Phenol Red Inhibits Arsenate Uptake by the Everted Gut Sacs of Mice and Reduces Its Toxicity

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Short title: Intestinal transport of arsenic is a target for phenol red

Keywords: dye; enterocytes; intestine; lactate dehydrogenase; lipid peroxidation; superoxide dismutase; viability.

Abbreviations used: LDH, lactate dehydrogenase; LPO, lipid peroxidation; SOD, superoxide dismutase; TBARS, thiobarbituric acid reacting substance.

Summary

Uptake of arsenate by the everted intestinal sac of mice was found to be reduced by phenol red. Presence of the dye on the serosal side was more effective. The intestinal damage assessed by the leakage of LDH and the level of lactate, was also lessened by the presence of phenol red. Chronic administration of arsenic was found to cause an increase in LPO and a decrease in SOD levels in many organs. Simultaneous administration of the dye significantly reduced the LPO in the intestines, kidney, and liver of the arsenate treated animals. However, the decrease in SOD activity was restored to normal by phenol red in all the organs tested in the arsenate treated animals. These findings suggest that phenol red is capable of reducing arsenate toxicity in mice.

Introduction

The concentration of arsenic in unpolluted surface water and ground water are typically in the range of 1-10 µg/ L. Elevated arsenic concentrations (> 1 mg As/ L) in groundwater was found in many parts of the world- India (1), Bangladesh (1), Chile (2), North Mexico (3) Argentina (4) and Taiwan (5). So far there is no known cure for the toxicity of arsenic. Preventive measures are mainly aimed at reducing the levels of arsenic in the drinking water (6). Gut remains the main portal of entry of this metal into the human body. Inorganic arsenicals in pentavalent and trivalent forms are rapidly and extensively absorbed from the gastrointestinal tract (7, 8). It is known that arsenate and phosphate share the same transport mechanism, a secondary active carrier-mediated system dependent on Na^+ and H^+ gradients in the intestinal mucosa (8, 9). Phenol red is an effective inhibitor of phosphate transport in everted gut sacs of mice (10). Will phenol red inhibit arsenate transport? If yes, will such an inhibition lead to mitigation of toxic effects of arsenate? With a view to answer these questions we have conducted the following experiments.

Materials and Methods

Chemicals and Reagents

Sodium arsenate ($Na_2HAs_5O_4\,7H_2O$; molecular weight 312.01) was procured from Sigma –Aldrich Chemicals (Bengaluru, India). All other analytical laboratory chemicals and reagents were purchased from Merck (Germany) or Sigma (Bengaluru, India). Lactate dehydrogenase and lactate determinations were performed using the kit procured from Euro Diagnostic Systems Pvt. Ltd. (Chennai) and DiaSys Diagnostic Systems (Holzheim, Germany) respectively. All the samples were stored and refrigerated in desiccators to avoid oxidation and thermal decomposition.

Animals

All experiments were performed on healthy, adult, male Swiss albino mice, weighing approximately 25-31 g obtained from Pasteur Institute of India, Coonoor and maintained at the institutional animal house. All animals received humane care in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The Institutional Animal Ethical Committee (IAEC) of Dr MGR Medical University, Tamil Nadu, India also approved the protocols for the experiments. Prior to testing, they were acclimatized for 7 days to light from 06:00 to 18:00 h, alternating with 12 h darkness. The mice were housed in sanitized polypropylene cages containing sterile paddy husk as bedding. The animals were maintained under controlled conditions of temperature (25 ± 2°C), humidity (50 ± 5%) and a 12-h light–dark cycle. Mice were allowed standard laboratory commercial feed (crude proteins 20%, ether extract 4%, crude fiber 4%, ash 8%, calcium 1%, phosphorus 0.6% and nitrogen free extract 55%) obtained from Gold Mohur Animal Feeds (Bangalore, India) throughout the experiment and water *ad libitum*.

