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Complexation of 1,2,4-Benzenetriol with Inorganic and Ferritin-Released Iron *in Vitro*

Sarfaraz Ahmad*,[1] and G. S. Rao†

**MD-68, Environmental Carcinogenesis Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711; and*
†Industrial Toxicology Research Centre, P.O. Box 80, Lucknow, 226001, India

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The reactive metabolite(s) responsible for the expression of benzene toxicity is not clearly known despite extensive information on the metabolism and hematotoxicity of benzene. It is now widely believed that hematotoxicity of benzene is due to the concerted action of several metabolites which arise from multiple pathways of benzene. In our earlier study, we proposed iron polyphenol chelates as possible toxic metabolites of benzene due to their prooxidant activity. In continuation, we demonstrate the formation of an iron and 1,2,4-benzenetriol (BT) complex, when added together in an acetate buffer, 0.1 M, pH 5.6, by sephadex G-10 column chromatography. It was also observed that iron released from ferritin in the presence of BT formed a complex with BT.

Key Words: **1,2,4-benzenetriol; iron; ferritin; iron-1,2,4-benzenetriol complex.**

Occupational exposure to benzene has been associated with various blood dyscrasias and myelogenous leukemia in human beings (1,2). Benzene requires biotransformation to express its toxic effects (3). Although the metabolism of benzene is very well understood, the intermediate(s) responsible for hematotoxicity is still under investigation. It is widely believed that the hematotoxic effects are likely to be exerted through multiple metabolites on multiple end points through multiple biochemical pathways (4). An overall hypothesis for benzene-induced leukemia involving a number of metabolites which can produce mitotic recombination and chromosomal aberrations has been proposed (5). Polyphenolic metabolites of benzene tend to accumulate in the bone marrow of experimental animals exposed to benzene (6,7). In our earlier study, accumulation of iron in bone marrow was demonstrated (8,9) and proposed that a chelate of polyphenolic metabolites of benzene with iron, which has been shown to exhibit prooxidant activity, as a possible toxic intermediate of benzene, in bone marrow (10). In the present study we investigated the complex formation of 1,2,4-benzenetriol (BT) with iron in view of accumulation of iron and BT in bone marrow during benzene exposure. Complexes of iron with phenolic compounds from soybean nodules and other legume tissues which showed prooxidant and antioxidant activities have been demonstrated (11). Recently, Herman et al. (12) showed that antineoplastic agents losoxantrone and piroxantrone from iron drug complexes to cause oxidative damage to various tissues. We report in the present study that BT forms complex with iron and also with the iron released from ferritin by BT (13). We also report a methodology to fractionate such complexes by sephadex G-10 column chromatography.

MATERIALS AND METHODS

Chemicals. Sephadex G-10, horse spleen ferritin and bathophenanthroline disulfonic acid were obtained from Sigma Chemical Company (St. Louis, U.S.A.). 1,2,4-Benzenetriol was obtained from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals used were of analytical grade.

Sephadex G-10 column chromatography. Sephadex G-10 (4.0 gm/200 ml) in 0.1 M acetate buffer saline, pH 5.6 was soaked and swollen overnight at room temperature. It was loaded on a glass column to prepare a gel column of 12 cm × 1 cm dimensions and 12 ml volume. The column was washed three times with an acetate buffer. Iron (as ferrous sulfate, $FeSO_4 \cdot 7H_2O$, 1 ml of 1 mM solution), BT (1 ml of 1 mM solution) or BT:iron (1ml of 1mM:1mM mixture) were applied to column and eluted with 0.1 M acetate buffer pH 5.6. The flow rate of 0.5 ml/min was maintained and thirty fractions were collected at every 5 min interval. To study the complex formation of BT with iron released from ferritin by BT, 1.0 ml reaction mixture containing 100 μg horse spleen ferritin, 2 mM BT with or without the presence of 1 mM ethylenediaminetetraacetic acid (EDTA), was incubated for 10 min. and passed through the column and thirty fractions were collected at every 5 min interval.

