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Distribution of Di(2-ethylhexyl) Phthalate and Products in Blood and Blood Components

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In order to impart flexibility, plastic medical devices incorporate liquid plasticizers into their structure. Data from several laboratories, including ours, have shown that these compounds leach from blood bags and tubing during collection of blood, storage of various blood components and during kidney dialysis and cell and plasma apheresis procedures. After the plasticizer di(2-ethylhexyl) phthalate leaches from poly(vinyl chloride) blood packs, it is converted by a plasma enzyme to a more toxic metabolite, mono(2-ethylhexyl) phthalate. Blood fractionation products from outdated plasma contain mono(2-ethylhexyl) phthalate, the highest level being found in normal serum albumin.

Recently, we have reported that di(2-ethylhexyl) phthalate actually binds to the red blood cell membrane and reduces its osmotic fragility. Current methods of red cell storage, which permit utilization up to 35 days after collection, are not possible without this membrane stabilization. Platelets are now stored for 5 days in the Fenwal PL 732 polyolefin bag. Although stated to be essentially free of liquid plasticizers, a significant level of leaching from this bag into the extracts of stored platelet concentrates was observed.

Introduction

The development of flexible plastic bags and multiple pack systems has revolutionized blood banking, permitting the rapid and easy preparation of blood components for therapeutic use. However, for some time it has been known that the plasticizer di(2-ethylhexyl) phthalate (DEHP), which is added to poly(vinyl chloride) (PVC) plastics to increase flexibility, can leach from the plastic during exposure to blood and blood components (*1–6*). PVC plastic is used in a great variety of medical devices, such as tubing for whole blood collection, bags for collection and storage of red cells, platelet concentrates (PC), or plasma, hemodialysis setups, tubing for intravenous injections, cytapheresis, plasma exchange and plasmapheresis.

DEHP plasticizer accounts for 30 to 40% of the weight of the formulated material and exists in the PVC matrix with a loose attachment to the resin in a semisolid or gellike structure. This loose arrangement is responsible for the leachability of the plasticizer upon contact with biological fluids. In 1967, Guess et al. (*7*) first reported the presence of DEHP as a contaminant of human blood stored in PVC bags. Subsequent work has demonstrated that DEHP accumulates in stored whole blood, in platelet concentrates (PC) (*3*), in cryoprecipitates (*8*), and in plasma derivatives (*9*) where there is a particular association with albumin.

An earlier publication had indicated that platelets were responsible for the leaching and subsequent plasma accumulation of DEHP. However, work in our laboratory demonstrated that a plasma protein which is not associated with platelets is responsible for the accumulation of DEHP, and that the DEHP is, for the most part, bound to plasma proteins (*6*). In any blood component where DEHP is found, the hydrolysis product mono(2-ethylhexyl) phthalate (MEHP), is also found to a lesser extent. The accumulation of MEHP was shown to be a direct result of the hydrolysis of DEHP by a plasma enzyme (*6*). This enzyme cofractionates with albumin, and some commercial preparations of normal serum albumin (NSA) contain significant amounts of both DEHP and MEHP (*9*). MEHP accumulation is of concern because MEHP has been shown to be 20 times more toxic than DEHP in rats (*10*).

The recent introduction of non-DEHP-containing bags for blood component production and storage was greeted with enthusiasm, since it was reasoned that DEHP, and therefore MEHP, exposure would be greatly reduced; however, PVC bags are still the most widely used for the collection and storage of red cell concentrates (RCC). We have recently found that DEHP binds to the red cell

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membrane and decreases its osmotic fragility (*11*). When RCC are stored beyond 21 days in the absence of DEHP considerable hemolysis occurs. Further, the *in vivo* survival is decreased. Thus, the long-term (beyond 21 days) storage of RCC is not possible without the stabilizing effect of DEHP.