Measurement of uptake of arsenate or arsenite

This was assessed by usage of everted gut sacs prepared from the mice. Under anaesthesia the intestine was excised carefully and fat and mesenteric attachments were removed. Everted sacs of 6 cm length were prepared from the duodenum according to the method described by Wilson and Wiseman (9). The distal end of the sac was tied with a ligature (000 Ethilon Black braided nylon). A ligature was placed loosely around the proximal end. After weighing, the empty sac was filled with 0.5 ml of the desired

incubation medium (serosal compartment) using a micro syringe (Gas tight syringe 1750, Hamilton Company, USA) fitted with a blunt needle. The filled sacs were slipped off the needle carefully and the loose ligature on the proximal end was tightened. After weighing, the distended sacs were placed in 5 mL of the same incubation medium contained in a 25 mL Erlenmeyer (siliconized) flask. After gassing for 1 min with 100% oxygen, the flasks were tightly stoppered and incubated at 37 ^{0}C for 1 h in a metabolic shaker bath (Techno India Ltd, Pune, India) at a frequency of 90-110 shakes/ min. At the end of the 1 h incubation period, the sacs were removed from the flasks, blotted and weighed again. The incubation medium contained (in mM) NaCl 135, KCl 11 and $CaCl_2$ 0.04 dissolved in phosphate buffer of desired concentration (KH_2PO_4 and Na_2HPO_4) at pH 7.4. Sodium arsenate was added to give a final concentration of 2 mM. In some experiments sodium arsenite was used at a similar concentration instead of arsenate. Phenol red was incorporated at a concentration of 100 µM in either mucosal or serosal compartments. Phenolphthalein was added in similar manner in relevant experiments.

Estimation of arsenate and arsenite

After incubation in a water bath at 37 ^{0}C with shaking for varying periods, the sacs were emptied and samples of fluid from the mucosal and serosal compartments were collected. The final arsenate concentrations of mucosal and serosal fluids were determined. The amounts of arsenate and arsenite removed from these fluids were calculated and characterized as ‘mucosal uptake’ and ‘serosal uptake’ respectively. These were expressed as µmol/ gm tissue wet weight/ hr. Arsenate and arsenite were estimated by using Hydride Generation-Atomic Absorption Spectrophotometer (HG-AAS, GBC Instruments Pvt. Ltd., Australia, Model-916) (12)

Enterocyte Viability Test

Enterocytes were isolated by mechanically vibrating the emptied gut sacs at the end of 90 min incubation. The cells were then incubated with 0.2% trypan blue solution at 37 ^{0}C to check the viability (13). The sample was taken into consideration only if 80% or more of mucosal cells excluded the dye showing their viable nature.

Biochemical assay

The emptied everted gut sacs were homogenized (as described below) and their lactate content and LDH activity were measured. Similar measurements were carried out on samples of serosal and mucosal fluids.

Measurement of Lactate

The lactate levels in the samples and tissue homogenates were measured using the kit procured from DiaSys Diagnostic Systems, Holzheim, Germany, based on the spectrophotometric method David and Sacks (14) with the help of Merck 200 (semi-autoanalyzer). The absorbance was recorded at 340 nm.

Lactate Dehydrogenase Assay

The lactate dehydrogenase (L-lactate-NAD^{+} oxidoreductase, LDH; EC 1.1.1.27) activities in the samples and tissue homogenates were measured using the kit procured from Euro Diagnostic Systems Pvt. Ltd., Chennai based on the spectrophotometric method Moss and Henderson (15) with the help of Merck 200 (semi-autoanalyzer) . The absorbance was recorded at 340 nm.

Assessment of toxicity

The animals were divided into 4 groups consisting of 10 mice each and were treated as below for 20 days:

Group I – control (received normal water);

Group II – intra-peritoneal administration of aqueous solution of 1.45 mg/ kg body wt/ day phenol red;

Group III – 2.5 mg/ kg body wt/ day arsenate as sodium arsenate, fed by oral gavage;

Group IV – intra-peritoneal administration of aqueous solution of phenol red (1.45 mg/ kg body wt/ day) followed 1 h later, by oral administration of 2.5 mg/ kg body wt/ day of sodium arsenate.

After 20 days, the experiment was terminated. Six mice from each group were sacrificed under light ether anesthesia, after overnight fasting. Heart, intestine, liver, lungs, kidney and brain were removed, washed with normal saline and all the extraneous materials (mesenteric and other adhering tissues) were removed before studying various parameters.