[1] To whom correspondence should be addressed. Fax: (919) 541-0694. E-mail: AHMAD.SARFARAZ@epamail.epa.gov.

Abbreviations used: BT, 1,2,4-benzenetriol; EDTA, ethylenediaminetetraacetic acid; Fe, iron.

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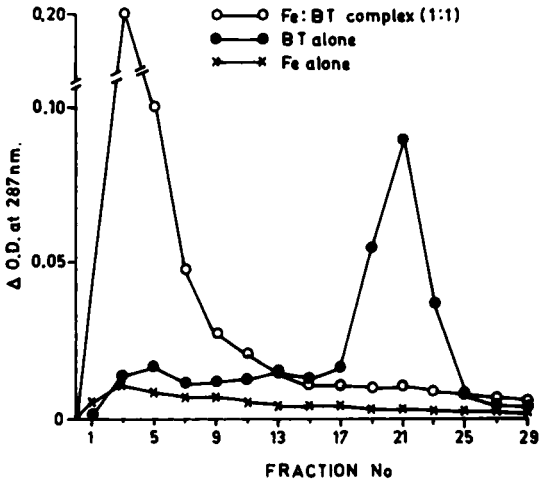

FIG. 1. Elution profile of Fe alone (x----x), BT alone (●----●) and Fe:BT mixture (○----○) subjected to Sephadex G-10 column. Fractions were collected at every 5 min. interval (flow rate 0.5 ml/min.) and were scanned at 287 nm.

Estimation of BT and iron in the sephadex G-10 column eluates. BT estimation in all the fractions was followed by recording the optical density at its absorption maximum (287 nm) in a spectrophotometer. Iron estimation in all the fractions was done according to the method of Peters et al. (14) by using bathophenanthroline disulfonic acid. Briefly, the assay system (total volume 2.0) contained 0.5 ml of eluate, 1.5 ml of acetate buffer (0.1 M, pH 5.6) with 200 μg ascorbic acid and 50 μg bathophenanthroline disulfonic acid. Reaction mixture was kept at room temperature for 10 min and the absorbance was recorded at 535 nm.

RESULTS

The elution profile of iron, BT and BT:iron mixture was studied by sephadex G-10 column chromatography (Fig. 1). With iron alone, the eluted fractions did not show any absorption in any of the fractions (1-30) at 287 nm. Whereas, with BT alone, eluted fractions showed a peak in fraction number 21and with BT:iron mixture, a peak was observed in fraction number 3 concurrent with disappearance of an absorption peak in fraction number 21. In terms of iron content in each eluted fraction (Fig. 2), sephadex G-10 column chromatography with BT alone, did not show any absorbance at 535 nm in any fraction (1-30) but, iron alone and BT:iron mixture showed an absorption peak at 535 nm in fraction number 3.

The elution profile of BT:iron complex formation in the absence of EDTA showed an absorption peak (287 nm) in fraction number 3 indicating complexation with the iron released from ferritin by BT. In addition to fraction number 3, a small peak of unreacted BT was also observed in fraction 21. In the presence of iron chelator, EDTA, all the iron released from ferritin by BT was chelated with EDTA and eluted in fraction number 3 with a large peak (O.D. of more than 1.0). This is consistent with our earlier observation that

