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PRELIMINARY COMMUNICATION

THE REQUIREMENT OF THE GUT FLORA IN NITROBENZENE-INDUCED METHEMOGLOBINEMIA IN RATS

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It has been clearly shown that methemoglobinemia produced by nitrobenzene is associated with the formation of the reduction products, nitrosobenzene and phenylhydroxylamine (1). The carcinogenic activity of nitrofurazone (2,3) and 4-nitroquinoline-1-oxide (4,5) is believed to be associated, at least in part, with the formation of hydroxylamines. Certain aromatic and heterocyclic hydroxylamines have been shown to be cytotoxic, mutagenic and carcinogenic in certain animals (6). Thus the biological reduction of nitroaromatic and heterocyclic compounds appears to play an important role in the toxicities elicited by various nitro compounds. However, the important question that remains unanswered is the localization of the nitroreductase activity that is responsible for the reduction of the nitro compounds involved in the observed toxicities.

The reduction of aromatic and heterocyclic nitro compounds by several mammalian enzyme systems has been documented. Cytochrome P-450 (7), cytochrome c reductase (8,9), xanthine oxidase (2,9,10), and aldehyde oxidase (11) have all been shown to catalyze this reaction in vitro, although their substrate specificities do differ. These reductions appear to occur only in the absence of oxygen (7-11). Since it is not possible to have complete anaerobic conditions in various tissues for any length of time, the question has been raised whether the nitro reductases present in the tissues play any important role in the nitro reduction of aromatic compounds (11,12).

The microbial flora of the gastrointestinal tract can also reduce aromatic and heterocyclic nitro compounds via hydroxylamine intermediates (13). In comparison to the mammalian nitro reductases, the bacterial nitro reductase has been shown to carry out nitro reduction even in the presence of oxygen (13,14). Since the gut flora consists predominantly of

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anaerobic and facultative anaerobic bacteria, the existence of comparatively anaerobic conditions in the gastrointestinal tract may favor the reduction of nitro compounds.

Recently Zachariah and Juchau have shown that the gut flora accounts for most of the reduction of para-nitrobenzoic acid (14) even though this substrate is rapidly reduced by liver microsomes under anaerobic conditions in vitro (7). However, there have been no convincing reports on the role of nitro reduction by the gut flora on the toxicities elicited by nitro compounds. In order to determine whether nitro reduction by the gut flora can play an important role in chemical toxicity, we have measured methemoglobin formation after the administration of nitrobenzene to germ-free rats and to germ-free rats acclimatized to the normal animal room. We also employed antibiotic pretreatment of the animals which is known to reduce the microbial flora of the gut (15).

When nitrobenzene (200 mg/kg in sesame oil) is administered to normal male Sprague Dawley rats intraperitoneally, about 30-40% of the hemoglobin in blood is converted to methemoglobin within 1-2 hr after administration. When the same dose of nitrobenzene is administered to either male Sprague Dawley germ-free or antibiotic pretreated rats, no measurable methemoglobin formation occurs even when measured up to seven hours after administration. However, if male litter mates of the germ-free animals are acclimatized in the normal animal room for seven days, nitrobenzene induces methemoglobin to the same degree as in normal animals (Fig. 1).

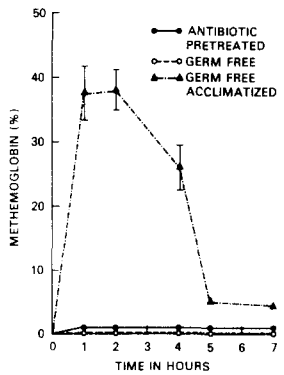

Fig. 1. Methemoglobinemia induced by nitrobenzene (200 mg/kg, ip). Three animals were employed in each group and methemoglobin was determined by the method of Evelyn and Malloy (16). The results are expressed as means ± S.E.

In order to test whether the nitro reductase present in various tissues is altered markedly by acclimatization of germ-free animals, the rate of aniline formation from nitrobenzene was measured in the homogenates of various tissues from germ-free, germ-free acclimatized and normal animals. As shown in Table I, there were no marked differences among normal, germ-free and acclimatized animals in the nitro reductase activity of various tissues

The only difference was a marked decrease in the nitro reductase activity in the gut contents of the germ-free animals. Since germ-free animals are free from microbial flora, it seems likely that the gut flora present in the normal and acclimatized animals is responsible for the reduction of nitrobenzene *in vivo* and subsequent methemoglobin formation

Table 1. REDUCTION OF NITROBENZENE BY VARIOUS RAT TISSUE HOMOGENATES*

Tissue	Nitroreductase Activity†		
	Germ-Free	Germ-Free Acclimatized	Control
	Aniline Formed (nmoles/mg protein/hr)		
Liver	2.0 ± 0.2	2.5 ± 0.4	3.3 ± 0.4
Kidney	0.5 ± 0.1	0.8 ± 0.1	0.7 ± 0.4
Gut Wall	2.0 ± 0.4	2.0 ± 0.6	2.4 ± 1.0
Gut Contents	0.2 ± 0.0	15.2 ± 2.7	11.1 ± 3.3

*The tissues were homogenized in two parts (w/v) ice cold 0.1 M potassium phosphate buffer (pH 7.35) and 0.4 ml of each homogenate was incubated anaerobically with 1.5 mM nitrobenzene for 1 hr in a final volume of 2.7 ml. Aniline formed was estimated by the method of Bratton and Marshall as described by Zachariah and Juchau (14). The reactions were linear with time. Addition of an NADPH generating system did not alter markedly the amount of aniline produced in any of the tissues.

†Results are expressed as means ± S.E. of determinations on three animals in each group. Each assay was performed in triplicate.

Similar investigations with other toxic aromatic and heterocyclic nitro compounds are needed to determine whether other nitro compounds are reduced *in vivo* mainly by bacterial flora.

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