtures

The following DEHP mixtures were prepared: stirred DEHP / phosphate buffered saline (PBS) (for incubation with RBCs), sonicated DEHP/PBS (for incubation with RBCs at higher DEHP concentrations), and stirred DEHP/water (for analysis by liquid chromatography (LC) / tandem mass spectrometric analysis (MS/MS)). PBS was made up from tablets (VWR) to give a solution with 137 mmol/l sodium chloride, 2.7 mmol/l potassium chloride, and 10 mmol/l phosphate buffer pH 7.4. The stirred DEHP/PBS was prepared by adding 5 µl of DEHP (Sigma-Aldrich, St. Louis, MO, USA) to 100 ml of aqueous medium and then stirring overnight. The sonicated DEHP/PBS was prepared from the stirred mixture, which was subjected to 2 × 25 min sonication in an Elmasonic S 60 (H) water bath. For the LC-MS/MS analysis, the stirred DEHP was prepared as described above, but with water in place of the PBS, due to the requirement of low salt in the analyte.

Determination of DEHP Concentration by LC-MS/MS

The concentration of DEHP in water was determined using LC (Agilent 1100 HPLC system from Agilent, Germany) followed by MS/MS with an API 4000 quadrupole mass spectrometer (Applied Biosystems / MDS Sciex, Foster City, CA, USA), equipped with an electrospray ionization source (see supporting information for details, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>).

Preparation of PAH Adhesion Layer for RBCs

Glass microscope slides were modified with polyallylamine hydrochloride (PAH) as described previously [19]. The PAH (Sigma-Aldrich, average M_w 15,000 Da) was prepared by stirring overnight in 0.5 mol/l NaCl at a concentration of 0.1 g/ml. The source of the PAH was found to be important for the reproducibility. Glass slides with a single depression (Häberle, Lonsee-Ettleschieß, Germany) were cleaned following the procedure described in the LC-MS/MS section, followed by a 30 min incubation in the PAH solution. The slides were then soaked in water overnight and stored in water until the day of the RBC experiments. The PAH-coated slides were soaked in PBS or DEHP/PBS on the day of the experiment for at least 3 h before addition of the RBCs.

Blood Samples

Blood was collected from volunteer healthy donors after informed consent using a 20 gauge needle and a 10 ml EDTA Monovette (Sarstedt, Nümbrecht, Germany). Ethical approval was granted by the Medical Ethics Committee of the university. Initial experiments with other anticoagulants did not show any observable differences of the morphological effects described below (data not shown). Whole blood was centrifuged at $420 \times g$ for the flow cytometry experiments, after which the plasma and buffy coat were discarded. Additional rinsing and incubation steps were as described in the different sections. The blood samples were centrifuged at $60 \times g$ for the morphological studies. Experiments in which RBCs prepared for flow cytometry were used in morphological studies showed that the centrifugation procedure did not affect the later results. The RBCs used for the Annexin A5 experiments were collected on the same day as the experiments unless otherwise specified and were cooled before use. For the morphological tests, the RBCs were collected, cooled and stored in upright collection tubes for up to 4 days before use. Storage time for the samples is specified in the figure captions, or where appropriate in the results section.

Some experiments were carried out with RCCs collected at the German Red Cross Blood Service Baden-Württemberg – Hessen dedicated to quality control analyses. The RCCs were prepared from whole blood anticoagulated with ACD (acid citrate dextrose solution) and processed by buffy coat removal and leukocyte filtration to reduce leukocytes and platelets. The RCCs were stored in saline-adenine-glucose-mannitol (SAG-M) in PVC storage bags, with DEHP as a plasticizer. These RBCs are noted in the results as ‘transfusion bag’ samples.

Observation of RBCs

Unless otherwise specified, RBCs that had been washed three times in PBS or DEHP/PBS (5 µl packed RBCs rinsed 3× in 1 ml buffer, then resuspended in

Fig. 2. Stomatocytes formed in 2% HSA in PBS, with RBCs stored for 4 days prior to the experiment. Late stage stomatocytes identified by the central linear slit are shown in figure 2a, marked LS; an early stage stomatocyte distinguished by its diameter and brightness is circled; an early stage stomatocyte adsorbed edge-on to show the distinctive cup shape is shown in figure 2b, marked S. Scale bars indicate 5 μm . The full field of view for 2a is shown in the supporting information as figure S1 (available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>).

1 ml) were added to 80 μl of PBS in the PAH-coated depression of a glass microscope slide, with no added coverslip in order to avoid the shape-changing effects of glass in contact with the buffer medium [21]. The RBCs were left to settle onto the PAH layer and were then rinsed gently in order to remove some of the suspended cells and to replace the buffer with fresh medium. The RBCs were observed under phase contrast, using a Zeiss Axiovert A1 microscope (40 $\times$ objective, NA = 0.55) with a Zeiss AxioCam ICc1 camera, or bright field, using a Zeiss Axiovert 100 microscope (40 $\times$ objective, NA = 0.60) with a Zeiss AxioCam MR3 camera (Zeiss, Oberkochen, Germany). For images showing multiple cells, a cropped 50 $\times$ 50 μm region is shown in the figures; some of images have had the brightness and contrast adjusted. Sample-unmodified images showing the whole field of view are shown in the supporting information (available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>). The data files showing the whole field of view are available as image files or as the original Zeiss files from either corresponding author. The observation period started immediately after the addition of the RBCs to the slides. Control experiments with PBS only were acquired for comparison with the DEHP results, using RBCs treated in the same manner apart from the exposure to DEHP.

