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

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Supplemental Material

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Supporting Information

1. Determination of DEHP concentration by LC-MS/MS

Mixing DEHP with water does not give a well-defined value, due to the wide range of solubilities that have been reported. The concentration of DEHP in water for stirred preparations was therefore determined using LC-MS/MS analysis, at 1.4±0.8 ng/ml. This value (0.0014 mg/l) is towards the lower end of the range of the previously determined solubilities. The variability in the measured values has been attributed to the formation of suspensions rather than true solutions; some differences may be associated with impurities in the water which enhance the formation of the suspension, and some differences may be due to different measurement techniques. The error limits from the LC-MS/MS values imply that the distribution of the suspended particles is such that there is significant variability in the 20 µl sample size, another indication that the DEHP is present as a suspension rather than as a true solution.

The liquid chromatography (LC) ensures sample separation; the tandem mass spectrometry is used to determine concentration. An initial precursor ion at an MW of 391.2 (the $(M+H)^+$ peak for DEHP) was then selected; out of the secondary peaks from this precursor ion, the most sensitive peak (at 167) was selected for quantification. A standard curve was prepared using DEHP solutions that had been diluted in water from an initial 1000 ppm stock solution in acetonitrile. An Agilent 1100 HPLC system (Agilent, Germany) was used for sample separation on a Gemini C-18, 3 µm column (30×2 mm). The injection volume for all samples was 20 µl. Isocratic conditions of acetonitrile and 1% acetic acid (70:30) were used at a flow rate of 0.4 ml/min. The MS experiments were carried out in the positive ionisation mode using an ion spray voltage of 4800 V, a declustering potential of 30 V and and an entrance potential of 10 V. MS/MS experiments were generated using the compound optimisation mode from the software Analyst V 1.6. Mass spectrometric analysis was done using an API 4000 quadrupole mass spectrometer (Applied Biosystems / MDS Sciex, Canada), equipped with an electrospray ionisation source. The MS spectra were generated by infusion experiments using a syringe pump (Harvard apparatus Inc., South Natick, USA). Nitrogen (quality 5.0) was used as a curtain gas, nebuliser gas and collision gas. Glassware used to prepare solutions for analysis was cleaned using a solution prepared from 50 ml of H_2O added to 10 ml ammonium hydroxide (VWR), heated to 70 °C, followed by addition of 10 ml 30% H_2O_2.

Water was selected as the diluent for preparing the standard curve in order to minimise the matrix effects when determining concentrations of aqueous samples. The standard curve thus obtained was linear but with some scatter in the data points, resulting in $R^2 = 0.993$. The concentration of DEHP mixtures prepared by stirring the DEHP with water was determined to be 1.4±0.8 ng/ml using the standards diluted in water, based on duplicate measurements of four samples. Variation was seen between duplicates as well as between different samples. The DEHP suspension was found to be stable with respect to centrifugation at 1800 g.

2. Positive control for stomatocyte formation: full field of view.

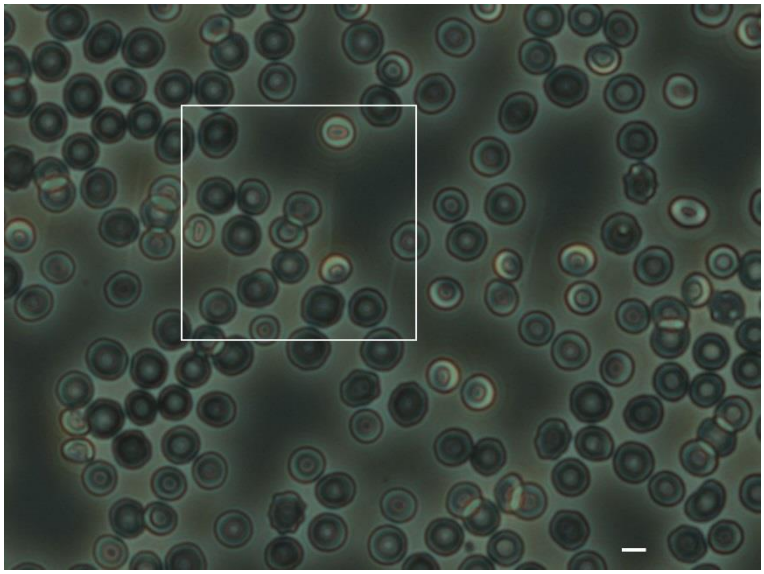


Figure S1: Stomatocytes formed in 2% HSA in PBS. This image shows the full field of view for the sample in figure 1a. The brightness and contrast have not been adjusted, and no modifications have been made to the original image other than the addition of the scale bar, which indicates 5µm, and the outline indicating the cropped region shown in figure 1a.

