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A Mechanistic Study of Aromatic Hydroxylamine Rearrangement in the Rat

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A modified crossover experiment was conducted to determine the mechanism of arylhydroxylamine rearrangement in the rat. When 1-hydroxy-1-phenyl-3-methylurea was incubated with fractionated liver homogenates or injected intraperitoneally in rats, 1-methylbenzimidazol-2-one was isolated as the major metabolite. Small amounts of the anticipated aminophenol, 1-(*o*-hydroxylphenyl)-3-methylurea were also isolated. Evidence is presented suggesting that hepatic isomerase-catalyzed rearrangements of hydroxylamines proceed via pathways analogous to those described for chemical model systems. Isomerization appears to be intermolecular involving the generation of a resonance-stabilized nitrenium ion capable of binding to amino acids and nucleotide bases.

Neoplasia induction by specific aromatic amines and amides is believed to involve the interaction of these chemical carcinogens in a metabolically modified form with tissue nucleic acids and protein (*1*). *N*-Hydroxylated metabolites of these amines have been cited as proximal carcinogens (*1*, *2*). However, further metabolic transformation of the hydroxylamine resulting in the production of a reactive electrophilic intermediate is postulated (*3*) as a prerequisite for combination with macromolecules and as being ultimately responsible for the tumor-inducing action. The observed enzymatic rearrangement of aromatic hydroxylamines and hydroxamic acids in liver to yield

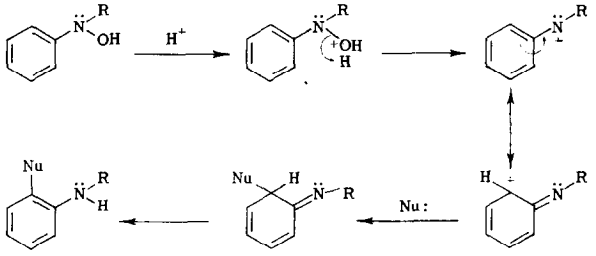

FIG. 1. Nonconcerted isomerization mechanism of arylhydroxylamine derivatives.

corresponding *o*-aminophenols (*4*) may serve to generate electrophilic species capable of covalently binding with nucleophilic sites on nucleic acids or protein. This hypothesis is predicted on the supposition that hydroxylamine rearrangement is not concerted, but

proceeds with the formation of a resonance-stabilized nitrenium cation. Centers of low electron density would be created on the aromatic nucleus which could subsequently combine with water to form aminophenol or alternatively would be capable of arylating nucleophilic sites (e.g., methionine or guanine) on protein and nucleic acids.

The production of resonance-stabilized nitrenium ions during the acid-catalyzed rearrangement of *N*-hydroxyamines (*5*), capable of covalently binding to amino acids (*6*) and nucleotide bases (*7, 8*) is well documented in chemical systems, and while it is suggestive of possible pathways by which enzymatic combination might occur, little is known concerning the mechanism of hydroxylamine isomerization in liver. A classical procedure to distinguish between an inter- and intramolecular mechanism is a crossover experiment. In the present context this involves incubating the hydroxylamine under the conditions of isomerization in the presence of a competing nucleophile. If the reaction is concerted, the hydroxyl moiety and aromatic ring never become completely independent and the competing nucleophile is not found in the rearranged product. Conversely, an intermolecular (nonconcerted) mechanism allows for incorporation of either hydroxide or the competing nucleophile into the product. A mechanistic study of enzymatic isomerization of aromatic hydroxylamines is complicated by uncertainty regarding the disposition of the competing nucleophile; that is, whether the competing nucleophile is sufficiently proximal to the activated substrate at the enzyme surface to permit their interaction. Accordingly, we have studied both the *in vivo* and *in vitro* metabolism of 1-hydroxy-1-phenyl-3-methylurea [HPM] (*1*), in the rat. This substrate is an aromatic hydroxylamine derivative into which a weak alternative nucleophile (ureido-nitrogen) is chemically incorporated into a side chain to assure juxtaposition of the arylhydroxylamine and competing nucleophile at the enzyme surface.

MATERIALS AND METHODS

Phenylhydroxylamine was synthesized by reduction of nitrobenzene with zinc (*9*) and condensed with methyl isocyanate (*10*) to yield HPM, which was subsequently used as substrate in enzymatic reactions. 1-Methylbenzimidazol-2-one was prepared by fusing *N*-methylphenylene diamine with urea (*11*) or alternatively by the acid-catalyzed rearrangement of HPM (*12*). 1(*o*-Hydroxyphenyl)-3-methylurea was synthesized by initial condensation of *o*-aminoanisole with methyl isocyanate to form 1-(*o*-methoxyphenyl)-3-methylurea and subsequent *O*-demethylation with boron tribromide at −80°C to yield the desired product as described by McOmie and Watts (*13*). Physical constants of all products were in agreement with literature values.

