THE EFFECT OF CERTAIN AROMATIC COMPOUNDS ON ENZYMES INVOLVED IN--ETC(U)

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Benzene, toluene and some of their nitro, amino and chloro derivatives often become environmental pollutants during the process of munitions manufacture. The full assessment of the ecological impact made by these compounds inadvertently released into the environment has not been made.

Because of the quantities manufactured, we focused much of our attention on the trinitrotoluenes and dinitrotoluenes. Both trinitrotoluene and dinitrotoluene are found in “pink water,” the water soluble effluent from factories making 2,4,6-trinitrotoluene. The 2,4,6-trinitrotoluene is the military high explosive produced in greatest quantity (1-2). This study was undertaken because benzene, trinitrotoluene and several other substituted benzenes and toluenes have been implicated as causative agents for both aplastic anemia (3-5) and leukemia (6-8). Benzene and, in some instances, toluene and xylene, have been shown to inhibit various steps in globin and heme synthesis in in vitro systems (9-10). We have utilized this property to study structure-activity relationships of benzene, toluene and many of their derivatives on two enzymes involved in heme synthesis. The enzymes are delta-aminolevulinic acid synthetase (ALAS) and heme synthetase (HS).

ALAS and HS were chosen for initial studies because they represent the starting and final enzymes in the synthesis of heme. ALAS is the first and presumably rate limiting enzyme in heme synthesis. Delta-aminolevulinic acid, (ALA), is the first stable product formed in the biosynthesis of heme. ALA is synthesized by the action
of ALAS on glycine and succinyl Coenzyme-A with a requirement for pyridoxal-5'-phosphate as a cofactor. Schulman and Richert (11), and Lascelles (12) were the first to demonstrate that pyridoxal-5'-phosphate was a required cofactor for the production of ALA. Observations within our laboratory confirm these earlier findings. In addition to its position in the scheme of the biosynthesis of heme, its short half-life of 1-2 hours makes ALAS ideally suitable to play a regulatory role in heme synthesis.

HS, (ferrochelatase or ferrolyase), E.C.4.99.1.1, is the final enzyme in the production of heme. It catalyzes the insertion of ferrous but not ferric iron into the protoporphyrin-IX ring to produce heme. The incorporation of ferrous iron into the porphyrin ring may occur non-enzymatically under certain mild conditions (13-14); however, the conditions under which we evaluated this system, only the enzymatic incorporation of iron occurred. Some of these conditions include:

1. Maintenance of anaerobic conditions and iron in the ferrous state.

2. A pH optimum near 8; we analyzed at pH 7.5.

3. De-esterification of the porphyrin ester into the corresponding free acid. (15)

Meso- and deuterophorphyrins also allow the incorporation of ferrous iron into their ring systems. Our observations confirmed those of Bonkowsky's (16) in that of the three porphyrin isomers, proto-, meso-, and deuter-, the meso- was the most active in the uptake of ferrous iron.

MATERIALS AND METHODS

The aromatic compounds studied (Figure 1) came from both commercial and "discreet" sources. Many of the compounds tested are available from the Aldrich Chemical Company, Milwaukee, Wisconsin 53233. We tested for impurities by subjecting each compound to thin-layer chromatography using two different solvent systems, benzene-ethyl acetate 1:4, and acetonitrile. Each compound tested showed only a single spot on migration on pre-coated silica gel plates (E.M. Laboratories, 500 Executive Blvd., Elmsford, N.Y. 10523). De-ionized water of at least 7 megohm resistance was used in the preparation of all aqueous reagents (17). All other reagents and solvents were ACS grade or better.
Male rats, Walter Reed strain, 275-400g, were fasted overnight but given water ad libitum. The animals were sacrificed by asphyxiation in a carbon dioxide saturated atmosphere. The livers were quickly excised, blotted with gauze, weighed and placed in ice-cold 0.25 mol/l sucrose - 0.05 mol/l Tris buffer, pH 7.5, containing 1% Tween-20 (16). All subsequent operations of the tissue prior to incubation at 37°C were carried out in the cold. The tissue samples were homogenized and sonicated to assure disruption of the mitochondrial membrane. After sonication, the volumes were adjusted to provide a 5% w/v suspension of tissue homogenate in the buffer.