3. Identification of stomatocytes.

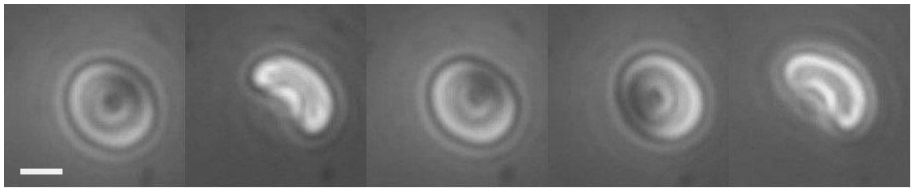


Figure S2: Sequence showing the changing orientation of the cell in figure 2b and 2f. The orientation of the lightly adherent stomatocyte is changed by in-situ rinsing. This set of images was taken over a 40 min period and shows a stomatocyte formed in DEHP/PBS. The surface of the PAH-coated slide was rinsed in-situ in between each of the images shown here using a pipette tip. The distinctive cup-shape of the stomatocyte can only be seen when the RBCs is adsorbed edge-on.

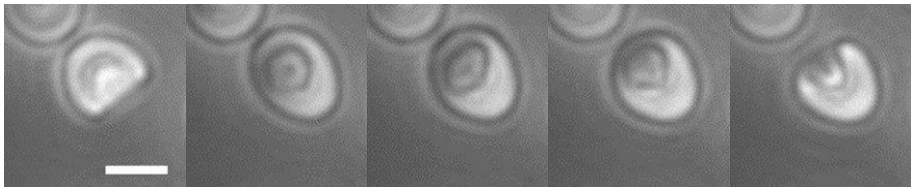


Figure S3: Sequence showing the changing orientation of the cell in figure 2c and 2g, collected as described above for figure S2 over a 12 min period.

4. Stomatocyte counts in sonicated DEHP: RBCs from different donors.

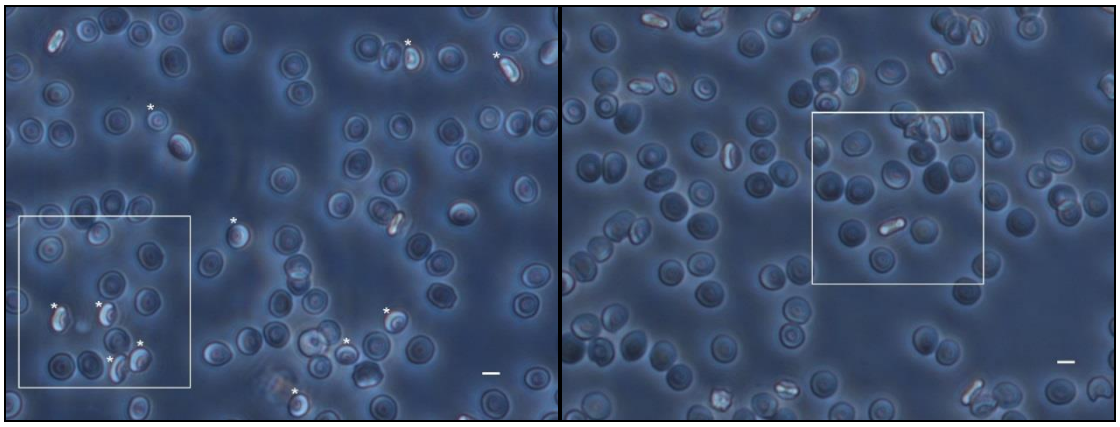
Incubation of RBCs with sonicated preparations of DEHP in PBS increased the numbers of stomatocyte present for samples from all donors.