Recently, a polyolefin bag, the Fenwal PL 732, has been introduced for the storage of platelets. This has extended the viability of the platelets from 3 to 5 days. Phospholipase A2 in platelet membranes is inhibited by DEHP (*12*). Perhaps the high level of DEHP which leaches out of the PVC bag is one of the causes of the platelet dysfunction found after three days of storage in the PVC bag; however, there may be another factor. Although the product is stated by the manufacturer to be essentially free of liquid plasticizers, we have recently found that there is a significant level of leaching of compounds into PC, platelet rich plasma (PRP), or platelet poor plasma (PPP) when these fractions are stored in the PL 732 bag (*13*). The function of platelets is better when stored in these bags, but aberrant morphological changes have also been observed (*14*).

Since compounds are leaching from PVC and polyolefin bags, and storage in these bags vastly improves the length of storage time of RCC and platelets, it would be extremely valuable to understand the mechanism of this increased stability, and fully define the biochemistry of interaction of the leachable materials with the RC and platelet membranes.

Results

One of the earliest studies done in our laboratory was to establish which blood component was responsible for DEHP leaching during storage of blood in PVC bags. After exposure to PVC plastics, DEHP levels in whole blood steadily increased for up to 4 weeks storage in PVC bags at 4°C (Fig. 1). Although it was previously thought (*1*) that platelets played an important role in the accumulation of DEHP in plasma, 72-hr PC, PRP, and PPP storage at 22°C showed similar concentrations of DEHP when corrections were made for volume differences (*6*) (Table 1). When plasma (which contained DEHP) was kept in glass, MEHP appeared with the concomitant disappearance of DEHP. Not all of the DEHP there originally was accounted for by MEHP accumulation. This

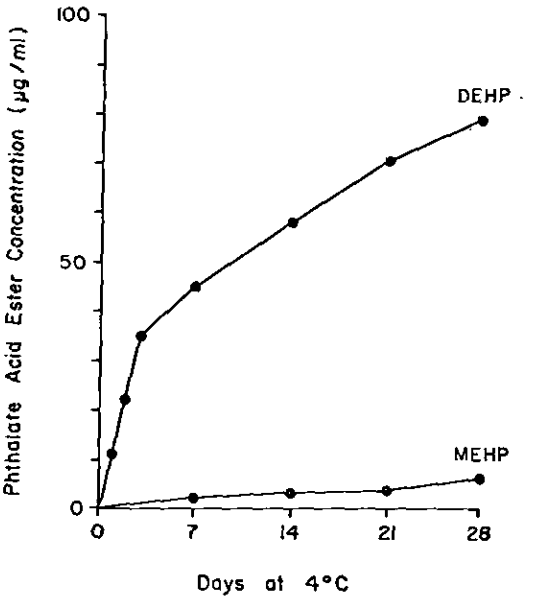

FIGURE 1. Accumulation of DEHP and MEHP during storage of whole blood in PVC bags at 4° C.

discrepancy was shown to be the result of the presence of other metabolites of MEHP, such as 2-ethylhexanol, 2-ethylhexanone, 2-ethylhexanoic acid, and some phthalic acid. These metabolites were identified by examination of the urine after ^{14}C-MEHP was administered orally to rats. More than 80% of the dose of radioactivity was found in the urine within 24 hr of ingestion (*10*). The liver was shown to be the major site of deposition, as well as the bladder and kidney, although the levels in the latter two tissues were probably high due to the excretory role played by these organs.

The accumulation of DEHP in stored blood is of concern for patients who receive multiple transfusions of blood products, although the long-term harmful side effects of this exposure have not been established. In animals the acute toxicity of phthalate esters is low with oral LD_{50} values in the range of 20 to 30 g/kg in mice and rats (*15*). Intravenous LD_{50} values in rats are in the range of 200 to 300 mg/kg. DEHP has been shown to elicit specific toxicological effects on the liver following oral administration to rats (*15*), with ultrastructural and biochemical changes having been observed (*15*). Recently, a study at

Table 1. Accumulation of DEHP and MEHP during storage at 22°C in various blood components.