The determination for ALAS activity was performed by a modification of the method of Ebert, et al (18). The difference was that we used labeled alpha-ketoglutaric acid instead of labeled succinyl Co-enzyme A. HS was analyzed by a modification of the technique of Bonkowsky, et al (16). The details of this modification are in preparation for publication elsewhere.

Initially, we evaluated some of these compounds at 0.2 mol/l, which was the concentration of the aromatic with respect to the reaction mixture. Recognizing that at higher concentrations, some effect would be manifested, we focused our attention on concentrations which might be reasonably expected to become physiologically achievable. We extensively studied the effects in the 10^-2 to 10^-6 mol/l concentration range.

RESULTS

Figure 1 shows the structures of the compounds studied, most of which inhibited ALAS and enhanced HS activities. It is recognized that in mammalian adults, heme acts as an inhibitor or by means of a heme-controlled repressor, controls the additional production of heme by limiting the amount of ALA produced. We have not ascertained through our experiments whether or not the decrease in ALAS activity under the influence of the chemical is due to the presence of the chemical alone, additionally produced heme alone, or a combination of these two factors.

Table 1 gives the relative sensitivity of these enzymes toward the compound studied. The strata of sensitivity to ALAS are the dinitrobenzenes > trinitrotoluenes > dinitrotoluenes > amino-dinitrotoluenes > chlorotoluenes > benzene. For HS, the strata of sensitivity are the dinitrobenzenes > dinitrotoluenes > amino-dinitrotoluenes > trinitrotoluenes > nitrobenzene. As a class of compounds, the monochlorotoluenes had little effect on the enzyme systems. The addition of one nitro group to the benzene ring had little effect on ALAS.
and HS activities. The addition of a second nitro group had a marked effect on the activities of both enzymes, especially at $10^{-4}$ mol/l. At this concentration, the dinitrobenzenes gave an average increase of 48% on HS activity. This effect could be demonstrated at a concentration as low as $10^{-5}$ mol/l in which one gets an average enhancement of 35% for this enzyme. Conversely, at $10^{-3}$ mol/l, the activity of ALAS was decreased an average of 44% and at $10^{-5}$ mol/l, the average was essentially the same as the control value.

The enhancement/inhibition effect of placing the second nitro group on the aromatic ring is also evident with 4-nitrotoluene and the dinitrotoluenes. Toluene and 4-nitrotoluene have little effect on ALAS and HS activities; however, when the second nitro group is introduced to the ring, there is an average decrease of 37% in ALAS activity and an average increase of 26% in HS activity.

One may note in Table 1 that on replacing the nitro group by an amino group, there is a tendency to further inhibit ALAS and enhance HS activities. The reserve trend occurs in the tri-substituted toluenes in which one nitro group has been replaced by an amino group.

Figure 2 shows the comparison of the activities of the dinitrobenzenes on ALAS and HS activities. It may be noted that in concentrations greater than $10^{-5}$ mol/l, as HS activity increases, ALAS activity decreases. The differences between ALAS and HS activities at $10^{-3}$ mol/l cannot be solely attributed to aqueous solubilities of the isomers because their rank in water solubilities is $m^p > p^m$ isomers respectively, whereas their effect on the enzymes is $p^m > m$.

The effect may be due to steric specificity and/or spatial arrangements of the molecules involved.

Figure 3 shows the comparison of the effect of the dinitrotoluenes on the enzymes studied. At the same concentrations with respect to the dinitrobenzenes, the effect is not as pronounced which suggests that there may be some interaction between the methyl and nitro groups which offsets the enhancement/inhibition effects. A similar trend is noted with the trinitrotoluenes and the amino-dinitrotoluenes which is shown in Figure 4. The addition of an amino group to the aromatic ring appears to lessen the decrease in ALAS activity.