For *in vitro* studies, male Sprague–Dawley rats weighing 150–200 g were sacrificed by decapitation and livers were immediately excised. Tissue homogenation and subsequent subcellular fractionation was carried out as previously described (*14*). Typical reaction mixtures containing hepatic enzyme (9000*g* or 105 000*g* supernatant fraction) equivalent to 7.5 g of wet liver and 0.25 mmoles of HPM dissolved in 180 ml of 0.02 *M* Tris–HCl buffer (pH 7.4) were incubated aerobically in a Dubnoff metabolic incubator at 37°C for 60 min. Reactions were terminated by the addition of 50 ml of methanol and mixtures were extracted with four 100-ml portions of ethyl acetate. Ethyl acetate extracts were purified by thin-layer chromatography on 1-mm silica gel GF-254 plates using

ethyl acetate as solvent system. All separated components were characterized from ir and mass spectra.

For *in vivo* studies, isotonic sodium chloride solutions of HPM (600 mg/kg body wt) were administered intraperitoneally to four male Sprague–Dawley rats (150–200 g). Urine from individually housed animals was collected over a 24-hr period as a run-off from metabolism cages with sodium fluoride as a preservative. Urine samples were combined and extracted with two 60-ml portions of ethyl acetate. The ethyl acetate extracts were treated using the procedure described form the characterization of metabolites from liver homogenates. Urine from saline-treated animals was similarly processed and served as control.

RESULTS

When HPM was incubated aerobically with only buffer or inactivated (boiled) liver homogenate, thin-layer chromatography of the residue obtained from protein-free incubations revealed only one band (R_f 0.74) corresponding in R_f value to unchanged HPM. Identification was confirmed by comparison of the mass spectrum of product with that of authentic HPM. However, when HPM was incubated aerobically with a viable 9000*g* or 105 000*g* supernatant fraction of rat liver in the absence of cofactor (NADPH; ATP) two new brands were detected after tlc of incubation mixture extracts, in addition to the band (R_f 0.74) corresponding to unreacted HPM. An intense band (R_f 0.35) located under uv light was identified as 1-methylbenzimidazol-2-one [MBO] (*2*), by comparing its mass spectrum with a synthetically prepared sample of MBO. Both spectra showed a prominent molecular ion (*m/e* 148) and the spectral fragmentation pattern of the metabolite (peaks with intensities greater than 10% of the base peak) corresponded with that of the authentic material. A second band (R_f 0.50) was further purified by rechromatographing the eluted spot using ethyl acetate–chloroform (99:1) as developing solvent. This permitted complete separation of the benzimidazolone metabolite (MBO) from this second minor metabolite. The resultant weak band (R_f 0.75) was identified as the corresponding *o*-aminophenol product, 1-(*o*-hydroxyphenyl)-3-methylurea [HMU] (*3*), by comparison of its mass spectrum with an authentic sample of HMU (Fig. 2).

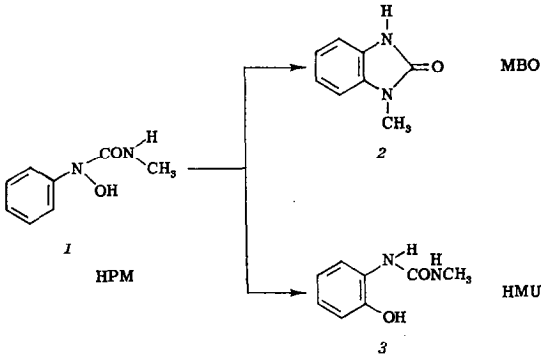

FIG. 2. Metabolic fate of 1-hydroxy-1-phenyl-3-methylurea.

1-Methylbenzimidazol-2-one was also identified as the major urinary metabolite from whole animal studies. Confirmation of structure was given based on comparison of the infrared and mass spectra of metabolite with authentic MBO.