Homogenization

The organs (brain, duodenum, heart, kidney, liver and lungs) were rinsed thrice with ice cold physiological saline (0.9%), dried by pressing between the folds of blotting paper, weighed and stored in a freezer. 5% (w/v) of the homogenate in 0.1 M acetate buffer at pH 5.0 was prepared using Potter- Elevehjam type homogenizer at 0 ^{0}C. The unbroken cells and cell debris were removed by centrifugation at 700 X g for 10 min in a refrigerated centrifuge. The homogenate was kept at 4 ^{0}C until use. Prior to biochemical analysis the required volume of tissue homogenate was incubated at 37 ^{0}C for 1 h in a stoppered Erlenmyer flask with constant stirring.

Assessment of lipid peroxidation

The extent of lipid peroxidation was determined by the method described by Ohkawa et al (16). Briefly, 5 ml of tissue homogenate was centrifuged at 3000 rpm for 10 min in presence of 20 % trichloroacetic acid (TCA). To 2 ml of the supernatant, 2ml of 0.67 % aqueous thiobarbituric acid reagent was added. After boiling for 10 min and cooling, absorbance was read at 535 nm. A pre-determined absorption coefficient of 1.56 X 10^5 cm^{-1} M^{-1} was used to calculate the malondialdehyde formed and the activity was expressed as nmol of MDA formed min^{-1} gm^{-1}tissue protein.

Superoxide dismutase assay

Superoxide dismutase activity was studied in tissue homogenate following the method of Marklund and Marklund (17). To 2.8 ml Tris buffer, 0.1 ml of sample was added and mixed and the reaction was started by adding 0.1 ml of pyrogallol solution. The absorbance was read after 90 s and 210 s at 420 nm. In controls, instead of tissue homogenate equal amount of pyrogallol solution was added to 2.9 ml Tris buffer. Superoxide dismutase activity was expressed as U/ mg of protein.

Total protein determination

The protein concentration of each sample was determined by the biuret method (18). In brief, 2.9 ml of biuret reagent was added to 0.1 ml of tissue homogenate and incubated for 10 min at 37 ^{0}C. After cooling the absorbance was recorded at 540 nm. In controls, instead of tissue homogenate equal amount of phosphate buffer was added. Paired samples were compared with standard curves prepared on the day of each experiment using known concentrations of Bovine Serum Albumin (BSA).

Statistical Analysis

Data were expressed as mean ± SEM of 10 values. One-way ANOVA test was performed to find whether the values of different groups differ significantly. To test the inter group significance, Student's unpaired t-test was performed. $P < 0.05$ was considered to be significant.

Results

Arsenate uptake was found to be decreased significantly in the duodenal sacs of mouse intestine in the presence of phenol red.

