Inhibition of membrane Na\(^+\)-K\(^+\) ATPase of the brain, liver and RBC in rats administered di(2-ethylhexyl) phthalate (DEHP) a plasticizer used in polyvinyl chloride (PVC) blood storage bags

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Significant amounts of di(2-ethylhexyl) phthalate (DEHP) leach out into blood stored in DEHP plasticized polyvinyl chloride (PVC) bags resulting in the exposure of recipients of blood transfusion to this compound. The aim of this study was to find out whether DEHP at these low levels has any effect on the activity of membrane Na\(^+\)-K\(^+\) ATPase, since a decrease in this enzyme activity has been reported to take place in a number of disorders like neurodegenerative and psychiatric disorders, coronary artery disease and stroke, syndrome-X, tumours etc. DEHP was administered (ip) at a low dose of 750 µg/100 g body weight to rats and the activity of membrane Na\(^+\)-K\(^+\) ATPase in liver, brain and RBC was estimated. Histopathology of brain, activity of HMG CoA reductase (a major rate limiting enzyme in the isoprenoid pathway of which digoxin, the physiological inhibitor of Na\(^+\)-K\(^+\) ATPase is a product), intracellular concentration of Ca\(^{++}\) and Mg\(^{++}\) in RBC (which is altered as a result of inhibition of Na\(^+\)-K\(^+\) ATPase) were also studied. (In the light of the observation of increase of intracellular Ca\(^{++}\) load and intracellular depletion of Mg\(^{++}\) when Na\(^+\)-K\(^+\) ATPase is inhibited). Histopathology of brain revealed areas of degeneration in the rats administered DEHP. There was significant inhibition of membrane Na\(^+\)-K\(^+\) ATPase in brain, liver and RBC. Intracellular Ca\(^{++}\) increased in the RBC while intracellular Mg\(^{++}\) decreased. However activity of hepatic HMG CoA reductase decreased. Activity of Na\(^+\)-K\(^+\) ATPase and HMG CoA reductase, however returned to normal levels within 7 days of stopping administration of DEHP. The inhibition of membrane Na\(^+\)-K\(^+\) ATPase activity by DEHP may indicate the possibility of predisposing recipients of transfusion of blood or hemodialysis to the various disorders mentioned above. However since this effect is reversed when DEHP administration is stopped, it may not be a serious problem in the case of a few transfusion; but in patients receiving repeated blood transfusion as in thalassemia patients or patients undergoing hemodialysis, possibility of this risk has to be considered. This inhibition is a direct effect of DEHP or its metabolites, since activity of HMG CoA reductase, (an enzyme which catalyses a major rate limiting step in the isoprenoid pathway by which digoxin, the physiological inhibitor of Na\(^+\)-K\(^+\) ATPase is synthesized) showed a decrease.

**Keywords**: DEHP, HMG CoA reductase, Intracellular Ca\(^{++}\) and Mg\(^{++}\) concentration. Membrane Na\(^+\)-K\(^+\) ATPase, Polyvinyl chloride

DEHP [di(2-ethylhexyl)phthalate], a widely used plasticizer in blood storage bags, leaches out in appreciable amounts into blood (about 10-15 mg/100ml)\(^1\) resulting in exposure of recipients of blood transfusion to this compound\(^2\).\(^3\)\(^4\). Various reports indicate the toxicity of DEHP, particularly in liver and reproductive organs\(^5\)\(^6\) but all these studies used large doses (up to 2g or more/kg body weight) and oral route of administration, which are not relevant to the intravenous administration during blood transfusion or the low amounts of DEHP present in blood. We had earlier reported that administration of DEHP at low doses (corresponding to transfusion of 2,4,6 and 10 units of blood) to rats did not produce any histopathological changes in the liver or testes or any significant alteration in many of the biochemical parameters studied like γ-glutamyl transferase, alanine aminotransferase, CPK, LDH, alkaline phosphatase β-glucuronidase of the liver, plasma and testes, serum albumin, acid phosphatase of the testes etc\(^1\). There was however decrease in concentration of vitamin E in the liver and testes which however returned to normal within 72 hr to 7 days\(^1\). Decrease in the concentration of vitamin E in the blood stored in DEHP plasticized PVC bags was also observed by us\(^1\).