Treatment of RBCs with HSA to Form Stomatocytes

In addition to the DEHP/PBS treatment of RBCs described above, RBCs were exposed to albumin, a treatment known to cause stomatocyte formation [22, 23]. The RBCs were rinsed in PBS and then observed in human serum albumin (HSA; Biowest, Nuaille, France) at a concentration of 20 mg/ml in PBS, following the same procedure described above for observation of RBC morphology.

Annexin A5 Detection of Exposed PS by Flow Cytometry: Effect of DEHP, Buffer and Vanadate on Native RBCs and Positive Controls

Packed native RBCs were rinsed 3 $\times$ in 0.154 mmol/l NaCl and then resuspended in PBS or sonicated DEHP/PBS, at a volume ratio of 10 μl packed RBCs to 10 ml solution. After 5 min incubation, cells were centrifuged at 420 $\times$ g, rinsed once with NaCl solution and then resuspended in annexin-binding buffer (10 mmol/l HEPES, 140 mmol/l NaCl, 2.5 mmol/l CaCl_2 ; BD Bioscience, Heidelberg, Germany). The cell suspension was incubated with annexin A5-FITC for 15 min in the dark before the fluorescence was measured using a flow

cytometer (BD FACS Canto II; BD Bioscience). Positive controls were prepared by resuspending the RBCs after the initial saline rinse step in 0.9 wt% NaCl + 2.5 mmol/l CaCl_2 for 3 min, followed by addition of ionomycin (Santa Cruz Biotechnology, Dallas, TX, USA) to a concentration of 4 $\mu\text{mol/l}$ and a 1 h incubation at 37 $^\circ\text{C}$ [24]. The RBCs were rinsed with NaCl (0.9 wt%) + 2.5 mmol/l EDTA followed by three rinses with NaCl (0.9 wt%) + 1% BSA (Applichem, Darmstadt, Germany) and one rinse with NaCl (0.9 wt%). The positive controls were then treated in the same way as the native RBC samples with respect to subsequent incubations with DEHP/NaCl and annexin A5-FITC staining. The effect of vanadate on the flow cytometry results was determined by preparing a stock solution of sodium orthovanadate (VWR) at 0.2 mol/l; vanadate was then added from this stock solution at a 1:1,000 dilution to all the solutions listed above for a final concentration of 0.2 mmol/l in order to inhibit the ATP-dependent enzymes [14].

Results

DEHP Concentration

The concentration of DEHP mixtures prepared by stirring the DEHP with water was found to be 1.4 ± 0.8 mg/ml.

Positive Control for Stomatocyte Formation: HSA and Identification of Stomatocytes

Stomatocytes formed after the addition of albumin, a known stomatogenic agent [22, 23], are illustrated in figure 2. When RBCs are observed in dried films, stomatocytes are identified by a central linear slit [25], such as that seen on the late stage stomatocytes marked LS on figure 2a. In addition to this advanced stage of stomatocytes, there are many intermediate forms that can be identified. As RBCs progress through stomatocytosis, discocytes initially decrease in diameter [22] and then develop cup-shaped forms before developing the form associated with the linear slit and then

Fig. 3. Top views (top row) and the corresponding side views (bottom row) for a discocyte shape and for progressively later stages of stomatocytes. The discocyte pair (**a**, **e**) represents different cells, both selected from examples that were seen frequently; the stomatocyte pairs (**b**, **f**) and (**c**, **g**) both show individual cells that have been rotated by gentle flow to show the cell shapes at different orientations, as illustrated in more detail in figures. S2 and S3 of the supporting information (available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>); the stomatocyte pair (**d**, **h**) represents a possible match of views of the relatively infrequent late stage stomatocytes. Scale bar 5 μ m.

Fig. 4. RBCs from five different donors incubated with sonicated DEHP/PBS. The top row (**a–e**) shows cells incubated with DEHP/PBS, with stomatocytes indicated by an asterisk; the bottom row (**f–j**) shows the corresponding controls from the same RBC samples incubated in PBS only. The scale bars indicate 5 μ m. Images b–e and g–j were acquired using a Zeiss Axiovert A1 microscope and images a and f in bright field, with a Zeiss Axiovert 100 microscope. The RBC sample used for a and f above was washed following the procedure described for flow cytometry, with centrifugation steps at 420 g. The DEHP-exposed RBCs shown in d and the corresponding control sample in i both show unusual features, which are indicated by arrows: the control sample had elliptocytes as well as echinocytes and small spherical cells, while the stomatocytes formed after exposure to DEHP showed some degree of budding. Experiments were carried out on the same day that the samples were collected (**a–d**) or the day after sample collection (**e**). The full fields of view for figures 3e and j are shown in figure S4 of the supporting information (available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>).