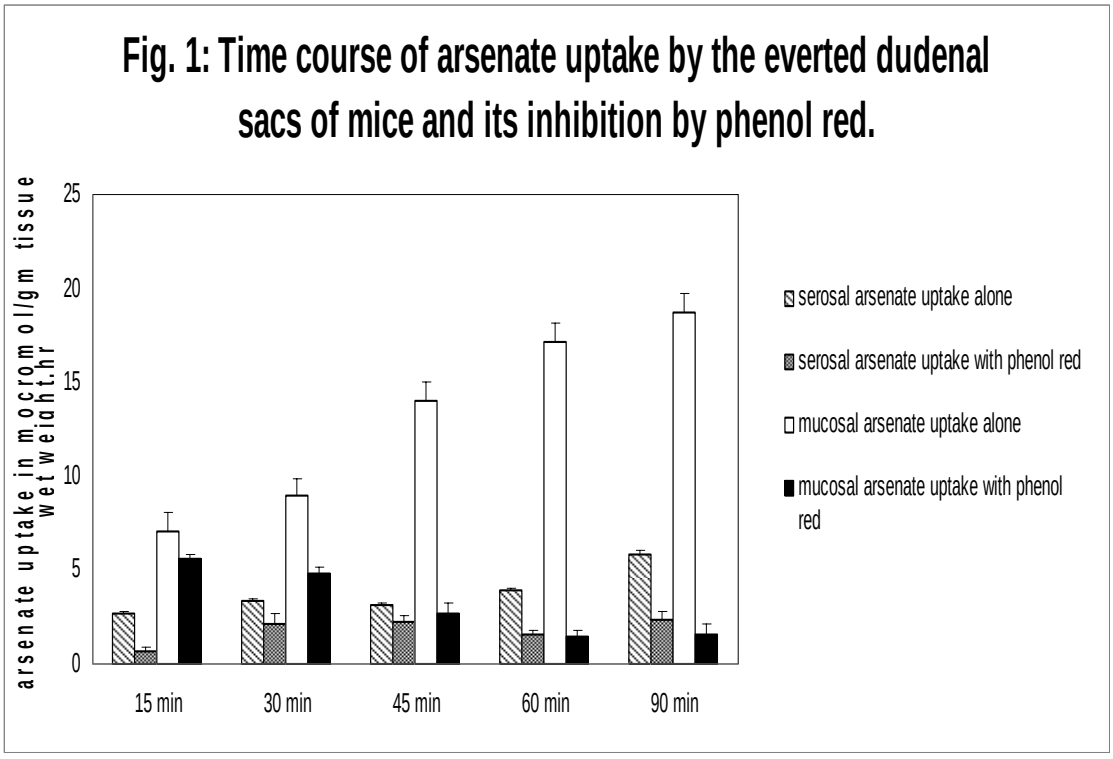


Fig 1 : Time course of arsenate transport by everted proximal intestinal sacs of mice and its inhibition by phenol red.

Fig shows that the serosal and mucosal As V uptake steadily increases with time.The AV concentration of the medium was 2 mM and the preferred segment of the intestine was the proximal segment (duodenum). The sacs were incubated in the medium for 1 h at 37 ^{o}C under an atmosphere of 100% pure oxygen. HG-ASS was used for As V estimation in samples. The drained sacs at the end of 90 min were tested for viability using trypan blue test, 80-90% of the mucosal cells showed an exclusion of the stain showing their viable nature. Both serosal and mucosal uptakes of As V (2 mM) were significantly reduced by phenol red (100 µM).

The serosal uptake when expressed as a percentage over mucosal uptake tends to remain at 37% and decreases to about 22% at the end of 60 min. At the end of 90 min incubation, the serosal uptake becomes 31%.Both serosal and mucosal uptake of arsenate (2mM) was significantly reduced by phenol red (100µM).

As shown in table 1,The inhibition was more when the dye was added to the serosal compartment.

Table 1. Effect of phenol red on As V transport in everted gut sacs of mouse intestine

When phenol red at a concentration of 100 µM was placed in the serosal or mucosal compartments, As V uptake was found to be decreased significantly in the everted sacs of mouse duodenum . The inhibition was more when the dye was added to the serosal compartment. As V uptake is expressed as µM/ g tissue wet weight/ h. Values are mean ± SEM; n = 6. †P<0.001, highly significant; *P<0.05, significant.

	As V uptake in µM/ g tissue wet weight/ h	
	Serosal	Mucosal
Control	2.57 ± 0.36	17.12 ± 0.51
Phenol red on serosal side	1.62 ± 0.22 *	2.46 ± 0.46 †
Phenol red on mucosal side	2.08 ± 0.18	10.13 ± 0.84 †

As shown in table 2,phenolphthalein (100 µM) placed in the serosal compartment failed to inhibit arsenate uptake.