The normal values obtained for rat liver ALAS were 1–3 nmol/g protein/0.5 hr and 8–14 μmol/g protein/0.5 hr for HS.
DISCUSSION

We have utilized the sensitivity of two enzymes involved in heme synthesis to selected organic compounds as a means of assessing pharmacological effects of aromatics of interest on the activity of these enzymes. ALAS and HS were chosen for these studies because they represent the initial and final enzymes in the biosynthetic pathway of heme. In addition, both have regulatory roles in the production of heme.

Several investigators have explored the effect of benzene on heme synthesis. It has been shown by Forte, et al (10) and Wildman, et al (9) that benzene, at a final concentration of 0.113 mol/l, inhibits rabbit reticulocyte heme synthesis in vitro at the ALAS step. Lee, et al (19) have shown that a single subcutaneous dose of benzene in mice decreases the incorporation of $^{59}$Fe into erythrocytes, 440 mg/kg body weight produced a 27% and a dosage of 2200 mg/kg producing a 50% inhibition. A smaller dose, 88 mg/kg had no effect on the incorporation of $^{59}$Fe and no effect on the hematocrit.

Other groups have examined structure-activity relationships of various classes of compounds with respect to their effect on ALAS and other related enzymes. Marks, et al (20), investigated the porphyrin-inducing activity of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethyl pyridine, a compound known to induce porphyria by enhancing the synthesis of ALAS, and several analogues of it and the corresponding pyridines. They determined the porphyrin-inducing activity of these compounds by feeding them to guinea pigs and estimating the amount of ALA and porphobilinogen excreted in the urine. Poland and Kende (21) investigated a number of halogenated di-benzo-p-dioxins for their ability to stimulate two hepatic enzymes: (1) ALAS and (2) aryl-hydrocarbon hydroxylase, a cytochrome P-450 mediated microsomal mono-oxygenase. The potency of the halogenated di-benzo-p-dioxins to induce ALAS and aryl-hydrocarbon hydroxylase corresponded precisely with their lethal, teratogenic and acnegenic potency, to the extent that toxicological data were available.

In summary, we have used techniques of measuring two enzymes involved in heme synthesis to study in vitro effects of certain organic chemicals on these enzymes as a means of determining structure activity relationships for in vivo predictions. We have attempted to use physiologically attainable concentrations of the chemicals in question and have demonstrated that an effect can be registered routinely at concentrations as low as $10^{-6}$ mol/l and in some cases as low as $10^{-8}$ mol/l.

10 to the minus 6th power
WILLIAMS, JOHNSON, HAUT, ALTSTATT

The utility of these techniques lie in the versatility of measuring the levels of these enzymes not only in liver hemogenates, but also in bone marrow aspirates and reticulocyte rich blood. Bone marrow or whole blood measurements may obviate the need for surgical procedures when monitoring the pharmacological effect of the compounds of interest.

REFERENCES


Table 1. The effect of the compounds investigated on the two enzyme systems studied.

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<th>Aromatic</th>
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<th>Concentration of Aromatic</th>
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<td>10^{-3} mol/1</td>
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Fig. 1. Structures of the compounds under investigation.
THE DINITROBENZENES

![Graph showing the effect of m-nitrobenzene on ALAS and HS activities.](image)

Fig. 2. The effect of the dinitrobenzenes on ALAS and HS activities. Note the inverse relationship at higher concentrations resulting in a "V" shaped curve.
Fig. 3. A comparison of the effect of the dinitrotoluenes on ALAS and HS activities. Note the coincidence of activity in the $10^{-5}$ to $10^{-4}$ mol/l concentration range of the aromatic resulting in an "X" shaped curve.
Fig. 4. A comparison of the effect of tri-substituted toluenes on the enzyme systems studied producing a "Y" shaped curve.