DISCUSSION

In chemical systems, aromatic hydroxylamines rearrange to aminophenols under mildly acidic conditions (*12*). Reaction is intermolecular (*15*) and proceeds with the generation of electrophilic sites on the aromatic ring which are susceptible to attack by available nucleophiles (Fig. 1). Similar hydroxylamine rearrangements occur *in vivo* (4). A hydroxylamine isomerase enzyme system present in rat liver cytosol has been characterized (*4*). This enzyme requires no obligatory cofactor (NADP or ATP) and activity is enhanced by an inducible microsomal component (*16*). It was initially postulated that enzymatic isomerization followed a course analogous to that elucidated for chemical systems, i.e., a process involving initial formation of a resonance-stabilized cation. Several lines of evidence support a nonconcerted mechanism. Simple esterification of the hydroxylamine promotes chemical and enzymatic isomerization (*6*, *8*) apparently by facilitating cleavage of the N—O bond with concomitant generation of a nitrenium cation. Isomerization is also stimulated by phosphorylating (ATP) and sulfurating coenzymes (*17*) which are presumed to enzymatically esterify the hydroxylamines. In addition, aromatic hydroxylamines bind covalently to nucleophilic sites on guanine (*8*) and methionine (*6*). These species serve as competing nucleophiles in hydroxylamine rearrangement reactions and their incorporation into the arylamine product supports an intermolecular mechanism.

More recently, Gutmann (*18*) studied the enzymatic isomerization of *N*-hydroxyl-2-fluorenylacetamide in which the hydroxyl group was labeled with ^{18}O. Determination of the isotope content of the rearranged product indicated that the hydroxyl group was transferred from nitrogen to an aryl carbon with retention of the isotopic label. He concluded from these data that isomerization was intramolecular (concerted) involving initial abstraction of a hydride ion from an aromatic ring by the enzyme to generate an aryl cation. Processes involving removal of a hydride ion from an sp^2 hybridized carbon atom are energetically unrealistic and certainly unprecedented in biochemical literature. The incorporation of ^{18}O into the rearranged product does not exclude a nonconcerted mechanism. For example, isomerization may involve heterolytic cleavage of the N—O bond with formation of a caged ion-pair, or alternatively the hydroxide ion lost from the hydroxylamine may become enzyme bound and recombine with the nitrenium cationic intermediate to yield the observed labeled aminophenol. In both cases the mechanism is nonconcerted but retention of the ^{18}O label is observed.

To substantiate the hypothesis of an intermolecular enzymatic rearrangement of hydroxylamines, a crossover experiment was conceived in which a competing nucleophile (urea nitrogen) was covalently incorporated into the hydroxylamine molecule. When exposed to the hepatic isomerase, HPM underwent a Bamberger-type rearrangement (*12*) to yield small amounts of the anticipated *o*-aminophenol, HMU; the *para* isomer was not detected. The major product, however, was 1-methylbenzimidazol-2-one, which apparently forms as a result of attack by the urea nitrogen at an electrophilic

site on the arene generated after the loss of hydroxide from the hydroxyurea. A concerted mechanism involving direct attack on a deactivated aromatic nucleus by either the weakly nucleophilic urea nitrogen or hydroxyl group seems unlikely. Intramolecular (concerted) hydroxide migration is very improbable since it must proceed through a highly strained, energetically unfavored, four-membered cyclic transition state.

The distribution of products obtained from enzymatic rearrangement of HPM is a reflection of a propinquity effect, i.e., formation of the aminophenol involves the interaction of two separate entities in solution while the reactive components responsible for benzimidazole formation are juxtaposed within a single compound. A similar product distribution was obtained with chemical model systems. Under the acidic conditions of the Bamberger rearrangement (*5*), HPM yielded MBO as the only product. Such acid-catalyzed reactions involving arylhydroxylamine derivatives proceed with the initial loss of hydroxide followed by attack of proximal nucleophiles; all of which implies a similar mechanism may occur with the corresponding enzymatic rearrangement.

These experiments disprove a concerted intramolecular process, but cannot distinguish between an intermolecular mechanism and an intramolecular process proceeding through a pi complex or ion pair. The ureido nitrogen may compete effectively with a loosely held π-bonded hydroxyl moiety resulting in the formation of a new π-complex and the major product. Other experiments have demonstrated (*6*) the ability of *N*-phenylhydroxamic acid derivative to bind covalently to electron-rich sites on amino acids and nucleoside bases (serving as competing nucleophiles) at physiological pH. When coupled with these experiments, observations described in this manuscript support the hypothesis of an intermolecular enzymatic hydroxylamine isomerization and suggest that a resonance-stabilized nitrenium ion is generated during rearrangement which may attach proximal nucleotide bases or protein, perhaps initiating a neoplastic response.

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