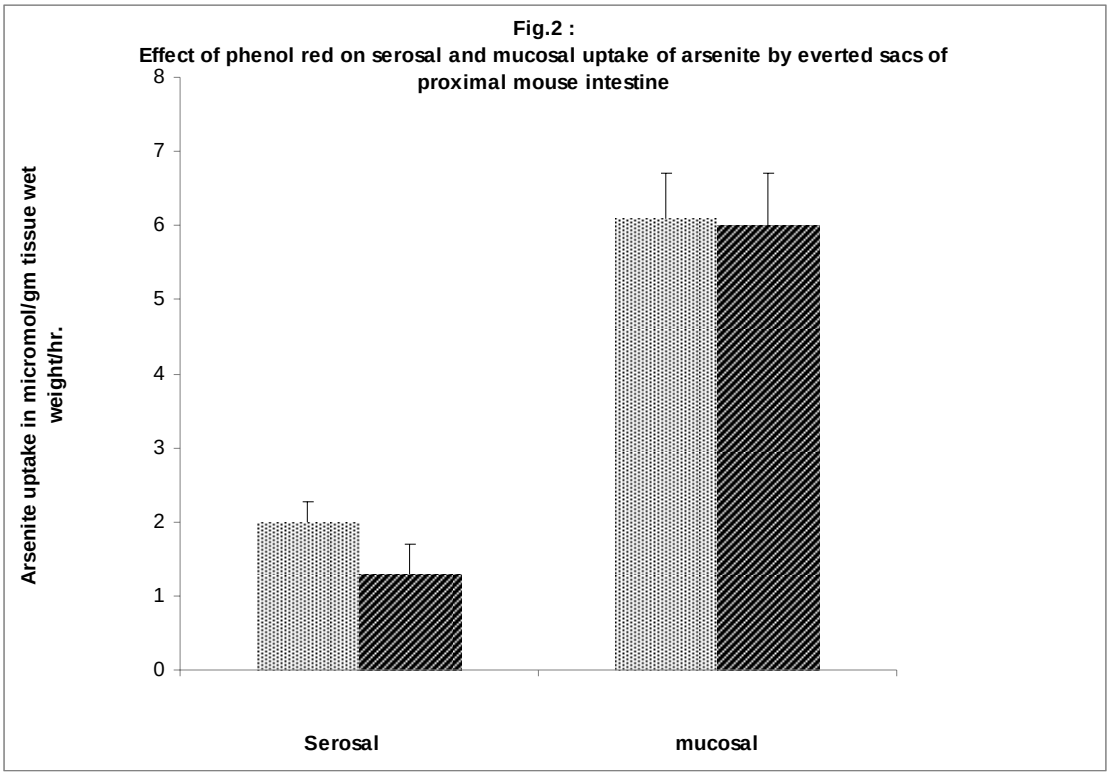
Table 2. Effect of phenolphthalein on As V transport.

This dye failed to affect the As V transport by the everted sacs of mice.

	As V uptake in µM/ g tissue wet weight/ h	
	Serosal	Mucosal
Control	3.56 ± 0.25	16.61 ± 0.82
Phenolphthalein serosal side	4.30 ± 0.53	16.97 ± 0.43

The presence of arsenate resulted in an increase in the LDH activity and in the lactic acid content, of serosal fluid, mucosal fluid and of the intestinal homogenate. Addition of phenol red (100 µM) to the serosal side resulted in a significant decrease in leakage of LDH and lactate into the fluids induced by arsenate. Phenol red (100 µM) by itself did not affect the LDH and lactate levels in the serosal and mucosal.

As shown in figure 2, phenol red failed to affect the arsenite transport.



Lighter column indicates only arsenite uptake.

Brighter column indicates arsenite uptake with phenol red.

When phenol red at a concentration of 100 µM was placed in the serosal or mucosal compartments, arsenite uptake was found to be unaffected in the everted sacs of mouse duodenum.

As shown in table, the presence of arsenate in the incubation medium on both sides of the gut resulted in the increase in the leakage of LDH and lactic acid content of serosal fluid, mucosal fluid and the intestinal homogenate. Addition of phenol red (100 µM) to the serosal side resulted in the decrease in the leakage of LDH and lactate levels and the fluids and tissue homogenate, induced by arsenate, significantly. Phenol red by itself did not affect the LDH and lactate levels in the serosal and mucosal fluids.

Table 3. The effect of arsenate and phenol red on leakage of lactate dehydrogenase and lactate in everted gut sacs of mice.

