Metabolic Conversion of Fluorenone Oxime to Phenanthridinone by Hepatic Enzymes

YECHIEL GOLANDER and LARRY A. STERNSON ˣ

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Abstract □ Fluorenone oxime is converted to phenanthridinone by enzymes present in rat liver homogenates. The reaction is analogous to the chemical Beckman rearrangement. The oxime–amide rearrangement enzyme is localized primarily in the microsomes, with some activity in the cytosol. The reaction requires reduced nicotinamide adenine dinucleotide phosphate and observes Michaelis–Menten kinetics. The reaction is relatively slow (V_{max} = 7.75 ± 2.01 nmoles of phenanthridinone formed/100 mg of liver/15 min), but the enzyme reaches maximum velocity at relatively low substrate concentrations (K_m = 3.90 ± 1.85 × 10^{-5} M). The reaction is strongly competitively inhibited by 1-decylimidazole (K_I = 3.75 ± 1.77 × 10^{-7} M) and inhibited to a lesser extent by the chelating agents bipyridyl (K_I = 1.33 ± 0.21 × 10^{-3} M) and ethylenediamine tetraacetate (K_I = 1.00 ± 0.28 × 10^{-3} M) and the sulfhydryl binding agent *p*-chloromercuribenzoate (K_I = 2.71 ± 0.07 × 10^{-4} M). Studies also suggest that the reaction mechanism does not involve initial enzymatic substrate esterification through acetylation, glucuronidation, phosphorylation, or sulfation.

Keyphrases □ Fluorenone oxime—metabolic conversion to phenanthridinone by hepatic enzymes, *in vitro*, kinetics □ Phenanthridinone—produced from fluorenone oxime by hepatic enzymes, *in vitro*, kinetics □ Enzymes, hepatic—conversion of fluorenone oxime to phenanthridinone, *in vitro*, kinetics

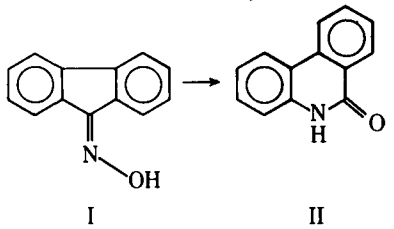
Oximes are metabolic products formed by liver microsomal oxidation of primary aliphatic amines (1, 2). Although these products were considered to be metabolically unreactive, they were susceptible to enzymatic reduction to the corresponding hydroxylamine and amine in several animal species (3, 4). Fluorenone oxime (I), when administered intravenously to rats, is converted (Scheme I) to phenanthridinone (II), which is subsequently excreted in the urine (5). The enzymes responsible for this transformation are localized primarily in the liver microsomes (6), with minor activity in the nuclear and mitochondrial fractions.

This report describes the enzymatic transformation kinetics and provides information to help characterize the oxime–amide rearrangement enzyme.

EXPERIMENTAL

Apparatus—High-performance liquid chromatography (HPLC) was performed on a component system[1], and analytes were detected with an absorbance detector operated at 254 nm.

Materials—Fluorenone oxime and phenanthridinone were synthesized by literature methods (7). Liver enzymes were prepared from male Sprague–Dawley rats, 200–250 g, as described previously (8).



I II

Scheme I

Enzymatic Assay—Incubation mixtures contained enzyme equivalent to 100 mg of liver, a reduced nicotinamide adenine dinucleotide phosphate (III)-generating system [nicotinamide adenine dinucleotide phosphate (1 μmole), glucose-6-phosphate (20 μmoles), glucose-6-phosphate dehydrogenase (1 unit), and magnesium chloride (100 μmoles)], fluorenone oxime, and phosphate buffer (0.05 M, pH 7.4) adjusted to a final volume of 5 ml.

Incubations were carried out for 15 min with agitation in a Dubnoff metabolic incubator at 37°. All reagents in the system used to generate III were in excess with respect to requirements for III during the entire incubation.

Analytical Methodology—Incubations were terminated by the addition of 10 ml of chloroform to the reaction mixture. The mixture was shaken for 20 min and centrifuged at 2500×*g*. The organic layer was recovered [extraction efficiency of II ≥98% (6)] and evaporated to dryness under nitrogen. The mixture was dissolved in 200 μl of dimethylformamide containing 20.8 nmoles of phenanthrene, present as an internal standard.

Aliquots (10 μl) of this mixture were analyzed by HPLC on a 4-mm o.d. × 30-cm column[2]. Components were separated using methanol–methylene chloride–water (60:5:35) as the mobile phase (flow rate of 2 ml/min). Under these conditions, the retention volumes (V_R) were: phenanthridinone, 7.1 ml; fluorenone oxime, 10.4 ml; and phenanthrene (added as internal standard), 26.7 ml. Quantitation of II was based on comparison of the peak height ratio (analyte–internal standard) with a standard curve prepared after analysis of liver homogenate samples containing known amounts of I and II in the 8.6 × 10^{-6}–1.4 × 10^{-4} M range. This response was related linearly to concentration, yielding the line $y = 0.05x - 0.005$ (zero-order correlation 0.99) after least-squares analysis.

