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## S-methylation, oxidation, hydroxylation and conjugation of thiophenol in the rat

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THIOPHENOL is an important chemical intermediate utilized in the manufacture of lubricating oil additives, antioxidants, resins, insecticides, herbicides and pharmaceuticals.

Parke[1] reported that rabbits excreted no free thiophenol after its oral administration, but instead the urinary products were thiophenyl glucuronide and, possibly, phenylthiosulfuric acid. Oxidation of thiophenol to diphenyldisulfide (DPDS) by spores of the fungus, *Myrothecium verrucaria*, was reported by Mandels.[2] Recently, Gessner and Acara[3] described the S-glucosylation of thiophenol in four insect species *in vivo* and *in vitro*. In this communication we present evidence indicating a third, major biotransformation pathway for thiophenol in the rat.

Thiophenol-$^{35}$S (PSH), 14·7 mc/m-mole, from the Radiochemical Centre, Amersham, England, was of a radiochemical purity of greater than 98 per cent as determined by gas chromatography. Methylphenylsulfide-$^{35}$S (MPS) and methylphenylsulfone-$^{35}$S ($MPSO_2$) each with a specific activity of 1·13 mc/m-mole, from Stauffer Chemical Company, Western Research Center, Richmond, Calif., were radiochemically pure samples after purification by preparative-scale thin-layer chromatography (TLC). Authentic unlabeled samples were from the following sources: PSH from Pitt-Consol, Newark, N.J.; MPS and $MPSO_2$ from Aldrich Chemical Company, Milwaukee, Wis.; methylphenylsulfoxide (MPSO) and DPDS from Stauffer Chemical Company, Richmond, Calif.

Four rats of the Long–Evans strain (two males and two females, 116–124 g) were orally dosed with 6 mg/kg of PSH-$^{35}$S. Each of two male rats weighing 280–300 g was similarly treated with 2·5 mg/kg of either MPS-$^{35}$S or $MPSO_2$-$^{35}$S. The treated rats were housed in glass metabolism cages.[4] Urine samples were collected for 60 hr in the case of PSH-$^{35}$S and for 64 hr in the case of MPS-$^{35}$S or $MPSO_2$-$^{35}$S. Nonradioactive PSH (200 $\mu$g) was added to 70 ml of freshly collected urine from untreated rats and processed in the same manner as the experimental urine, thus serving as a control. Urine from each group was pooled and extracted twice with benzene to separate the polar (aqueous phase) and nonpolar (benzene phase) metabolites. Polar fractionated urine samples were hydrolyzed by refluxing for several hours with 3 N $H_2SO_4$. Hydrolyzed radioactive products were extracted with diethyl ether for subsequent chromatography.

TLC analysis of the benzene-soluble and water-soluble metabolites, before and after hydrolysis, involved Silica gel H (250 $\mu$ thick) precoated plates (Analtech, Inc., Wilmington, Del.) and chloroform:ethyl acetate(1:1) mixture (CEA) for development. $R_f$ values in this system were as follows: PSH,0·95; MPS, 0·90; $MPSO_2$, 0·83; MPSO, 0·34. The benzene-soluble metabolite and $MPSO_2$ were chromatographed in two additional solvent systems: benzene: diethyl ether(7:3) mixture (BE) and *n*-hexane:chloroform(3:2) mixture(HC). Water-soluble metabolites were also resolved on Silica gel F-254 (250 $\mu$ thick) precoated plates (Brinkmann Instrument Company, Inc., Westbury, Long Island, N.Y.) using *n*-butanol:acetic acid:water(2:1:1) mixture (BAW). They were also examined before hydrolysis in the CEA system. Radioactive products were detected by autoradiography using

Kodak No-screen X-ray film. Two reagents were used to detect MPS, MPSO and DPDS: spraying the developed plates with 0·5% 2,6-dibromo-*N*-chloro-*p*-quinoneimine (DCQ)[5] in cyclohexane solution detects MPS and DPDS; spraying with a saturated solution of $KMnO_4$ detects MPS and MPSO. $MPSO_2$ is detected with iodine vapors or by examining developed TLC plates (Silica gel F-254) under ultraviolet light (2540 Å). Aliquots of the benzene-soluble phase of control urine were subjected to TLC separation in the BE system. Regions on the TLC plate, where $MPSO_2$ and DPDS would occur if present, were scraped free of the glass support and eluted with acetone. Appropriate aliquots were analyzed by gas–liquid chromatography (GLC) using a Varian-Aerograph Hy-Fi model 600-D with a 3 ft × 1/8 in. i.d. Pyrex column coated with 10% DC-200 (1000 cs) on Varaport-30 support (80/100 mesh). The injection temperature was 150° and the column temperature was 170°.