Fig. 5. RBC incubated with DEHP/PBS. The scale bar indicates 5 μm . The sequence here shows the transition from a discocyte to a spherical shape, and the subsequent lysis. Elapsed time in minutes is shown on the images.

Fig. 6. Sequence showing the transition from an RBC ghost to a stomatocyte form, on an adherent layer of PAH; part of an intact discocyte may be seen to the lower right in the images. The ghost was formed after incubation of an RBC sample in DEHP/PBS. Images b-e were acquired at 15 min intervals, with a Zeiss Axiovert A1 microscope (40 $\times$ objective, NA = 0.55). Additional images from the sequence are shown in figure S6 of the supporting information (available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>). The scale bar on fig. 6a indicates 5 μm .

the final stage, which is a smooth sphere [26]. The cup-shaped form is distinctive when adsorbed on edge, as shown in figure 2b, for the cell marked S. The cup shape is not visible when the cells do not adsorb to the PAH edge-on, but the cells may be distinguished from the discocytes because they are both smaller in diameter and also thicker, making them brighter in phase contrast microscopy. The range of stomatocyte shapes present is summarized in figure 3, which shows both the edge and top views.

DEHP and Cell Morphology

Incubation of RBCs from different donors with the sonicated DEHP induced stomatocyte formation for all donors tested, as shown in figure 4. The control samples had low numbers of stomatocytes present (0.8–2.5%), while the samples incubated with DEHP had between 9.2 and 20.3% cells that could be identified as stomatocytes. In all cases, many cells clearly retained their discocyte shape. The RBCs from one of the donors (fig. 4d, i) had some unusual features both before addition of the DEHP (some ellipocytes and small spherical cells) and after the addition of DEHP (budding). The experiment pairs 4b, 4g and 4c, 4h were carried out on the same day with the same DEHP preparation. Full details of the stomatocyte counts are shown in table S1 of the supporting information (available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>).

DEHP also caused some RBCs to become spherical, as shown in fig. 4b (cell at top of the image, marked by an asterisk). Time lapse sequences show the transition from the discocyte to the spherocyte with no intervening stomatocyte forms (fig. 5; additional example: supporting information fig. S5, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>).

The spherocytes thus formed are susceptible to lysis (fig. 5). For the RBC ghosts remaining after the lysis, examples were seen in which the cells appeared to transform into a stomatocyte (see fig. 6 and additional images in supporting information fig. S6, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>).

Calculations of DEHP-to-Lipid Ratio

The ratio of DEHP to lipids in stored RBCs is calculated first based on literature values for the lipid content of RBCs [27] and for the amount of DEHP associated with the RBC membrane, which has been measured for RBCs stored as RCCs using radiolabeled DEHP [3]. Lipids can be extracted from RBCs with a yield of 3.15 μmol cholesterol and 3.90 μmol phospholipid per milliliter of packed cells [27], for a total lipid content of approximately 4.3 mg/ml, if we assume an approximate molecular weight of 800 Da for the phospholipids. The DEHP associated with the membrane at day 28 of the RCC storage is 11.6% of the 80 $\mu\text{g}/\text{ml}$ of DEHP that is extracted from the blood bag [3], or 9.3 μg DEHP/ml.

The DEHP-to-lipid ratio that can be calculated for RBCs will then depend on the volume fraction of RBCs in the suspension. If we calculate based on a 60% hematocrit, a possible value for RCCs, the lipid content will be $0.60 \times 4.3 \text{ mg lipid}/\text{ml}$, giving us a DEHP to lipid ratio of 0.36%. At the end of the storage period, the DEHP is therefore present at a value of more than 0.1% by weight of the membrane lipid.

In order to calculate the DEHP-to-lipid ratio in the cell morphology studies, experiments were carried out with the stirred DEHP preparations, for which the DEHP concentration had been

Table 1. Effect of DEHP on the % annexin A5-positive cells as determined by flow cytometry^a

Donor	No DEHP % annexin A5-positive cells	With DEHP % annexin A5-positive cells
Donor 1	0.48 ± 0.08	3.04 ± 0.38
Donor 1	0.50 ± 0.11	2.65 ± 0.28
Donor 2	1.09 ± 0.22	2.10 ± 0.30
Donor 3	0.26 ± 0.08	1.68 ± 0.52

^aValues in the table represent averages and standard deviations (for stained cells minus unstained controls), for four samples.

determined. After exposure to these DEHP preparations, between 5 and 7% of the cells were identified as stomatocytes, compared to control samples in PBS with between 0.2 and 0.9% stomatocytes. The full details of the counts are given in table S2 of the supporting information (available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>). During these experiments, 5 µl of RBCs were exposed to 5.6 ng of DEHP over the course of the washing procedure (4 × 1 ml at 1.4 ng/ml). The total DEHP incubated with the RBCs in this case is 0.02% by weight of the membrane lipids (5 µl at 4.34 mg/ml = 21.7 µg; 5.6 ng = 0.026% of 21.7 µg).