We have now carried out further detailed studies on the effect of DEHP and investigated the changes in the membrane Na\(^+\)-K\(^+\) ATPase of the brain, liver and RBCs, since a decrease in this enzyme activity is known to be involved in the pathogenesis of a number of disorders like neurological disorders, hypertension, diabetes, coronary artery disease and stroke, tumors...
enzyme involved in the transport of various cations and the steroidal glycoside digoxin, now known to be synthesized by the mammalian hypothalamus. It is also known that an inhibition of this enzyme results in a depletion of intracellular Mg and increase in intracellular Ca load. The change in the intracellular levels of calcium and magnesium is believed to be the major factor in the pathogenesis of the various disorders mentioned above. If DEHP at the level present in blood stored in DEHP plasticized PVC bags causes any inhibition of Na-K ATPase, then the possibility of exposing a recipient of blood transfusion to a risk for the above-mentioned disorders, has to be reckoned with.

We therefore studied the changes in the activity of this enzyme in the membrane of brain, liver and RBCs in rats administered DEHP at a dose of 750 μg/kg body weight which corresponds to successive transfusion of 10 units of blood in a recipient. The intracellular Mg and Ca concentration was also studied in the RBCs. Since the physiological inhibitor of Na-K ATPase is digoxin, which is known to be synthesized by the hypothalamus, from acetyl CoA by the isoprenoid pathway, the activity of a key regulatory enzyme, viz. HMG CoA reductase of this pathway was also studied to ascertain whether DEHP acts via influencing this pathway. Concentration of cholesterol in the serum, which is also synthesized by this pathway, was also studied.

Necessary ethical clearance of this work was obtained by the ethical committee of the Institute.

Materials and Methods
Female, albino rats (Wistar strain, average body weight 150 g) were used for this study. The rats were grouped randomly into two groups with 16 rats in each group.

1. Group 1 — control rats
2. Group 2 — experimental rats

The experimental rats were administered DEHP at a dose of 750μg/kg body weight. Emulsion of DEHP was prepared in 2.2% glycerol containing 1.2% egg yolk lecithin by sonication under sterile conditions and was administered intraperitonially as described earlier. The control rats received the same volume of vehicle. The animals were caged individually in polypropylene cages and maintained on normal laboratory feed (Sai feed) at temperature 28°C ± 1°C. Food and water were available to the rats ad libitum.

Administration of DEHP was made on alternate days. A total of 7 injections were given and at the end of 14 days, 8 rats in each group were deprived of food overnight and blood was removed to citrated tubes for preparation of RBCs. They were then stunned by a blow at the back of neck and killed by decapitation. Brain and liver tissues were removed to ice-cold containers for various estimations.

The injection of DEHP was stopped in remaining 8 rats in group II at the end of 14 days and these rats along with control rats continued on their diet for another 7 days. They were then deprived of food overnight, and killed as above. Blood, liver and brain were collected for various estimations as before. This was done to ascertain whether the effect produced by DEHP was reversible or not, when DEHP administration was stopped.

Analytical Procedures

Estimation of membrane Na-K ATPase activity (EC 3.6.1.37)—Preparation of RBC membrane: RBCs were separated from citrated blood by centrifugation at 1300 rpm in a Sorvall super T1 centrifuge (USA) using rotor SL-50T for 15 min at 4°C. The erythrocyte fraction after removal of buffy coat and plasma was washed thrice by centrifugation and resuspended in 0.9% NaCl solution (3000 rpm for 10 min at 4°C). The cells were then lysed with distilled water and the cell membranes washed twice with 1 mM tris-HCl buffer (pH 7.4) containing 1 mM EDTA at 4°C followed by washing with 1 mM tris-HCl buffer containing 10 mM EDTA and finally thrice with 2 mM Tris-HCl buffer (pH 7.4). The RBC membrane was finally suspended in 0.0625M Tris-HCl buffer pH 7.4. The membrane bound enzyme was released by freezing the suspension and thawing it and repeating the process. This suspension was used for the assay of Na-K ATPase.

Preparation of the membrane from brain and liver tissues: Tissues were homogenized under ice-cold conditions in 0.25 M sucrose and centrifuged at 3500 rpm for 10 min at 4°C. The residue containing cell debris and unbroken cells was washed with normal saline twice and the rest of the procedure was the same as described for RBC.

Assay of Na-K ATPase activity: The following reaction mixture was used.