TABLE 1. EXCRETION OF METABOLITES OF THIOPHENOL-$^{35}$S (PSH), METHYLPHENYLSULFIDE-$^{35}$S (MPS) AND METHYLPHENYLSULFONE-$^{35}$S ($MPSO_2$) IN THE URINE OF RATS AFTER THEIR ORAL ADMINISTRATION

| | $^{35}$S compound administered | | |
|---|---|---|---|
| | PSH | MPS | $MPSO_2$ |
| Time after administration (hr) | 60 | 64 | 64 |
| $^{35}$S recovery in urine (% of administration): | | | |
| Benzene-soluble | 8 | 10 | 13 |
| Water-soluble | 37 | 35 | 32 |
| Nature of products (CEA system): | | | |
| Benzene-soluble, identity | $MPSO_2$ | $MPSO_2$ | $MPSO_2$ |
| Water-soluble, $R_f$ | | | |
| Before hydrolysis | 0·00* | | |
| | 0·39†‡ | | |
| | 0·42†‡ | | |
| After hydrolysis | 0·39‡ | 0·39‡ | 0·39‡ |
| | 0·42‡ | 0·42‡ | 0·42‡ |

* $R_f$ = 0·76 in BAW system.
† $R_f$ = 0·93 in BAW system.
‡ $R_f$ similar to that of *o*-hydroxy $MPSO_2$ and *p*-hydroxy $MPSO_2$.

Table 1 presents the quantitative data, the nature of the identified metabolites, and the $R_f$ values for those which are not identified. The benzene-soluble metabolite excreted is $MPSO_2$ and it increases in amount in the sequence of PSH, MPS and $MPSO_2$ for the administered compound. This metabolite is neutral, based on partitioning studies from acidic or basic solution using ether for extraction, it is stable to acid hydrolysis, and it cochromatographs in the CEA, BE and HC systems with known samples of $MPSO_2$. The respective $R_f$ values for $MPSO_2$ in these systems were as follows: 0·83, 0·50 and 0·04. Compounds not recovered in the benzene-soluble fraction, that would be present in this fraction if excreted, are PSH, MPS and DPDS. Trace amounts of what appears to be MPSO were evident on chromatograms (CEA system) of urine from rats treated with PSH and MPS but not $MPSO_2$.

Analysis by TLC and GLC of the benzene-soluble phase of control urine showed that approximately 30 per cent of the added PSH was converted to DPDS, an oxidation product of PSH, and no $MPSO_2$ was recovered. These results were obtained from retention time values and cochromatography (GLC) with known samples of DPDS and $MPSO_2$.

The water-soluble metabolites consist of three products as resolved in the CEA system and these have $R_f$ values of 0·00, 0·39 and 0·42. The BAW system moves these products towards the front but resolves only two components, the upper spot being a mixture of the products of $R_f$ 0·39 and 0·42 in the CEA system. Acid hydrolysis does not alter the 0·39 and 0·42 products, but cleaves the 0·00 product to give the 0·39 and 0·42 products. The $R_f$ 0·39 and 0·42 products are acids, readily extracting into ether from acidic water (pH 1), but not from alkaline (pH 12). The $R_f$ 0·00 product, apparently a conjugate, fails to extract into ether from either acid or basic solution. Preliminary studies indicate

the $\rho$- and $o$-hydroxy $MPSO_2$ are similar in chromatographic mobilities to the $R_f$ 0·39 and 0·42 metabolites respectively.

The same products are formed from PSH, MPS and $MPSO_2$ and so each product must form ultimately from $MPSO_2$. These findings suggest that PSH readily undergoes S-methylation *in vivo*, followed by oxidation of the sulfide (MPS) to the corresponding sulfone ($MPSO_2$). Since DPDS and not $MPSO_2$ was detected in the fortified control urine, it indicates that S-methylation and subsequent sulfoxidation are the biotransformation steps clearly associated with metabolism of PSH in the living rat. Although trace amounts of MPSO might occur in urine, it probably forms as a transient intermediate which is readily oxidized to $MPSO_2$. Methylation of a terminal sulfhydryl group, attached to a heterocyclic ring through a methylene bridge, and subsequent oxidation to the sulfoxide or sulfone (or both) *in vivo* is known with the insecticides Menazon[6] and Supracide.[7] It is known that mice and guinea pigs[8] dosed with ethyl mercaptan excrete ethyl methyl sulfone. S-methyl sulfoxide and sulfone formation also occurs *in vivo*[9] in the metabolism of thiamine tetrahydrofurfuryl disulfide in rats. S-methyl oxidation also occurs in animals with 2,2-dichloro-*N*-[$\beta$-hydroxy-$\alpha$-(hydroxy-methyl)-$\rho$-(methylsulfinyl)phenethyl] acetamide[10] and its sulfide derivative.