Sample	Initial volume, mL	DEHP/MEHP concentration after various storage times, µg/mL			
		0	24 hr	48 hr	72 hr
DEHP					
PC	30	—	76 ± 7*	302 ± 22	491 ± 36
PRP	120	5 ± 2	34 ± 2	136 ± 4	181 ± 7
PPP	90	—	52 ± 2	197 ± 28	285 ± 40
MEHP					
PC	30	—	15.0 ± 7.7	28.5 ± 1.6	76.1 ± 11.5
PRP	120	2.7 ± 0.8	5.9 ± 0.5	13.2 ± 1.2	31.0 ± 0.9
PPP	90	—	8.5 ± 0.9	20.0 ± 0.8	53.6 ± 3.9

End Table 1, Chapter 41

the National Institutes of Health documented carcinogenicity in rats and mice following oral administration of large doses of DEHP (*16*). Atrophy of the testis and cessation of spermatogenesis have also been observed (*15*). At the cellular level, DEHP is lethal for chick embryo beating heart at a concentration of 4 μg/mL (*15*). It is highly toxic to mice fibroblasts in culture and causes significant growth inhibition of human diploid fibroblasts in tissue culture (*15*).

These toxicity figures are not necessarily applicable to blood and blood products; however, some typical amounts of DEHP exposure can be calculated. Based on rates of leaching of DEHP of 0.25 mg/100 mL/day into whole blood stored at 4°C (*15*), a 70-kg man given a single exchange transfusion (3 L) of whole blood which has been stored for 3 weeks at 4°C would receive approximately 250 mg DEHP, a dose equivalent to 3.6 mg/kg. Administration of various products to the "multi-transfused" patient results in average exposures to DEHP in the range of 1 mg per patient per day for whole blood (*9*), 0.6 mg per patient per day for packed cells, and 3 mg per patient per day for platelet concentrates.

Hemophiliacs are one of the patient populations at risk of considerable exposure, since they may receive many transfusions of blood products in a short period of time. Each cryoprecipitate contains approximately 20 μg/mL of DEHP (*17*). The higher purity VIIIC concentrates contain no DEHP and < 1 μg/mL MEHP (*9*). In a study carried out at the Ottawa Centre, DEHP blood levels were measured before and after infusion with cryoprecipitate into hemophiliac patients, and all of the DEHP was cleared from the blood 10 min after infusion. Although constant exposure to DEHP can result in the induction of liver microsomal enzymes along with other structural changes (*15*), all of our patients had normal liver function.

Patients undergoing renal dialysis have also been studied as another high risk group. Several reports (*18*) have demonstrated the extraction of 1.5 mg of DEHP from hemodialysis tubing into the blood of a patient dialyzed for 5 hr (*19*), with the levels peaking within the first few minutes of hemodialysis. The MEHP concentration increases gradually. When we carried out a similar study, we measured pre- and post-dialysis whole blood levels of DEHP in a series of 14 patients. The concentration of DEHP ranged from 1 to 14.5 μg/mL. MEHP was detected in seven of these patients before dialysis, while the post-dialysis blood samples of all 14 patients contained 0.2–4 μg/mL of the compound. The high levels of DEHP in the predialysis samples in some patients were found to be due to leaching from the Terumo AVF extension set. Our study showed that DEHP leached from the artificial kidney; it was detectable in blood after only one passage through the dialysis machine. However, DEHP was rapidly removed from the circulatory system as none was found in blood after 15 min. MEHP also appeared in the blood after one passage through the artificial kidney, indicating that DEHP was rapidly converted to MEHP, the level of which remained elevated after 15 min.

If daily plasma exchange is required for 8 days, as is often the case, the total short-term exposure would be 29 mg DEHP. Exposure approaching this value could occur for cytopheresis or plasmapheresis donors, thereby putting the normal donor population at risk.

Whenever DEHP is present, MEHP also appears. The characteristics and site of this metabolic conversion were studied in our Centre. One site for this reaction to occur was shown to be plasma. A soluble enzyme which converts DEHP to MEHP was partially purified by high speed centrifugation of PPP (*20*). The enzyme responsible for the conversion has a pH optimum of 7.2 and a molecular weight of 50,000 to 100,000; it appears to exist in a complex with the oxidation system that further degrades MEHP. In its partially purified state, Mg^{2+} and ATP stimulate the activity, whereas adenine (0.13 mM) and detergents inhibit it (Fig. 2). The enzyme can be freed of 95% of the plasma albumin by passage through an Affigel Blue column; however, polyacrylamide gel electrophoresis of the partially purified enzyme still indicates a significant contamination by albumin.