Reagents:
(1) NaCl-0.25 M, MgSO4-0.00138 M and KCl-0.0125 M — 0.4 ml
(2) Na-ATP — 0.00125 M — 0.4 ml
(3) Tris-HCl buffer — 0.0625 M (pH 7.4), containing
3.12 x 10^{-4} M EDTA — 0.16 ml

Two sets of tubes, one test and the other control, containing the above reagents in a total volume of 0.96 ml, were preincubated at 37°C for 5 min and 0.2 ml of the enzyme preparation (20-25 mg/ml protein in the case of liver membrane, 0.8-1 mg/ml protein in the case of brain membrane and 4-5 mg/ml protein in RBC membrane) was then added to the test. The tubes were incubated at 37°C for 1 hr and were then transferred to powdered ice and ice-cold 12% TCA (1 ml) was added to both followed by 0.2 ml enzyme preparation to the control. The ATP remaining in the test (after the action of the enzyme) and in the control (enzyme inactivated) was then measured by using kit purchased from Sigma chemical, (USA). Concentration of the protein in the enzyme preparation was estimated after dissolving the TCA precipitate in 0.01 N NaOH. Lowry’s procedure was used for protein estimation.

Analysis of intracellular Ca^{2+} and Mg^{2+} in the RBC — For the analysis of intracellular Ca^{2+} and Mg^{2+} in the RBC, the cells were lysed with millipore deionised spectroscopy grade water and deproteinised with perchloric acid (final concentration-4%). The supernatant was neutralized with KOH and kept at 4°C overnight. The precipitated potassium perchlorate was removed by centrifugation and the supernatant was used for estimation of Ca^{2+} and Mg^{2+} using commercial kits (Ca^{2+}-Bayers and Mg^{2+}-Randox).

Activity of HMG CoA reductase (EC 1.1.1.34) — Activity of HMG CoA reductase in the liver was studied by the method of Rao and Ramakrishnan by measuring the ratio of HMG CoA to mevalonate. A low ratio indicates higher activity. Cholesterol in serum was estimated by using kit available from Randox.

For histopathological examination of the brain, the tissue was transferred to buffered 10% formalin and the sections were stained with haematoxylin and eosin.

Statistical analysis was carried out using ‘ANOVA’. SPSS statistical programme was used and the results were evaluated using analysis of variance utilizing the ‘t’ test. The results were presented as the mean ± SD. Differences among the means of the groups were assessed using the Duncan Procedure to determine which mean values were significantly different at \( P < 0.01 \) and \( P \) between 0.01 and 0.05.

Results

Histopathological evaluation — Histopathological examination of the brain revealed areas of degeneration (Fig. 1). Results of histopathological examination of liver was reported before. No gross changes were observed in the liver.

Activity of Na^{+}-K^{+} ATPase in the membrane of RBC, liver and brain cells (Table 1) — There was significant inhibition of the enzyme activity in the membrane of RBC, liver and brain in the rats administered DEHP, when compared to control rats. The effect was reversed and near normal value of enzyme activity was observed at the end of 7 days after withdrawal of DEHP.

Concentration of intracellular Ca^{2+} and Mg^{2+} in the RBC (Table 2) — Significant decrease in the Mg^{2+} in RBC was observed in the rats administered DEHP.

Fig. 1 — Histopathology of brain from rats receiving 750 \( \mu \)g/100 g body weight by successive administration. Sections stained with H&E. 200 \( \times \). [A — Control rats, B — Experimental rats, ↑ — Normal neuronal cells, ▲ — Neuronal degeneration]
when compared to control rats. This decrease was reversed after 7 days when the injection of DEHP was stopped, indicating that the effect is reversible.

On the other hand intracellular Ca\(^{2+}\) concentration in the RBC showed significant increase, which too returned to near normal levels after 7 days of withdrawal of DEHP administration.

Activity of hepatic HMG CoA reductase and concentration of serum cholesterol (Table 3) — Activity of hepatic HMG CoA reductase and concentration of serum cholesterol showed significant decrease in the liver of the rats administered DEHP, when compared to control rats. Both these also returned to very near normal levels when the administration of DEHP was stopped.

Discussion
The observation that administration of DEHP on alternate days at low levels (corresponding to the successive transfusion of about 10 units of blood in a recipient) causes degenerative changes in the brain is important. Previous studies carried out in this laboratory however showed no gross histopathological changes in liver or testes at these levels\(^1\).

The results obtained indicate that administration of DEHP at low levels caused significant inhibition of membrane Na\(^+\)-K\(^+\) ATPase in the brain, liver and RBC. This is an important observation in view of the reports of a significant decrease in this enzyme activity in the RBC membrane in many neurological and psychiatric disorders (epilepsy, Parkinson's disease (PD), schizophrenia, manic-depressive psychosis (MDP), CNS glioma, multiple sclerosis (MS), sub acute sclero encephalitis (SSPE))\(^{15,17,30}\), CAD (coronary artery disease) and stroke, syndrome-X and tumors). Inhibition of this enzyme activity was also observed by us in the heart in isoproterenol induced myocardial infarction in rats (unpublished report) and in the brain in quinolinic acid induced neurodegeneration in rats\(^1\).