Kinetic Measurement—The Michaelis constants (K_m) and the maximum velocity (V_{max}) for the conversion of I to II were determined from double-reciprocal (Lineweaver–Burk) plots of initial velocity against substrate concentration (9), which varied over the range of 62–1000 μM. The line of best fit was determined by least-squares regression analysis. In experiments to determine the effect of various chemicals on enzyme activity, the test compounds were added to the incubation mixture concurrently with the substrate (I). Microsomes isolated by centrifugation of the 9000×*g* supernate of liver homogenates at 105,000×*g* were not subjected to additional purification.

RESULTS AND DISCUSSION

A previous report (6) showed that the conversion of I to II in liver homogenates is an enzymatic process. Now it is shown that the major oxime–amide rearrangement enzyme responsible for the rearrangement of fluorenone oxime (present at concentrations of 10^{-4} M) is present in liver microsomes (6.04 ± 1.25 nmoles of phenanthridinone formed/100 mg of liver/15 min), with the cytosol exhibiting ~38% of this activity (2.32 ± 0.02 nmoles of phenanthridinone formed/100 mg of liver/15 min).

Minor activity was observed in nuclear and mitochondrial subcellular liver fractions. Repeated washings (six times) of the microsomal pellet (resuspending the pellet in the buffer and then centrifugating at 105,000×*g* for 45 min) caused no loss in enzyme activity, suggesting that the enzyme is bound relatively tightly to the membrane surface. Essentially no oxime–amide rearrangement was observed in the buffer used to wash the microsomal pellet.

Compound III was required as a cofactor for activity in both microsomes and cytosol (6). Neither oxidized nor reduced nicotinamide adenine dinucleotide nor oxidized nicotinamide adenine dinucleotide phosphate (NADP) was an effective cofactor for these enzymes. The role of III in

[1] Waters Associates, Milford, Mass.

[2] μBondapak C-18, Waters Associates, Milford, Mass.

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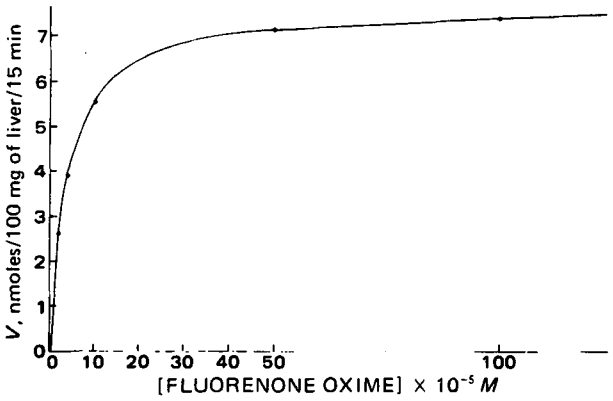


Figure 1—*Rate of fluorenone oxime rearrangement to phenanthridinone determined as a function of the substrate concentration.*

the transformation of I to II has not been elucidated. Conversion of I to II results in no net change in the oxidation state, so the involvement of III in a redox role is questionable.

Incubation of I with tritiated III resulted in the formation of II with no incorporation of tritium in the product. Compound III was consumed during both the microsomal- and cytosol-catalyzed rearrangement of I, as determined by monitoring the reaction spectrophotometrically at 340 nm. Although this result could suggest that the cofactor plays an electron-transfer role in the oxime–amide rearrangement enzyme-catalyzed reaction, the experiment was inconclusive because HPLC analysis of incubation mixtures showed that I was converted to products other than II (particularly in microsomes), and these transformations probably required III. This supposition is supported by the fact that III was consumed more rapidly than can be attributed solely to the enzymatic formation of II. Biotransformation of I occurred with III consumption, but its role in the reaction under study could not be segregated from the total substrate biodegradation.

Microsomal oxime–amide rearrangement enzyme-catalyzed reaction kinetics were determined by measuring the initial I–II conversion rate at substrate concentrations of 10^{-3}, 5×10^{-4}, 10^{-4}, 4×10^{-5}, and 2×10^{-5} *M*. Since microsomes were used without further purification, the observed K_m and K_i values may have been influenced by nonspecific substrate binding to proteins other than the oxime–amide rearrangement enzyme(s). Double-reciprocal plots of initial velocity data *versus* substrate concentration yielded the straight line $v^{-1} = 5.03 \times 10^{-6} S^{-1} + 0.13$ ($r = 0.99$). The plot linearity indicates Michaelis–Menten kinetics (10).

Values of K_m and V_{max} extracted from these plots were $3.90 \pm 1.85 \times 10^{-5}$ *M* and 7.75 ± 2.01 nmoles of II formed/100 mg of liver/15 min (for eight determinations), respectively. Therefore, the reaction was quite slow, but the enzyme reached maximum velocity at relatively low substrate concentrations. Figure 1 shows reaction rate as a function of substrate concentration.