It appears that S-methylation of PSH followed by oxidation and conjugation are the primary biotransformation steps in the metabolism of PSH in the rat. Sarcione and Sokal[11] showed that rats utilized methylation of the thiol group of 2-thiouracil, to form 2-methylthiouracil, as a key detoxication mechanism. Ring hydroxylation and conjugation of $MPSO_2$ apparently also occur. The metabolic pathway thus appears to be as described in Fig. 1.

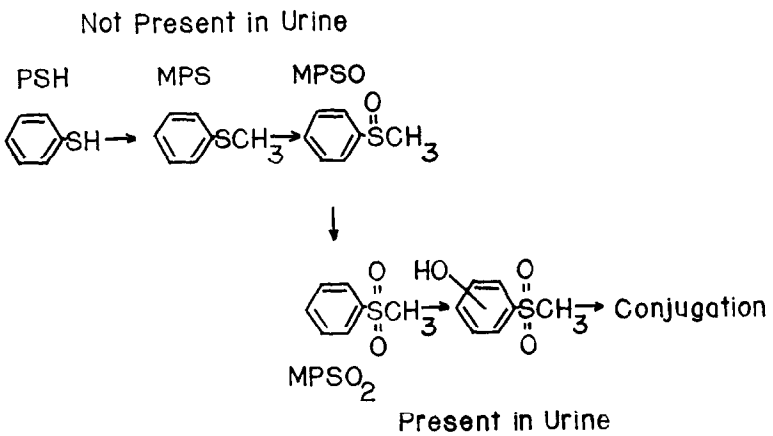


FIG. 1. Proposed metabolic pathway of thiophenol in the rat.

Toxicity data further support our finding that initial S-methylation is the important detoxifying step for MPS, since the acute oral $LD_{50}$ of PSH in rats is 65 mg/kg while those of MPS and $MPSO_2$ are 891 mg/kg and 1470 mg/kg respectively.*

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# The estimation of monoamine oxidase using $^{14}$C-labelled substrates

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SEVERAL micro-methods have recently been published for the estimation of tissue monoamine oxidase (MAO) activity based on the measurement of labelled metabolites of $^{14}$C-tryptamine,[1,2] $^{14}$C-tyramine,[1,3,4] $^{14}$C-5-hydroxytryptamine,[1] $^{14}$C-dopamine[3] and $^{14}$C-benzylamine.[1] These methods depend on the isolation of the labelled compounds by either solvent extraction with toluene,[2] ethyl acetate[3] or anisole,[4] or by ion-exchange procedures.[1] In the present work, some of the limitations of these procedures are reported.

The partitioning of $^{14}$C-tryptamine between an aqueous solution and ethyl acetate, toluene and anisole was measured at pH values of 1·0, 7·4 and 8·8; extraction was least at acid pH value with each solvent (Table 1). In addition, the solubility of indole-3-acetic acid and indole-3-acetaldehyde in the three solvents was determined at the two lower pH values using the assay procedure described by Larsen and Klungsöyr.[5] It appears that the degree of extraction of the two compounds differs, the acid being more soluble in all three solvents at acid pH (Table 1). In an attempt to verify these results, rat liver aldehyde dehydrogenase was prepared[6] and solubilized MAO was also prepared from rat liver, using the method described by Youdim and Sandler.[7] In some tubes, $^{14}$C-tryptamine was incubated with MAO in the presence of phosphate buffer for 20 min, after which 0·4 ml of 2N HC

TABLE 1. EFFECT OF pH ON THE EXTRACTION OF TRYPTAMINE, INDOLE-3-ACETIC ACID AND INDOLE-3-ACETALDEHYDE BY ETHYL ACETATE, TOLUENE AND ANISOLE

| | Solvent | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ethyl acetate | | | Toluene | | | Anisole | | |
| pH | 1·0 | 7·4 | 8·8 | 1·0 | 7·4 | 8·8 | 1·0 | 7·4 | 8·8 |
| Tryptamine | 11·3 | 25·6 | 56·3 | 0·45 | 3·6 | 15·6 | 0·44 | 9·1 | 35·4 |
| Indole-3-acetic acid | 30·0 | 13·9 | — | 74·2 | 21·0 | — | 75·0 | 70·2 | — |
| Indole-3-acetaldehyde | 9·3 | 26·9 | — | 4·8 | 37·0 | — | 17·7 | 32·7 | — |

The values given represent percentage recovery.