A comparison of whole blood stored in citrate-phosphate-dextrose (CPD)-adenine blood packs shows that the presence of adenine actually causes a decrease in the amount of DEHP converted to MEHP; however, the use of CPD-adenine packs to increase the storage life of RCC past 21 days also means a higher final level of DEHP, since the concentration of DEHP gradually increases up to 46 days when whole blood is stored at 4°C (*21*).

Since plasma contains the enzyme system for the leaching and conversion of DEHP to MEHP and because this system is increasingly active at higher temperatures, the conditions used for the storage and transport of outdated plasma used for fractionation are of great significance. Low levels of DEHP contamination have been found in a number of Cohn fractions including fibrinogen, gamma-globulin, and albumin (*22*). Low levels of MEHP were

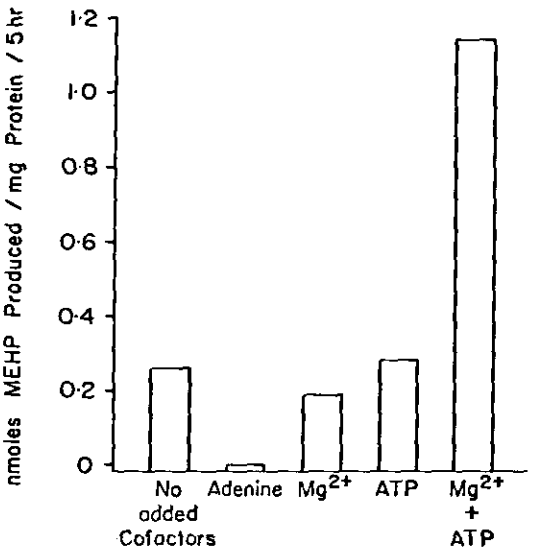

FIGURE 2. Purified DEHP-hydrolyzing enzyme activity with added cofactors.

Table 2. DEHP and MEHP levels in blood products from three different companies.

	Phthalate acid ester concentration, μg/mL[a]					
	Company A		Company B		Comapny C	
Blood product	MEHP	DEHP	MEHP	DEHP	MEHP	DEHP
NSA (5%)	1 ± 0	0	Trace	0	N/A	N/A
NSA (25%)	10 ± 1	0	13 ± 10	0	330 ± 48	0
PPF (5%)	5 ± 1	0	Trace	0	N/A	N/A
Factor VIII	1 ± 1	0	0	0	N/A	N/A
Factor IX	0	0	0	0	3 ± 2	23 ± 16
Immune serum globulin	0	0	0	0	0	0
Rh immune globulin	N/A	N/A	N/A	N/A	0	0

[a] N/A = not available; trace = < 1 μg/mL; n = 4 for each product.

also found in fibrinogen and immunoglobulins with higher levels in albumin (*23*).

The study carried out in our laboratory compared MEHP and DEHP in commercially available plasma fractionation products obtained from three different North American companies and related the level of the contamination to the conditions of plasma storage and transportation (Table 2). As might be expected, contamination of plasma with DEHP and MEHP resulted in a concomitant contamination of blood products prepared by fractionation of this plasma. Products from companies A and B, which used fresh frozen plasma as starting material, did not contain DEHP but did have low levels of MEHP in all of the albumin-containing fractions, as well as in some Factor VIII preparations. On the other hand, the 25% NSA from company C, which used out-dated unfrozen plasma as starting material, was contaminated by high levels of MEHP but no DEHP, while Factor IX preparations contained both MEHP and DEHP.

These data showed a direct correlation between temperature of storage or shipment of plasma and the level of contaminating phthalates. Since we have shown that the enzyme with DEHP hydrolyzing activity copurifies with albumin, even after Affi-gel Blue chromatography, it is not surprising that MEHP was present in all NSA and plasma protein fraction solutions. The MEHP, once formed, is tightly bound to albumin in the purified preparation and could not be removed by dialysis or ultracentrifugation. By freezing the plasma it was possible to prevent further leaching of DEHP or conversion to MEHP (Fig. 3) for 6 months. DEHP does not increase in frozen RCC stored for up to 2 years (*5*) or in plasma which is frozen for 5 weeks (*23*).