It is possible that the DEHP in our studies is present as a suspension and that only some RBCs encounter DEHP droplets. If we calculate the DEHP-to lipid ratio considering only the 6% of the cells that changed shape, this produces a number of 0.4%, similar to the value calculated for the transfusion bags at the end of the storage period.

RBCs and External PS: Effect of DEHP and Orthovanadate

For native RBCs collected in EDTA tubes, incubation with DEHP preparations increased the percentage of cells with exposed PS on the surface, as shown in table 1. Only a small fraction of the cells became annexin-positive. These annexin-positive cells showed a distribution of sizes (see FACS results in fig. S7, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>). There is no evidence here for a size-based selection of the RBCs, a point that is of interest because of the decrease in cell volume that is associated with RBC senescence [28].

Inhibition of the ATP-dependent translocases that maintain phospholipid asymmetry in the RBC membrane [14] through the addition of 0.2 mmol/l orthovanadate did not have a significant effect on the PS exposure in RBCs incubated in either PBS or sonicated DEHP/PBS (see table S3 in the supporting information, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>). The positive control samples produced with ionomycin and calcium were all late stage echinocytes, with 41 ± 3% annexin A5-positive cells based on four experiments; incubation of these cells with DEHP had variable effects, with the annexin-positive cell counts varying by 4%, 16%, 2.5% and –3%.

Tests were also done on one freshly collected transfusion bag (day 3 after collection), 6 outdated transfusion bags (after the 35-day maximum storage period) and samples from three donors col-

lected and stored in EDTA tubes (between 30 and 35 days). The RBCs collected in bags with added SAG-M had lower numbers of annexin-positive cells for comparable storage times: the day-3 transfusion bag samples had 0.05% annexin-positive cells (based on two aliquots taken from one bag, values 0.06 and 0.04%), as compared to the values shown in table 1; after the extended storage period, transfusion bag samples had 0.46 ± 0.16% annexin-positive cells, while the samples stored in tubes had much higher values that seemed to be donor specific (values of 4.7%, 13.9% and 16.5%).

Discussion

The action of DEHP on whole transfusion bag samples has been described previously, but there has been little focus on the molecular mechanisms by which DEHP exerts its effects. The DEHP is known to have a beneficial effect on RBCs that are stored for transfusion, reducing susceptibility to hemolysis [8–10, 29] and minimizing the formation of both echinocytes [10] and microparticles [8–11]. Hemolysis, vesicle formation, and the shape changes associated with echinocyte formation are associated with the membrane; together with the known association of DEHP with the RBC membrane [3], this makes the DEHP-membrane interactions a specific target to study. The focus of the work presented here is on the link between DEHP and RBC shape, a feature that is readily observable and that can be used to track both the interactions of added molecules with RBCs and the transverse movement of such molecules between the two leaflets of the lipid bilayer [14, 20].

One question to consider when characterizing the molecular effects of DEHP on RBCs is the medium in which the RBCs should be suspended for the studies. The greatest interest is in RBC samples that are stored for transfusions. The problem with studying RBCs in this environment is that multiple factors are known to affect the RBC shape during the storage period: in addition to the ATP depletion which leads to echinocyte formation [13] (and which is minimized by the SAG-M additives), RBCs in transfusion bags are subjected to pH values that decrease over the storage period [30], which favors stomatocyte formation [31], and increasing amounts of lysolipids in plasma [32] during storage can drive echinocyte formation [33]. The presence of albumin is also a complicating factor: added albumin can cause stomatocyte formation [22, 23], because of extraction of lipids from the outer membrane leaflet [34, 35]; albumin is also known to interact with DEHP [2]. The large number of variables in the transfusion medium over the course of the storage period therefore makes it difficult to isolate the effects of a specific additive. This problem can be minimized by studying RBCs in a simple system such as a buffer, although this in turn leads to the question of the applicability of the results in a medically relevant setting. The choice here has been to use the simple system on the principle that this is the best way to obtain structural information; the applicability of the results to transfusion bags is discussed later.

In these experiments, the RBCs were deposited on an adherent surface that has been shown previously to be capable of attaching

cells without distortion of either the discocyte or the echinocyte forms [19]. The surface is positively charged, and adheres to the negatively charged RBCs, in a manner similar to that of polylysine, a more familiar adherent polymer used for observation of cells. The procedure has several advantages: it allows the identification of early stage stomatocytes, unlike the standard clinical preparation of dried blood films that can be used for identification of late-stage stomatocytes [25]; it avoids the echinocyte-inducing effect of unmodified glass, associated with the elevated pH near the glass surface [21]; it also allows the observation of unfixed cells, thus making it possible to follow dynamic processes and also avoiding artefacts associated with the fixation process. The use of an adherent layer also allowed us to rinse the cells in situ, so that RBCs in suspension could be removed in order to improve the image quality and the buffer could be exchanged, in order to ensure fresh buffer for the cell medium. There are, however, potential problems: one of these is that there can be some inherent variability to positively charged surfaces, due to changes in the positive charge density when the samples are stored [36]; a second possible issue is that adsorption to a positively charged surface may lead to the formation of lipid domains [37]. In the results presented here, the stomatogenic effects of DEHP are evaluated by comparison to matched controls in which RBCs in buffer have been applied to the surfaces.