Thus the present observation may suggest that transfusion of blood stored in DEHP plasticized PVC

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### Table 1 — Effect of administration of DEHP to rats on the activity of Membrane Na\(^+\)-K\(^+\) ATPase in the brain, liver and RBC

<table>
<thead>
<tr>
<th>Group</th>
<th>Activity of membrane Na(^+)-K(^+) ATPase at the end of administration of DEHP</th>
<th>Activity of membrane Na(^+)-K(^+) ATPase after 7 days of stopping DEHP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
<td>Liver</td>
</tr>
<tr>
<td>Control rats</td>
<td>6.75 ± 0.981</td>
<td>0.461 ± 0.019</td>
</tr>
<tr>
<td>Test rats</td>
<td>3.24 ± 1.511</td>
<td>0.258 ± 0.064</td>
</tr>
</tbody>
</table>

\(P\) values: *\(< 0.01, ^{b}\)between 0.01 and 0.05 compared to corresponding controls.

### Table 2 — Effect of administration of DEHP to rats on the concentration of intracellular Ca\(^{2+}\) and Mg\(^{2+}\)

<table>
<thead>
<tr>
<th>Group</th>
<th>Conc. of intracellular Ca(^{2+}) &amp; Mg(^{2+}) at the end of administration of DEHP</th>
<th>Conc. of intracellular Ca(^{2+}) &amp; Mg(^{2+}) after 7 days of stopping DEHP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca(^{2+})</td>
<td>Mg(^{2+})</td>
</tr>
<tr>
<td>Control rats</td>
<td>0.67 ± 0.04</td>
<td>3.634 ± 0.186</td>
</tr>
<tr>
<td>Test rats</td>
<td>0.826 ± 0.05</td>
<td>3.097 ± 0.37</td>
</tr>
</tbody>
</table>

\(P\) values: *\(< 0.01, ^{b}\)between 0.01 and 0.05 compared to corresponding controls.

### Table 3 — Effect of administration of DEHP to rats on the Activity of HMG CoA reductase and concentration of serum cholesterol

<table>
<thead>
<tr>
<th>Groups</th>
<th>Activity of HMG CoA reductase at the end of administration of DEHP</th>
<th>Activity of HMG CoA reductase after 7 days of stopping DEHP</th>
<th>Con. of serum cholesterol at the end of administration of DEHP</th>
<th>Con. of serum cholesterol after 7 days of stopping DEHP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HMG CoA/Mevalonate ratio*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control rats</td>
<td>1.893 ± 0.085</td>
<td>1.910 ± 0.077</td>
<td>74.4 ± 2.59</td>
<td>74.58 ± 2.57</td>
</tr>
<tr>
<td>Test rats</td>
<td>2.38 ± 0.107</td>
<td>1.931 ± 0.086</td>
<td>70.5 ± 2.25</td>
<td>75.04 ± 2.62</td>
</tr>
</tbody>
</table>

*Higher the ratio, lower the activity

\(P\) values: *\(< 0.01, ^{b}\)between 0.01 and 0.05 compared to corresponding controls.
blood bags can expose a recipient to the risk of any of the various disorders mentioned above. However the fact that this inhibition of Na⁺-K⁺ ATPase activity is reversed when DEHP is withdrawn appears to be a saving factor. But with frequent or repeated transfusions of blood stored in DEHP plasticized PVC bags as in the case of thalassemia patients or patients undergoing hemodialysis, this risk factor has to be considered. In fact, in the recent National seminar on thalassemia held in New Delhi (28th April 2001), physicians treating thalassemia patients reported neurological, cardiac and other complications in those getting repeated transfusion of blood stored in DEHP plasticized PVC bags.

Membrane bound Na⁺-K⁺ ATPase is an important enzyme utilizing the energy of ATP hydrolysis for transport of several cations. It is known that an inhibition of this enzyme produces an increase in intracellular Ca²⁺ and a decrease in intracellular Mg²⁺. Wang et al. reported elevation of intracellular Ca²⁺ from 125 nM to 408 nM when Na⁺-K⁺ ATPase activity was inhibited. Yuan Wei et al. also recently confirmed that inhibition of Na⁺-K⁺ ATPase with strophanthidin increased intracellular Ca²⁺ to a very high level.