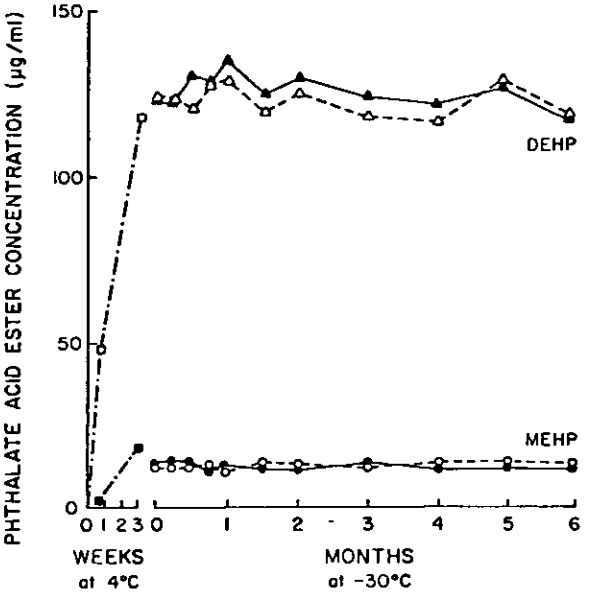

FIGURE 3. DEHP and MEHP levels in PPP stored at −30°C. Levels of DEHP and MEHP in PPP stored at −30°C: (▲,●) frozen in glass tubes; (△,○) frozen in PVC bags; (□,■) bags pooled and frozen.

The recommendation that was made at the time of this study (1981) was to freeze out-dated plasma to be used for fractionation as soon as possible prior to shipment to the fractionator. Unfortunately, this still is not currently the practice in several countries. A few months ago, we did a study in our Centre for a hospital which was routinely storing a liter of lactated Ringer's solution with albumin for 3 months in PVC bags. The DEHP levels slowly increased during this period; however, the initial level of MEHP in the 25% stock NSA solution used for the preparation of the IV solution was extremely high (300 μg/mL). The enzyme which converts DEHP to MEHP copurified with the albumin, and the MEHP, once formed in the plasma, bound to the albumin during purification. Immediate freezing of the out-dated plasma would eliminate both of these effects.

Despite the availability of non-DEHP plasticized bags, the collection and storage of RCCs is still done in DEHP plasticized packs for an extended period (35 days) of time. Recently, we have examined the interaction of DEHP

Table 3. Association of ^{14}C-DEHP with red blood cells.

	Association after storage at 4°C, %[a]	
Samples	No storage	7 days storage
Red cell concentrate before washing	100.0 ± 0*	100.0 ± 0
Wash supernatant	35.9 ± 1.6[b]	47.9 ± 4.6[c]
Platelet pellet from wash	6.3 ± 2.0	10.0 ± 3.4
Washed RBC	28.0 ± 2.4[d]	24.6 ± 5.5[e]
RBC cytosol	14.7 ± 0.8	13.0 ± 0.8
RBC membrane	11.6 ± 1.2	11.7 ± 2.0

[a] Mean ± SD (n = 4)
[b,c] significantly different at $p < 0.05$.
[d,e] not significantly different at $p < 0.05$ (unpaired Student's *T*-test).

with stored RCC (*11*) and found that ^{14}C-DEHP is incorporated into the RBC during storage at 4°C. There is an immediate binding of 28% of the available ^{14}C-DEHP to the RBC on day 0, with approximately equal amounts of ^{14}C-DEHP being incorporated into the cytosol and membrane fractions (Table 3). The total amount and relative distribution of the ^{14}C-DEHP does not significantly change over 7 days. A study by Estep et al. (*24*) showed that distribution of DEHP added as an emulsion to whole blood was complete within 24 hr. They found that about 5 to 10% of the total blood concentration of DEHP was associated with RBC; however, the DEHP concentration they were working with was 300 to 500 μg/mL, whereas ours was 50 μg/mL. The significant point of this work is that the stability of RBC is greatly improved by the addition of DEHP.