		Control	Phenol red	Arsenate	As V with phenol red
Serosal Fluid	LDH	2530 ± 178	2211 ± 189	10220 ± 1119 †	77 ± 13.89 †
	Lactate	8 ± 0.88	7.03 ± 1.12	34 ± 2.5 †	2.3 ± 0.37 †
Mucosal Fluid	LDH	1356 ± 48	1402 ± 92	2022 ± 49 †	338 ± 83 †
	Lactate	2 ± 0.31	3.12 ± 0.92	4 ± 0.55 *	0.43 ± 0.03 †
Intestinal homogenate	LDH	1946 ± 169	ND	3728 ± 259 †	47.6 ± 5.8 †
	Lactate	0.04 ± 0.01	ND	0.52 ± 0.03 †	0.14 ± 0.04 †

Arsenate (2 mM) was incorporated in the incubation medium bathing both serosal and mucosal sides. The LDH leakage and lactic acid levels in the serosal, mucosal fluids and intestinal tissue homogenates were determined. The presence of As V on the either side of the gut sac resulted in an increase in leakage of LDH and lactic acid contents in the serosal, mucosal fluids and intestinal tissue homogenates. Addition of phenol red (100 µM) to As V (2 mM) in the serosal side resulted in a decrease in leakage of LDH and lactate levels in the fluids and tissue homogenates significantly. Phenol red (100 µM) by itself in the serosal side did not affect the LDH and lactate levels in the serosal and mucosal fluids. The LDH leakage and lactic acid levels in the presence of phenol red (100 µM) in the serosal side were not determined in the intestinal tissue homogenates. LDH, Lactate dehydrogenase as UL^{-1}; lactate as mg dL^{-1}. Values are means ± SE; $n = 6$. † $P<0.001$, highly significant; * $P<0.01$, significant. ND, not done.

Effect of phenol red on LPO of tissues: As shown in table 4, The administrations of sodium arsenate for 20 days showed a statistically significant increase in LPO in intestine, heart, lungs and liver, whereas in brain and kidneys though an increase in LPO

was observed, it was not statistically significant. Phenol red treatment resulted in a significant decrease in LPO, showing that the dye protected the damage brought about by arsenate. The protection was observed only in intestine, kidneys and liver and not observed in other tissues.

Effect of phenol red on SOD activity of tissues: As shown in table 4, SOD activity of all the six tissue studied was significantly decreased in arsenate treated mice and phenol red treatment showed a statistically significant elevation in SOD activities in all the tissues. Phenol red by itself did not affect the enzyme activities when compared to control.

Tissues		Group I	Group II	Group III	Group IV
Intestine	LPO	0.04	0.05	0.23 ± 0.04 ‡	0.06 ± 0.01 ‡
	SOD	152.77 ± 6.58	155.17 ± 4.47	49.21 ± 4.11 †	148.4 ± 5.4 †
Heart	LPO	0.01	0.02	0.08 ± 0.01 †	0.06 ± 0.01
	SOD	108.82 ± 4.37	113.64 ± 8.7	75.49 ± 4.47 ‡	117.38 ± 5.41 †
Lung	LPO	0.05 ± 0.01	0.07 ± 0.01	0.1 ± 0.01*	0.07 ± 0.01
	SOD	190.27 ± 4.77	159.51 ±15.54	94.83 ± 2.83 †	145.03 ± 3.7 †
Kidney	LPO	0.06 ± 0.02	0.08 ± 0.01	0.12 ± 0.01	0.07 *
	SOD	229.58 ± 15.39	242.08 ± 12.82	124.99 ± 5.37 †	161.83 ± 3.5 †
Liver	LPO	0.17 ± 0.01	0.11 ± 0.02	0.25 ± 0.01 *	0.12 ± 0.02 †
	SOD	104.53 ± 2.89	103.8 ± 6.44	56.51 ± 6.52 †	106.62 ± 4.22 †
Brain	LPO	0.06 ±0.02	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
	SOD	215.33 ± 18.97	244.39 ± 13.76	112.86 ± 3.81 †	179.15 ± 5.82 †

Table 4. The effect of arsenate (2 mM) and phenol red (100 µM) on LPO and SOD in mice.

Effect of phenol red on LPO of intestinal tissue homogenate (in vitro): The everted gut sacs following the time study using phenol red, were used for estimation of lipid peroxides formed. The LPO of intestinal tissue homogenate was found to be increased by As V alone. Addition of phenol red to the serosal side along with As V decreased LPO levels, though it was not statistically significant.

Effect of phenol red on LPO of tissues (in vivo): The administrations of sodium arsenate for 20 days showed a statistically significant increase in LPO in intestine, heart, lungs and liver, whereas in brain and kidneys though an increase in LPO was observed, it was not statistically significant. Phenol red treatment resulted in a statistically significant decrease in LPO, showing that phenol red protected the damage brought about by As V. The protection was observed only in intestine, kidneys and liver and not observed in other tissues.