	PBS only			sonicated DEHP / PBS		
donor	% stomatocytes	total cells counted	fields of view	% stomatocytes	total cells counted	fields of view
figure 3a, 3f	1.4	71	1	13.6	95	1
figure 3b, 3g	2.5	527	4	20.3	305	3
figure 3c, 3h	1.5	202	2	11.4	448	4
figure 3d, 3i	1.0	103	1	9.2	415	6
figure 3e, 3j	0.8	362	5	13.3	248	5

Table S1. Counts of stomatocytes associated with figure 3, for five different donors. The donors are identified here by the associated figure 3 images. The full fields of view from these images were used to count the number of stomatocytes present, along with additional fields of view from the same experiments, as specified in the table.

5. Sample images from DEHP-induced stomatocyte formation: full field of view.

These images show the full field of view for the cropped selections in figure 3e (left; RBCs incubated with sonicated DEHP/PBS)) and 3j (right; control for 3e, with RBCs in PBS). The brightness and contrast have not been adjusted, and no modifications have been made to the original image other than the addition of the scale bars, which indicate 5µm, the asterisks marking the stomatocytes and the outline indicating the cropped regions shown in figure 3.



6. Transition from discocyte to spherical shape after incubation with DEHP.

The figure below shows a second example, additional to Figure 3 in the main text, of an RBC changing from a discocyte shape to a spherical shape, with no apparent intervening stomatocyte forms.

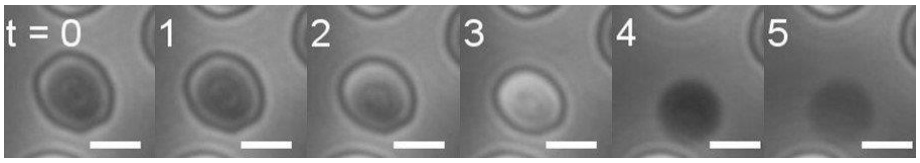


Figure S5: RBC incubated with DEHP/PBS on an adherent layer of PAH. The scale bar indicates 5 μm. The sequence here shows the transition from a discocyte to a spherical shape, and the subsequent lysis. Elapsed time in minutes is shown on the images.

7. RBC ghosts forming stomatocytes.

The spherical cells formed after exposure of the RBCs to DEHP lysed readily to form RBC ghosts. The example here shows additional images from the sequence shown in fig. 5, in which the ghost was seen to change shape to a form similar to a stomatocyte.

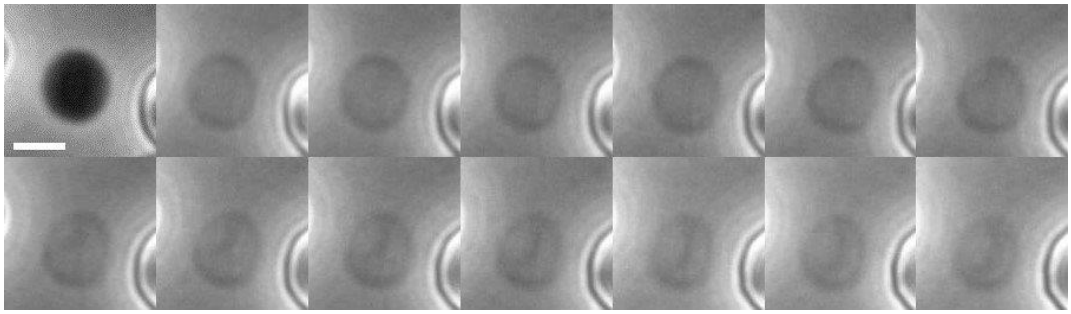


Figure S6: Sequence showing the transition from an RBC ghost to a stomatocyte form, on an adherent layer of PAH. The ghost was formed after incubation of an RBC sample in DEHP/PBS. The images shown here were acquired at 5 min intervals (apart from the first two images in the sequence) using phase contrast, with a Zeiss Axiovert A1 microscope (40× objective, NA = 0.55). The brightness and contrast have been adjusted. The scale bar on the first image indicates 5 μm.