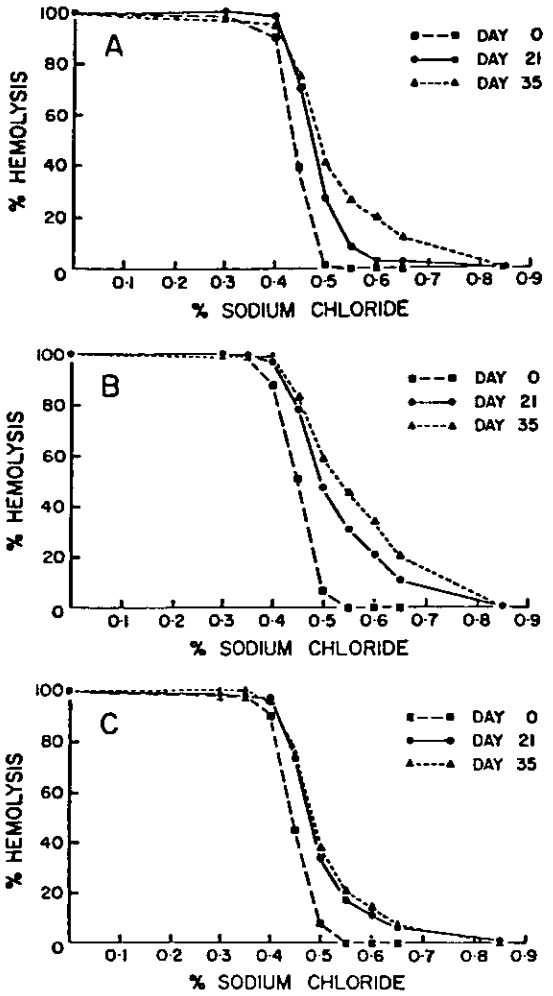

FIGURE 4. Reduced osmotic fragility of red blood cells. Red cells concentrates (*A*) contained DEHP-enriched plasma and were stored in polyolefin bags; (*B*) contained fresh plasma in polyolefin bags; (*C*) contained fresh plasma in PVC bags (n = 3). Red cell concentrates were prepared by adding either fresh plasma or DEHP enriched plasma to packed RBCs. Red cell osmotic fragility was determined on days 0, 21, and 35.

We have found increased osmotic fragility in RCC stored in polyolefin bags rather than in PVC bags, with significant release of hemoglobin after prolonged storage of cells in polyolefin bags (Fig. 4). When RBC were stored in DEHP-enriched plasma in polyolefin bags (series A) or in PVC bags (series C), the stability was considerably better than for RBC stored in polyolefin bags without the DEHP (series B). The decreased RBC fragility could be correlated directly to the DEHP concentration which in series A was 97.2 μg/mL, series B 30.0 μg/mL, and series C 34.2 μg/mL. By day 35, the DEHP concentration in the series C cells was 418.3 μg/mL.

The values for plasma hemoglobin in the series A, B, and C RCC storage groups agreed with the osmotic fragility studies, i.e., there was less hemoglobin in series C (stored in PVC packs) than in either of the two RCC stored in polyolefin packs (Table 4). The series A samples had the highest initial concentration of DEHP and showed the lowest plasma hemoglobin on day 0.

There was also decreased osmotic fragility in cell concentrates stored in glass tubes with added DEHP. The level of DEHP was 50.8 μg/mL on day 0 and 40 μg/mL on day 21. There was no detectable DEHP in the controls.

Estep et al. (*24*) were able to show a 20-fold improvement in the percentage of cells exhibiting normal morphology, a 60% inhibition in the accumulation of hemoglobin in plasma, a 50% reduction in the degree of erythrocyte microvesiculation, and an enhanced preservation of normal cellular osmotic fragility and filterability after 35 days of refrigerated storage when DEHP was added to the environment.

Two important conclusions can be drawn: one is that previous estimates of the dose of plasticizers which patients receive have probably been underestimated, since the calculations were based primarily on plasma concentrations, ignoring the uptake by RBC; the other conclusion has been observed by other groups (*24,25*) as well as ourselves, that *in vitro* stability of RCC is improved by exposure to DEHP.

Although PVC bags are still used for storage of RBC, platelets are now being stored in new bags which allow 5 day storage. When platelets were kept in PVC bags,

Table 4. Plasma hemoglobin values of stored red cell concentrates.