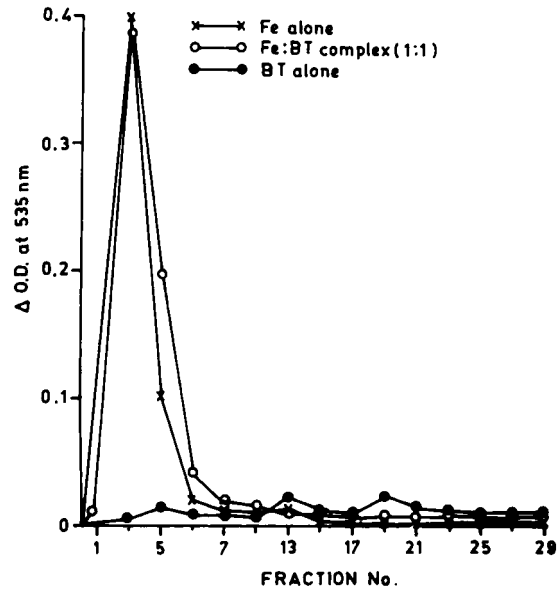

FIG. 2. Iron estimation using coloring reagent bathophenanthroline sulfonic acid in each fractions and absorbance was followed at 535 nm. Fe alone (x----x), Fe:BT complex (1:1 ratio) (○----○), and BT alone (●----●).

EDTA-Fe complex has an absorption in the UV-range of 250-300 nm (15). Simultaneously an increase in unreacted BT peak was observed in fraction number 21 at 287 nm (Fig. 3).

DISCUSSION

Phenolic metabolites viz. phenol, hydroquinone, catechol, BT and glutathionyl hydro-quinone and their

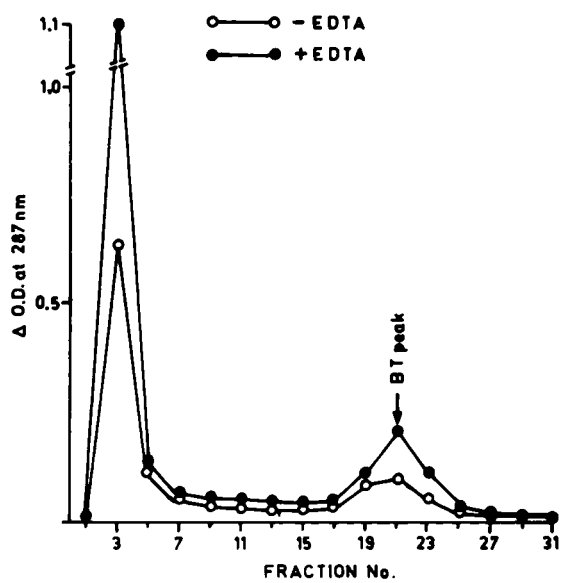

FIG. 3. Elution profile of Fe:BT complex formation during the iron release from ferritin by BT with or without the presence of EDTA. Fractions were analyzed at 287 nm.

accumulation in bone marrow after exposure to benzene have been demonstrated (6,7,16). Altered iron metabolism and distribution are associated with benzene exposure and recent studies have proposed the involvement of transition metal ions in the expression of hematotoxicity caused by benzene (17,18). We believe that the complex of Polyphenolic metabolites of benzene with iron is the low molecular weight iron component in bone marrow which has been reported to accumulate selectively during the exposure to benzene (9). Although, polyphenol:iron complexes have been identified in plant systems (11), existence of such complexes in animal systems have not been reported.

Oxidant mediated toxicity of BT is a complicated and multifactorial process. It involves the reaction of oxygen with BT, which is the most reactive Polyphenolic metabolite of benzene (19,20) and the resultant production of oxygen derived reactive oxygen species and quinones. However, the electron transfer to oxygen from BT is slow in the absence of transition metal ion. In this respect, the formation of BT:iron complex facilitates the transfer of electron from BT to oxygen. Chelate catalyzed autoxidation of iron(II) complexes and facilitation of iron(II) autoxidation by iron(III) complexing agents have been reported (21,22).

In our earlier study (10), it was demonstrated that the presence of BT enhanced iron-catalyzed bleomycin-dependent degradation of calf thymus DNA, which is mediated by reactive oxygen radical species. In the present study, we demonstrated a methodology to fractionate and characterize BT:iron complexes by sephadex G-10 column chromatography. Our results clearly indicate that BT forms a complex with iron and also with iron released from ferritin. These in vitro observations indicate a possibility of similar complexation in vitro to contribute to the expression of hematotoxicity of benzene. Further studies are in progress to demonstrate, identify and characterize such complexes in vivo during benzene exposure.

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