The challenge with studying DEHP was to establish a method for adding the DEHP to suspensions of cells in buffer. During standard storage procedures, this addition happens when the DEHP leaches slowly out of the bags during the period when the plastic is in contact with plasma [2, 3]. Introduction of DEHP to cell suspensions has been accomplished previously with the help of an emulsifier [10] and by addition of DEHP to plasma [8], but both the emulsifier [10] and the albumin found in plasma [22, 23] affect the RBC shape. For analysis of RBCs in the simple buffer system chosen here for the studies, we were limited to the amount of DEHP that could be suspended in an aqueous medium. The concentration of DEHP in stirred aqueous preparations was determined here to be 1.4 ± 0.8 ng/ml, towards the lower end of the very wide range of solubilities reported in the literature (0.0006–1.3 mg/l [38]). DEHP may be present as a suspension rather than as a solution (see supporting information Determination of DEHP concentration by LC-MS/MS for some additional discussion on this topic, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>).

The solubility of DEHP in water is much lower than in plasma, given that concentrations of 80 µg/ml have been demonstrated in blood bags [3]. This is as expected, because of the association between DEHP and albumin in the blood [2, 3]. The ratio of DEHP to RBC lipid was therefore increased in the experiments by decreasing the RBCs in suspension: RBCs were mixed with the DEHP preparations at numbers much lower than those found in suspension in transfusion bags, which are between 40 and 70% by volume packed RBCs [30]. Under the experimental conditions used, the stirred DEHP preparations caused between 5 and 7% of the RBCs to form stomatocytes. As calculated in the results section (see ‘Cal-

culations of DEHP-to-Lipid Ratio’ above), the DEHP-to-lipid ratio in the affected cells may be similar to that seen for RBCs stored in blood bags. The ratio found in blood bags, where DEHP is 0.1% or higher by weight relative to the membrane lipid, is potentially high enough to affect the lipid-based membrane physical properties such as phase transitions, which have been shown in model systems to vary strongly with small amounts of added DEHP [39].

The sonicated DEHP preparations resulted in higher numbers of cells becoming stomatocytes for RBCs from all donors, as shown in figure 4 and in table S1 of the supporting information (available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>) with the associated cell counts. The numbers of stomatocytes formed ranged from 9 to 20%, a variation that may reflect experimental conditions, differences in the donors, or else a combination of these factors. The existence of some differences between the donors is suggested by one experiment in which two donors were compared on the same day, with the same DEHP preparations and the same batch of PAH-modified slides (fig. 4b, c): the sample shown in figure 4b produced 20.3% stomatocytes after exposure to DEHP, while the sample in figure 4c produced 11.4% stomatocytes. Additional experiments would be required to confirm the existence and extent of any donor differences, although it is interesting to note that the donor sample with the highest stomatocyte count after DEHP exposure (4b) also had the highest stomatocyte count in the PBS control sample, at 2.5%. Donor differences in lipid profiles and cholesterol content are certainly possible, and may affect the cell response; donor differences in RBC membrane phase transitions have been noted previously, and have been attributed to differences in lipids [40].

In all cases where RBCs were exposed to DEHP, many of the cells retained their initial discocyte shape. As mentioned above, it is probable that the DEHP is a suspension rather than a true solution: it is therefore possible that the interaction with the DEHP is limited to the cells that come into contact with discrete DEHP droplets. This model is supported by the fact that sonicating the DEHP increases the numbers of affected cells, but does not appear to increase the extent to which individual cells are affected: as shown in figure 4, the affected cells are still largely early-stage stomatocytes. This is one area where experimental conditions differ from transfusion bag storage: it is not possible here to say that all cells have been exposed uniformly to the same concentration of DEHP. An alternate explanation for the differences in the RBC shapes after exposure to DEHP is that there are subpopulations of cells with differing degrees of susceptibility to DEHP; for example, older RBCs have been found previously to be less susceptible to chlorpromazine-induced stomatocyte formation [23]. This would provide one possible explanation for the differences between donors, but the flow cytometry results did not show any size-based selectivity in the annexin-positive cells. In the present case, we are limited to the conclusion that the addition of DEHP can affect at least some RBCs, and that it can lead to the formation of stomatocytes.