Red cell concentrates[a]		Plasma hemoglobin, mg/dL[b]		
		Day 0	Day 21	Day 35
A	Prepared with DEHP plasma, stored in polyolefin packs	5.7 ± 2.0	30.3 ± 3.1	118.3 ± 38.5
B	Prepared with fresh plasma, stored in polyolefin packs	26.7 ± 3.6	73.3 ± 13.4	181.7 ± 70.6
C	Prepared with fresh plasma, stored in PVC packs	21.2 ± 6.5	42.3 ± 8.4	90.3 ± 20.8

[a] The red cell concentrates were prepared from pooled RBCs and stored at 4°C. Immediately prior to assay they were diluted to a hematocrit of 40% with fresh plasma.

[b] Mean ± SD (n = 3).

they progressively lost functional integrity and were not used after 3 days. We have found that DEHP leaches from PVC bags into PC (*6*) and that the platelets bind 6 to 10% of ^{14}C-DEHP mixed with whole blood. In a recent study in our laboratory, the direct effect of the plasticizer DEHP on platelet phospholipase A2 (PLA2) activity was studied. PLA2 activity decreased continually during storage over 3 days in DEHP-containing bags to 61% of the activity on the day of collection. During this period DEHP levels increased from 35 to 270 μg/mL. Addition of DEHP directly to fresh platelets at concentrations of 67 μg/mL or higher resulted in a similar drop of PLA2 activity to 53% of control. The inhibition of PLA2 in platelets by accumulated DEHP would prevent arachidonic acid liberation and endoperoxide formation, rendering platelets unable to respond to physiological stimuli *in vitro* and *in vivo* (*12*).

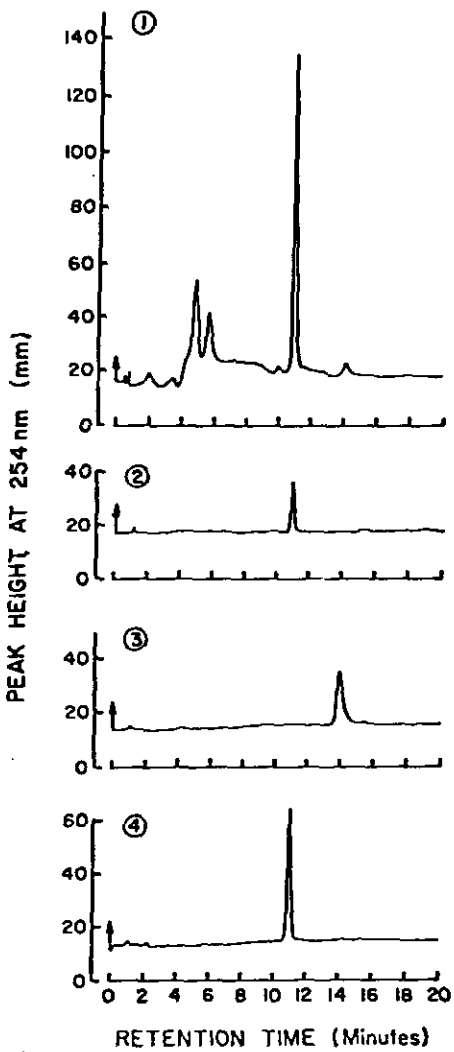

FIGURE 5. Leachable compound from the PL 732 Bag and stored plasma: (1) HPLC of the extract of the PL 732 bag; (2) extract of stored plasma (72 hr at 22°C); (3) BHBB standard (10 μg/mL); and (4) DEHP standard (10μg/mL) using an MCH-10 reverse-phase column eluting with a gradient of 70% acetonitrile:30% water increasing to 100% acetonitrile. The arrow indicates the time of injection.