Inhibition of membrane Na⁺-K⁺ ATPase results in increased release of Ca²⁺ from endoplasmic reticulum stores and increased entry of Ca²⁺ via the voltage gated calcium channel. Increased intracellular calcium displaces magnesium from binding sites and magnesium being freely permeable, leaks into serum. There is defective reabsorption of magnesium from the renal tubules because of increased tubular cell calcium. Similary increased intestinal epithelial calcium inhibits magnesium absorption from the gut. Thus inhibition of membrane Na⁺-K⁺ ATPase can lead to increased entry of calcium into the cell and produce cellular magnesium depletion. The present observation of decrease in intracellular Mg²⁺ and increase in intracellular Ca²⁺ in the RBCs in rats administered DEHP agrees with this. A direct consequence of inhibition of Na⁺-K⁺ ATPase is increase in concentration of intracellular Na⁺ and depletion of K⁺. Increase in intracellular Na⁺ in turn raises intracellular concentration of Cr²⁺ by stimulating Na⁺/Cr²⁺ exchanger. There is thus disruption of ion homoeostasis.

Increased intracellular Ca²⁺ can trigger apoptosis or cell death by several mechanism. It can activate Ca²⁺ dependant calcineurin signal transduction pathway which can produce T cell activation resulting in the secretion of TNF alpha (Tumour Necrosis Factor alpha) among other things. TNF alpha binds to its receptor and in turn activates the caspase cascade, especially downstream caspase-9 and produce apoptosis. Increased intracellular calcium can open the mitochondrial PT (permeability transition) pore causing collapse of the proton gradient across the inner membrane and uncoupling of the respiratory chain. This causes the rupture of the outer membrane of mitochondria resulting in the release of AIF (apoptosis inducing factor) and cyto C (cytochrome-C) to the cytoplasm. This results in activation of caspase-9, which produces cell death. In this connection Xiao et al. observed from their studies on cultured cortical neurons that inhibition of Na⁺-K⁺ ATPase resulted in neuronal death involving concurrent apoptotic and necrotic components, mediated by intracellular depletion of K⁺ and accumulation of Ca²⁺ and Na⁺ respectively. Disruption of ion homoeostasis was considered a major factor by them. We have earlier mentioned disruption of ion homoeostasis as a consequence of Na⁺-K⁺ ATPase inhibition. Apoptosis has been implicated in neuronal degeneration. Mg²⁺ deficiency, related ATP synthase defect, among other things can also contribute to mitochondrial dysfunction resulting in incomplete reduction of O₂ and increased production of free radicals. Increased Ca²⁺ level can also activate NOS (nitric oxide synthase) causing increased production of NO which combines with superoxide radical to form peroxynitrite ion promoting lipid peroxidation. Free radical damage has been implicated in the neuronal degeneration.

Increased intracellular Ca²⁺ also activates the signal transduction mechanism involved in NMDA transmission which results in increased glutamatergic excitotoxicity, important in neuronal degenerative disorders.

The inhibition of Na⁺-K⁺ ATPase may be caused directly by DEHP or its important metabolite MEHP or indirectly by its stimulation of the isoprenoid pathway of which digoxin, an inhibitor of this enzyme, is one of the products. In most of the diseases particularly epilepsy, PD, schizophrenia, MS, SSPE, CAD and stroke, syndrome-X, an increase in serum digoxin has been reported. The fact that HMG CoA reductase activity, the major rate limiting step in the isoprenoid pathway, is decreased in rats administered DEHP may argue against the possibility that DEHP acts via stimulating the endogenous synthesis of digoxin. The observed effect on
membrane Na"-K" ATPase may therefore be a direct effect of DEHP or its metabolites, particularly MEHP. In this connection, we had reported earlier that significant amount of DEHP is observed in the RBC membrane in blood stored in DEHP plasticized PVC bags. This indicates that DEHP gets incorporated into the cell membrane. We had further reported changes in the protein, glycosaminoglycans (GAG), carbohydrate components of glycoproteins, cholesterol, phospholipids and vitamin E in the RBC membrane in the presence of DEHP. The protein: GAG ratio, protein: carbohydrate ratio, cholesterol: phospholipid ratio as well as protein: lipid ratio, all showed significant increase in the RBC membrane in the presence of DEHP. It is clear from these observations that incorporation of DEHP into the RBC membrane results in significant alteration of the integrity of the membrane. This may possibly be responsible for the inhibition of the Na"-K" ATPase, which is a membrane, bound enzyme. Further work is in progress to elucidate the mechanism of this inhibition.

References