The shape change associated with the transition from discocytes to stomatocytes is due to an increase in the area of the inner leaflet of the membrane relative to the outer leaflet [17, 23, 41]. There are

Fig. 7. Three possible mechanisms by which DEHP could decrease the area of the outer lipid leaflet of the RBC membrane relative to that of the inner leaflet, thus driving the formation of stomatocytes. The top arrow illustrates the removal of lipids from the outer leaflet; the middle arrow illustrates selective insertion into the inner leaflet, causing the inner leaflet to expand; the bottom arrow illustrates the mixing of lipids from the two leaflets, which would be associated with some loss of the actively maintained membrane asymmetry. This third mechanism seems to be the most probable.

different possible mechanisms by which DEHP could cause this, as illustrated in figure 7: DEHP could insert selectively into the inner membrane leaflet, thus increasing the area directly; it could extract lipids from the outer leaflet; it could act as a synthetic scramblase, mixing up the lipids from the inner and outer leaflets; it could affect the active transport system that maintains lipid asymmetry. Selective insertion into the inner leaflet has been seen before for chlorpromazine [17, 23], and is based on the interaction of positively charged additives and the negatively charged lipids such as PS that are found predominantly on the inner leaflet. DEHP, however, is uncharged and oil-soluble, and would therefore be expected to reside in the hydrophobic interior of the membrane; selective insertion as a means of DEHP-induced stomatocyte formation therefore seems unlikely. Albumin causes formation of stomatocytes [22, 23] by extracting lipids from the outer leaflet [23, 34], but this is because the albumin is water-soluble, while still maintaining hydrophobic pockets that can interact with lipids. The DEHP has a very low solubility in water, and so this method of stomatocyte formation also seems unlikely.

The third possible mechanism for stomatocyte formation listed above, scramblase activity in which the DEHP mixes lipids from the inner and outer leaflets, was investigated by monitoring the effect of DEHP on PS. As summarized in table 1, DEHP did have a small effect on the PS distribution in the test samples of RBCs and a variable effect on the positive control samples. The DEHP-induced exposure of the PS does not seem to be dependent on the ATP-dependent translocases that maintain the lipid asymmetry because addition of orthovanadate, which inhibits the translocases, has little effect on the observed results, as shown in table S3 (available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>). DEHP therefore appears to be able to act as a synthetic scramblase.

If PS is the only lipid that moves from the inner to the outer membrane leaflet, then this would increase the area of the outer leaflet relative to that of the inner leaflet, an area change that is in the wrong direction to cause the formation of stomatocytes. It is, however, possible that DEHP has a non-specific effect with regard to lipid flip-flop and that other lipids also have increased transverse movement after RBCs are exposed to DEHP. The effect in which

stomatocyte formation is seen simultaneously with appearance of exposed PS, has been seen elsewhere, with the addition of nanoparticles to RBCs [42]; small molecules have also been shown previously to be able to cause lipid flip-flop between leaflets [43, 44].

Exposure of PS is associated with cell death, but this link does not appear to be well-defined for RBCs: the positive controls for the annexin test, in which an ionophore was added to allow calcium ions into the cells, caused 100% late-stage echinocyte formation, but much less than 100% PS exposure; conversely, incubation of the RBCs with DEHP was able to cause some PS exposure without forming any late-stage echinocytes. There is therefore no evidence that the PS exposure during the DEHP incubation was due to the DEHP causing cell death in the RBCs. The DEHP did, however, cause a small number of RBCs to become spherical and then lyse, as shown in figure 5, possibly due to larger droplets of DEHP or older cells. The lysed cells are clearly dead by any definition of cell death, but it is interesting to note that it was possible for them to continue changing shape, as shown in figure 6 and figure S6 (available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>), which show a lysed RBC forming a stomatocyte. This provides further evidence that the stomatocyte-forming effect is not due to the action of DEHP on the active transport system. Most of the stomatocytes that were formed after the DEHP incubation were, however, stable over the period of observation, as illustrated in figure S2 (available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=490502>), which shows a single cell over a 40-min period.

The next question to address is the applicability of these results to the storage of RBCs for transfusions. DEHP is added here rapidly, probably in the form of suspended discrete droplets. The kinetics of the interaction are clearly not the same as the gradual increase of DEHP that would be expected with transfusion bags; the distribution of the DEHP in the RBC population may also differ. The location of the DEHP within the lipid bilayer of the RBC membrane should, however, be determined by the structures of the DEHP and the lipids, regardless of the mode of addition. Measurements reported elsewhere on the extent of the DEHP interaction with the RBC membrane in transfusion bags [3] suggest that the

DEHP-to-lipid ratio is sufficiently high to affect the lipid-based membrane physical properties [39]; the ratio in the stomatocytes seen here may be in a similar range, as suggested by the calculation of stomatocyte counts formed after exposure to stirred DEHP. The RBC suspensions in transfusion bags have been shown to have a relatively high proportion of stomatocytes over the majority of the storage period [45]. This may be due to a combination of factors including the pH of the storage medium. DEHP would be low at the beginning of the storage period, but would increase with time, which would counteract the formation of echinocytes. The exposure of PS is potentially deleterious [46], but this effect appears to be much lower in RBCs collected and stored in EDTA tubes than in the transfusion bags, possibly because of additives that maintain the ATP levels within the cells, and thus help to maintain the activity of the translocase system that actively transports the PS to the inner leaflet [14].

In summary, DEHP in aqueous suspensions affects the RBC shape; these effects can be seen at relatively low DEHP concentra-

tions, provided that the cell count is correspondingly low; the DEHP appears to act as a synthetic scramblase, driving stomatocyte formation by increasing the flip-flop of lipids between the leaflets of the RBC membrane. DEHP is therefore actively driving the cell shape in a manner that counteracts the echinocytosis associated with the ATP depletion of long-term storage.