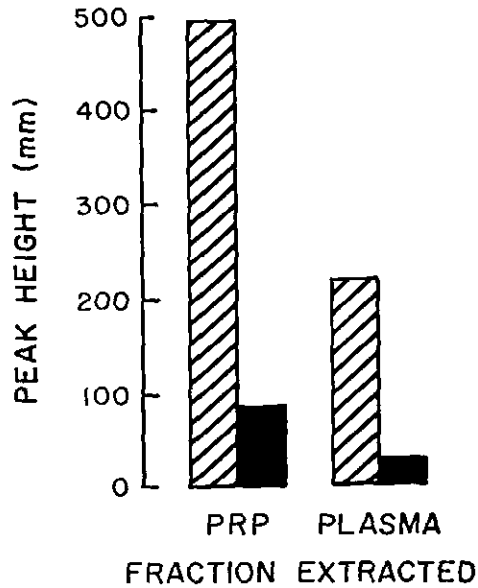

FIGURE 6. Day 6 ratio of "DEHP:MEHP-like" leachables: (▨) DEHP extraction procedure and HPLC system; (■) MEHP extraction procedure and HPLC system.

While it would seem that this problem is now solved by the storage of platelets in the new plastic bags, we have recently found that there is a significant level of leaching of bag-derived compounds into PC, PRP, or plasma when these fractions are stored in the Fenwal PL 732 at 22°C in a platelet rotator (*13*). Although stated by the manufacturer to be essentially free of liquid plasticizers, an antioxidant, 1,3,5-trimethyl-2,4,6-tris-(3,5-di-*tert*-butyl-4-hydroxybenzyl)benzene (BHBB), is used in the manufacture of the polyolefin plastic.

The major leachable compound observed has the same HPLC retention time as does DEHP. This compound can be extracted from the bag itself, in addition to a small amount of BHBB. The ratio of BHBB to the major leachable compound is 1:15; BHBB was not detected in the extract from any of the stored platelet fractions or from plasma (Fig. 5).

The amount of leachable material continued to increase linearly up to 72 hr storage. On day 6, the ratio of the two leachable compounds in PRP supernatant and plasma was determined. By using the extraction method for DEHP and MEHP (*6*), followed by analysis of the extracts using the HPLC system for DEHP and MEHP (*9*), it can be seen in Figure 6 that the ratio of compounds is 3:1 whether the fraction analyzed is PRP or plasma.

The recent observation of aberrant morphology of platelets following storage in these new bags may be due to these leachable compounds or to other factors associated with polyolefin bags. Fratantoni et al. (*14*) were able to stop the development of the unusual morphological alterations if the permeability of the container was inhibited and the pH kept below 6.7. In light of the documented effect of DEHP on red cell membranes, it may perhaps be the absence of high levels of DEHP which permits the platelet membrane to change shape.

Conclusions

As is usually the case with any technological advance, the development of flexible plastic bags, multiple blood pack systems, and plastic tubing for medical use has brought with it great benefits along with some risk. It is well established that DEHP leaches out of these bags and other medical devices into blood components, that the concentration increases with storage, and that DEHP is converted to MEHP, a more toxic metabolite (*1–6*).

Animal studies have demonstrated the toxicity of these compounds and more recently their carcinogenicity and teratogenicity (*16*). Although the results of these investigations cannot be extrapolated directly to humans, DEHP and MEHP have been found in blood products such as albumin and cryoprecipitate and have been identified in the sera and tissues of many patients (*8*). This has raised concerns about the amount of plasticizer received by hemodialysis patients, hemophiliacs, and other multi-transfused individuals. Non-DEHP plastics, such as polyolefin, are now available and are used for storing platelets, but these plastics are not generally used for RC storage.

The data we obtained on the binding of ^{14}C-DEHP to the red cell membrane suggest that the previous estimates of the dose of plasticizers which patients receive have been underestimated, since the calculations were based primarily on plasma concentrations, ignoring the uptake by the red cell. This difference has important implications, since it is RCC rather than whole blood which are stored in the majority of blood banks. Long-term storage in the absence of DEHP severely compromises red cell integrity and survival (*11*).

Platelets which were previously stored for 3 days in DEHP-containing PVC packs are now stored in polyolefin bags where they are stable for 5 days; however, we have found that material also leaches from these bags (*13*); other groups have reported aberrant morphology of platelets stored in these bags (*14*).

In order to assess the risk/benefit question for blood and blood products stored in plastic containers, the mechanism of interaction of the plasticizer with the RBC and platelet membrane must be fully defined, as well as the effects on RBC and platelet metabolism.

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