Effect of phenol red on SOD activity of tissues (in vivo): SOD activity of all the six tissue studied was significantly decreased in Group III mice and phenol red treatment showed a statistically significant increase in SOD activities in all the tissues.

The malondialdehyde formed and its unit expressed as nmol of MDA formed min^{-1} gm^{-1}tissue protein. Superoxide dismutase activity is expressed as U/ mg of protein.

†P<0.001, very highly significant; ‡ P<0.01, highly significant; * P<0.05, significant.

Discussion

Our results clearly indicate that phenol red is able to inhibit the uptake of arsenate by the everted gut sacs of mice. Unlike phosphate (10, other), arsenate seems to be absorbed from both mucosal and serosal compartments of the sac. However, the uptake from the mucosal side is several-fold higher than the serosal uptake. When placed on the serosal side of the sac phenol red significantly curtailed the uptake of arsenate from both the compartments, the mucosal uptake being drastically reduced. But when the dye is on the mucosal side, the uptake of arsenate from the serosal side remained unaffected. Since phenol red has been shown to inhibit phosphate uptake by everted intestinal sacs of mice

(10) and since ample evidence exists, of similarity of transport mechanisms of these two anions (19), the ability of the dye to inhibit the arsenate transport is not surprising. However, the greater effectiveness of the dye when placed in serosal compartment is difficult to explain. Although considered unabsorbable (20), the dye has been shown to enter the enterocytes from the serosal compartment of everted sacs and gain entrance to the mucosal compartment through active transport involving multidrug resistance – associated protein 2 (Mrp2) (21). Like in renal tubules, in the intestine also secretion of substances may interfere with absorption (22,23). It is likely that such a mechanism is operative in the everted sacs explaining the greater effectiveness serosal phenol red in reducing the uptake of arsenate. The studies (24) on Xenopus oocytes have shown that, phenol red inhibits the phosphate transport, possibly by blocking the chloride channels. The dye inhibition of arsenate transport observed in our studies may be due to a similar mechanism.

Phenol red failed to decrease arsenite transport significantly. This observation clearly indicates that both arsenite and arsenate have different cellular uptake mechanisms. The cellular uptake of arsenite was reported to be four-fold higher than for arsenate (25). The initial uptake rates of arsenite in KB oral epidermal carcinoma cells were linearly correlated with its extracellular concentrations, suggesting that arsenite uptake is probably accomplished through simple diffusion perhaps through aquaglyceroporins (7). The above findings add support to the suggestion that phenol red might have a specific inhibitory effect on arsenate transport through Na^+- dependent carrier-mediated phosphate transport mechanism at the mucosal membrane.

Phenolphthalein failed to inhibit arsenate uptake. This dye closely resembles phenol red

except for the SO_3H, a group possessed by all phenolsulphophthalein dyes, which is replaced by COOH. This indicates that SO_3H group is crucial for inhibitiory effect of the dye on arsenate transport.

Phenol red was also successful in preventing the cellular damage induced by arsenate in the everted sacs, as evidenced by the reduction of elevated lactate production and LDH leakage in the intestinal homogenate and the serosal and mucosal fluids. Surprisingly the dye reduced these parameters to levels significantly less than the controls. The dye alone did not show this effect on the mucosal and serosal fluid levels of LDH or lactate. These findings indicate a likely intracellular effect of phenol red on arsenate, which remains to be explored.

The toxic effects of arsenate are mainly due to its ability to generate reactive oxygen species (ROS) that lead to lipid peroxidation (LPO) and inhibition of superoxide dismutase (SOD) in various tissues (26) – brain (27;28), heart (29) intestine (), liver (30;28;29;31), lungs (29) and kidney (30;29; 32; 31). Chronic administration of arsenate in our experiment has shown these effects in many organs. These were curtailed by simultaneous administration of phenol red. This might be due to inhibition of absorption of orally administered arsenate from the intestine, prevention of uptake of arsenate by the tissues or through a direct intracellular antagonism of arsenate effect. Further studies are needed to clarify the action of phenol red in preventing arsenate toxicity.

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