8. Stomatocyte counts in stirred DEHP preparations

	PBS only			stirred DEHP / PBS		
donor	% stomatocytes	total cells counted	fields of view	% stomatocytes	total cells counted	fields of view
a (1 day old)	0.2	441	7	5.4	456	8
b (1 day old)	0.5	206	2	6.9	303	4
b (4 days old)	0.9	117	2	6.6	136	2

Table S2. Counts of stomatocytes for stirred preparations of DEHP, for three experiments with two different donors.

9. Flow cytometry and phosphatidylserine: **distribution of RBC size for Annexin A5 positive cells**.

RBCs incubated with PBS or DEHP/PBS were stained with annexin A5-FITC and then analysed using a flow cytometer (BD FACS Canto II, BD Bioscience). Variations in the percentage of annexin A5-positive cells are reported in Table 1 in the main text of the paper. The annexin A5- positive cells had a distribution of sizes in both the control and the sample incubated with DEHP, as indicated by the variation in the extent of the forward scattered light (FSC-A). This shows that there is no obvious selectivity for RBC size.

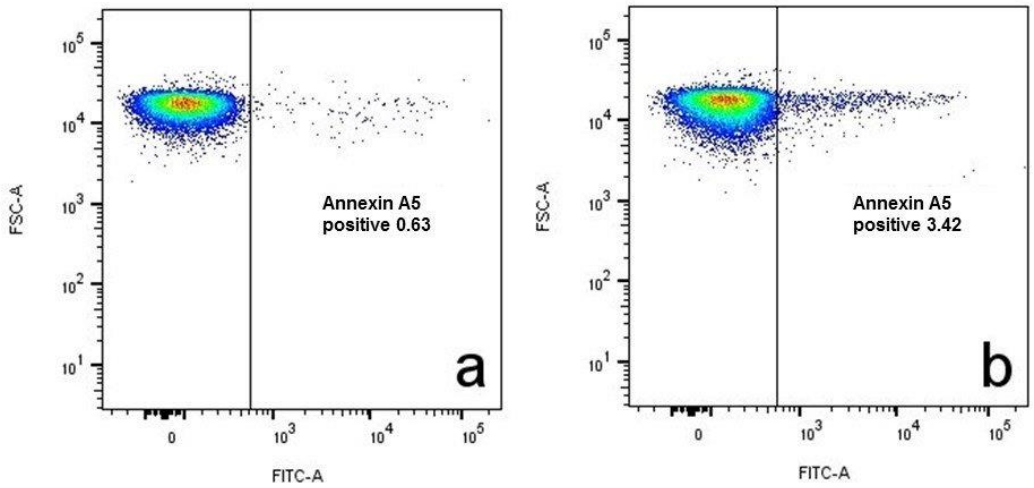


Figure S7: Forward scattering as a function of fluorescence intensity for RBCs incubated with PBS (a) and DEHP/PBS (b) and stained with annexin A5-FITC.

10. Effect of sodium orthovanadate on DEHP-induced phosphatidylserine exposure.

RBCs were incubated with PBS or with sonicated preparations of DEHP/PBS, both of these either with or without 0.2 mM sodium orthovanadate, and were then stained with annexin A5-FITC followed by analysis with a flow cytometer (BD FACS Canto II, BD Bioscience).

	no DEHP no vanadate	no DEHP with vanadate	with DEHP no vanadate	with DEHP with vanadate
experiment 1	0.25	0.24	1 (3.10 %)	1.07
experiment 2	0.00	0.03	1 (1.29 %	1.49
	0.27	0.13	1 (1.36 %)	1.07
experiment 3	0.05	0.05	1 (2.81 %)	0.78
experiment 4	-0.09	0.01	1 (0.95 %)	1.16
	0.04	0.10	1 (1.56 %)	1.13
	0.03	0.14	1 (1.16 %)	0.97
average	0.08	0.10	1 (1.75 %)	1.09
standard dev	0.13	0.08		0.22

Table S3. Effect of 0.2 mM sodium orthovanadate on the DEHP-induced phosphatidylserine exposure. Experiments were carried out with different DEHP preparations but with RBCs from one donor. The percentage of cells with exposed PS are given here relative to values for DEHP/PBS so that experiments can be compared. Measured values for DEHP/PBS are given in brackets. Negative numbers can be obtained because FACS results are given as stained minus unstained values.