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References

- 1 Rahman M, Brazel, CS: The plasticizer market: an assessment of traditional plasticizers and research trends to meet new challenges. *Prog Polym Sci* 2004;29:1223–1248.
- 2 Jaeger RJ, Rubin RJ: Plasticizers from plastic devices: extraction, metabolism, and accumulation by biological systems. *Science* 1970;170:450–451.
- 3 Rock G, Labow RS, Tocchi M: Distribution of Di(2-ethylhexyl) phthalate and products in blood and blood components. *Environ Health Perspect* 1986;65:309–316.
- 4 Sampson J, de Korte D: DEHP-plasticised PVC: relevance to blood services. *Transfus Med* 2011;21:273–283.
- 5 Koch HM, Rossbach B, Drexler H, Angerer J: Internal exposure of the general population to DEHP and other phthalates- determination of secondary and primary phthalate monoester metabolites in urine. *Environ Res* 2003;93:177–185.
- 6 Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, Gray, LE: The plasticiser diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol Sci* 2000;58:339–349.
- 7 Blystone CR, Kissling GE, Bishop JB, Chapin JR, Wolfe GW, Foster PMD: Determination of the di(2-ethylhexyl) phthalate NOAEL for reproductive development in the rat: importance of the retention of extra animals to adulthood. *Toxicol Sci* 2010;116:640–646.
- 8 AuBuchon JP, Estep TN, Davey RJ: The effect of the plasticizer di-2-ethylhexyl phthalate on the survival of stored RBCs. *Blood* 1988;71:448–452.
- 9 Bicalho B, Serrano K, dos Santos Pereira A, Devine DV, Acker JP: Blood bag plasticizers influence red blood cell vesiculation rate without altering the lipid composition of the vesicles. *Transfus Med Hemother* 2016;43:19–26.
- 10 Estep TN, Pedersen RA, Miller TJ, Stupar KR: Characterization of erythrocyte quality during the refrigerated storage of whole blood containing di-(2-ethylhexyl) phthalate. *Blood* 1984;64:1270–1276.
- 11 Serrano K, Levin E, Chen D, Hansen A, Turner TR, Kurach J, Reidel A, Boecker WF, Acker JP, Devine DV: An investigation of red blood cell concentrate quality during storage in paediatric-sized polyvinylchloride bags plasticized with alternatives to di-2-ethylhexyl phthalate (DEHP). *Vox Sang* 2015;110:227–235.
- 12 Hill HR, Oliver CK, Lippert LE, Greenwalt TJ, Hess JR: The effects of polyvinyl chloride and polyolefin blood bags on red blood cells stored in a new additive solution. *Vox Sang* 2001;81:161–166.
- 13 Nakao M, Nakao T, Yamazoe S: Adenosine triphosphate and maintenance of shape of the human red cells. *Nature* 1960;187:945–946.
- 14 Seigneuret M, Devaux PF: ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proc Natl Acad Sci U S A* 1984;81:3751–3755.
- 15 Devaux PF, Herrmann A, Ohlwein N, Kozlov MM: How lipid flippases can modulate membrane structure. *Biochim Biophys Acta* 2008;1778:1591–1600.
- 16 Ferrell JE, Huestis WH: Phosphoinositide metabolism and the morphology of human erythrocytes. *J Cell Biol* 1984;98:1992–1998.
- 17 Sheetz MP, Singer SJ: Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc Natl Acad Sci U S A* 1974;71:4457–4461.
- 18 Mukhopadhyay R, Lim G, Wortis M: Echinocyte shapes: bending, stretching and shear determine spicule shape and spacing. *Biophys J* 2002;82:1756–1772.
- 19 Melzak KA, Lazaro GR, Hernandez-Machado A, Pagonabarraga I, de Espada JCMD, Toca-Herrera JL: AFM measurements and lipid rearrangements: evidence from red blood cell shape changes. *Soft Matter* 2012;8:7716–7726.
- 20 Zachowski A: Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochem J* 1993;294:1–14.
- 21 Wong P: A basis of echinocytosis and stomatocytosis in the disc-sphere transformations of the erythrocyte. *J Theor Biol* 1999;196:343–361.
- 22 Jay AWL: Geometry of the human erythrocyte I. Effect of albumin on cell geometry. *Biophys J* 1975;15:205–222.
- 23 Reinhart WH, Chien S: Echinocyte-stomatocyte transformation and shape control of human red blood cells; morphological aspects. *Am J Hematol* 1987;24:1–14.
- 24 Stewart A, Urbaniak S, Turner M, Bessos H: The application of a new quantitative assay for the monitoring of integrin-associated protein CD47 on red blood cells during storage and comparison with the expression of CD47 and phosphatidylserine with flow cytometry. *Transfusion* 2005;45:1496–1503.
- 25 Bain BJ: *Blood Cells – a Practical Guide*, ed 2. Oxford, Blackwell Science, 1995.
- 26 Daleke DL, Huestis WH: Incorporation and translocation of aminophospholipids in human erythrocytes. *Biochemistry* 1985;24:5406–5416.
- 27 Dodge JT, Phillips GB: Composition of phospholipids and of phospholipid fatty acids and aldehydes in human red cells. *J Lipid Res* 1967;8:667–675.
- 28 Bosch FH, Werra JM, Roerdinkholder-Stoelwinder B, Huls TH, Willekens FLA, Halie MR: Characteristics of red blood cell populations fractionated with a combination of counterflow centrifugation and Percoll centrifugation. *Blood* 1992;79:254–260.
- 29 Draper CJ, Greenwalt TJ, Dumaswala UJ: Biochemical and structural changes in RBCs stored with different plasticizers: the role of hexanol. *Transfusion* 2002;42:830–835.
- 30 Högman CF, Meryman HT: Storage parameters affecting red blood cell survival and function after transfusion. *Transfus Med Rev* 1999;13:275–296.
- 31 Deuticke B: Transformation and restoration of biconcave shape of human erythrocytes induced by amphiphilic agents and changes of ionic environment. *Biochim Biophys Acta* 1968;163:494–500.
- 32 Vlaar APJ, Kulik W, Nieuwland R, Peters CP, Tool ATJ, van Bruggen R, Juffermans NP, de Korte D: Accumulation of bioactive lipids during storage of blood products is not cell but plasma derived and temperature dependent. *Transfusion* 2011;51:2358–2366.
- 33 Truong HTN, Daleke DL, Huestis WH: Human erythrocyte shape regulation: interaction of metabolic and redox status. *Biochim Biophys Acta* 1993;1150:51–56.

- 34 Khairy K, Foo J, Howard J: Shapes of red blood cells: comparison of 3D confocal images with the bilayer-couple model. *Cell Mol Bioeng* 2008;1:173–181.
- 35 Tyteca D, D'Auria L, Van Der Smissen P, Medts T, Carpentier S, Monbaliu JC, de Diesbach P, Courttoy PL: Three unrelated sphingomyelin analogs spontaneously cluster into plasma membrane micrometric domains. *Biochim Biophys Acta* 2010;1798:909–927.
- 36 Melzak KA, Janzen J, Brooks DE: Electrostatic effects on the adsorption and carbodiimide-mediated coupling of ferrichrome A to amine-modified silica. *J Coll Interface Sci* 1995;174:480–489.
- 37 Sackmann E, Feder T: Budding, fission and domain formation in mixed lipid vesicle induced by lateral phase separation and macromolecular condensation. *Mol Membr Biol* 1995;12:21–28.
- 38 European Chemicals Bureau, Institute for Health and Consumer Protection (Joint Research Centre), Toxicology and Chemical Substances: Summary risk assessment report bis (2-ethylhexyl) phthalate (DEHP). <https://publications.europa.eu/en/publication-detail/-/publication/322f90a7-4766-4b8c-8d2f-564e32ae7199/language-en> (last accessed October 11, 2018).
- 39 Bonora S, Fini G, Piccirilli B: DSC study on the interaction between bis-2-(ethylhexyl) phthalate and other o-phthalic acid esters and dipalmitoyl phosphatidylcholine liposomes. *J Therm Anal Cal* 2000;61:731–743.
- 40 Wolkers WF, Crowe LM, Tsvetkova NM, Tablin F, Crowe JH: In situ assessment of erythrocyte membrane properties during cold storage. *Mol Membr Biol* 2002;19:59–65.
- 41 Lim G, Wortis M, Mukhopadhyay R: Stomatocyte-discocyte-echinocyte sequence of the human red blood cell: evidence for the bilayer-couple hypothesis from membrane mechanics *Proc Natl Acad Sci U S A* 2002;99:16766–16769.
- 42 Ran Q, Xiang Y, Liu Y, Xiang LX, Li FJ, Deng XJ, Xiao YN, Chen L, Chen LL, Li ZJ: Eryptosis indices as a novel predictive parameter for biocompatibility of Fe₃O₄ magnetic nanoparticles on erythrocytes. *Sci Rep* 2015;5:16209.
- 43 Boon JM, Smith BD: Facilitated phosphatidylcholine flip-flop across erythrocyte membrane using low molecular weight synthetic translocases. *J Am Chem Soc* 2001;123:6221–6226.
- 44 Boon JM, Lambert TN, Sisson AL, Davis AP, Smith BD: Facilitated phosphatidylserine (PS) flip-flop and thrombin activation using a synthetic scramblase. *J Am Chem Soc* 2003;125:8195–8201.
- 45 Roussel C, Dussiot M, Marin M, Morel A, Ndour PA, Duez, J, Le Van Kim C, Hermine O, Colin Y, Buffet PA, Amireault P.: Spherocytic shift of red blood cells during storage provides a quantitative whole cell-based marker of the storage lesion. *Transfusion* 2017;57:1007–1018.
- 46 Lu J, Shi H, Yu H, Hou J, Zhou J: Procoagulant activity of long-term stored red blood cells due to phosphatidylserine exposure. *Transfus Med* 2011;21:150–157.