The microsomal oxime–amide rearrangement enzyme system was further investigated by assessing the ability of certain compounds to inhibit the conversion of I to II. Three compounds that are structurally similar to I, carbazole, 2-nitrofluorene, and 9-aminofluorene, had no effect on the reaction in question when I was incubated with the microsomal system at a 10^{-4} *M* concentration and the potential inhibitors were present in a concentration of 10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} *M*. At substrate (I) concentrations of 10^{-4} *M*, ethyl 2,2-diphenylvalerate failed to inhibit the reaction when present at concentrations below 10^{-5} *M*; however, when this potential inhibitor was added at concentrations of 10^{-3} *M*, reaction was inhibited 62% during a 15-min incubation period.

N-Decylimidazole, a known inhibitor of microsomal mixed-function oxidases (11), was a potent competitive inhibitor of the microsomal oxime–amide rearrangement system (Table I), inhibiting the conversion of I to II by 77% when the substrate and inhibitor were present at concentrations of 10^{-4} and 10^{-3} *M*, respectively. However, cytochrome P-450 was not involved in the reaction since conversion of I to II was not effected by either carbon monoxide (6) or microsome preexposure to UV light. The imidazole inhibitory role in this reaction is, therefore, probably not that normally associated with this class of inhibitors.

Chelating agents bipyridyl and edetate sodium also inhibited the biotransformation of I to II (Table I), suggesting that metal ions may play a role in the enzymatic reaction. However, when the substrate was incubated with microsomes and III (*not* a reduced nicotinamide adenine dinucleotide phosphate-*generating* system but III itself), the rearrangement was not inhibited when magnesium ion was totally excluded from the incubation mixture. Thus, the observed inhibitory effects of chelating agents on the enzymatic I → II conversion apparently do not result from an inhibition of III formation (when an NADPH-generating system is used) due to complexation of the magnesium ion required for III formation.

Table I—Inhibition Constants for Various Compounds toward Microsomal Oxime–Amide Rearrangement Activity [a,b]

Inhibitor	Inhibitor Concentration, *M*	$K_I \pm SEM$[c], *M*
N-Decylimidazole	10^{-3}, 10^{-6}, 5×10^{-7}	$3.75 \pm 1.77 \times 10^{-7}$
Bipyridyl	10^{-3}	$1.33 \pm 0.21 \times 10^{-3}$
Edetate sodium	10^{-3}	$1.00 \pm 0.28 \times 10^{-3}$
p-Chloromercuribenzoate	10^{-3}	$2.71 \pm 0.07 \times 10^{-4}$

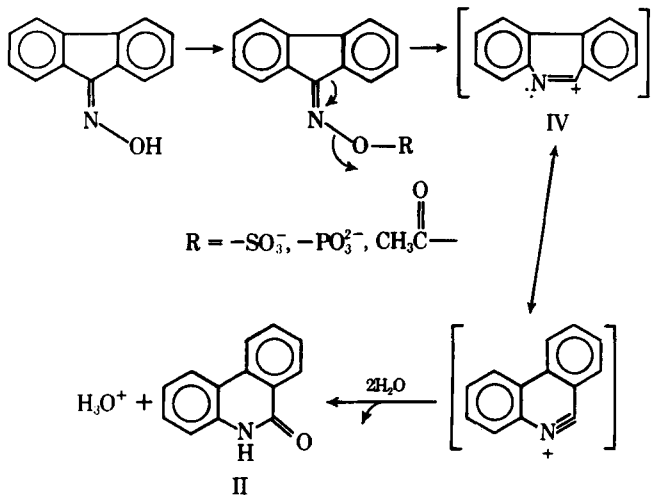
[a] Oxime–amide rearrangement activity assayed as described previously (6) using fluorenone oxime as the substrate. [b] Fluorenone oxime incubated at concentrations of 20, 40, 100, and 500 μ*M*. [c] Each inhibitor showed linear competitive kinetics, and inhibition constants were calculated from Lineweaver–Burke plots (9, 15).

Microsomal oxime–amide rearrangement enzymes also were sensitive to the sulfhydryl binding reagent *p*-chloromercuribenzoate; this reagent also competitively inhibited the rearrangement (Table I), suggesting the possible involvement of a thiol linkage in the transformation. Chelating agents and sulfhydryl binding agents rarely exhibit competitive kinetics with regard to reactions such as the one under investigation. No explanation is offered at this time for this observation.

Microsomal oxime–amide rearrangement activity also was studied as a function of pH. With a substrate concentration of 5×10^{-4} *M* ($13 K_m$), maximum activity was observed at pH 7.1 with diminished activity on either side of this maximum (Fig. 2) over the pH 6.0–8.2 range. All of the experiments in the study were carried out at pH 7.4 to compare oxime–amide rearrangement activity with other microsomal enzyme (mixed-function oxidase) processes acting on fluorenone oxime and to provide an environment for the microsomal suspension that was most similar to that encountered *in vivo*.

It was postulated (5, 6) that the role of the oxime–amide rearrangement enzyme may be to esterify the oxime, producing an intermediate with a high propensity for formation of a transitory nitrenium ion (IV), which undergoes facile rearrangement to yield the amide (II, Scheme II). This mechanism is identical with one that occurs during the Beckman rearrangement (12) of I to II. Four enzymatic esterifying systems were investigated as potential catalysts for the reaction: acetylation, glucuronidation, phosphorylation, and sulfation. Incubation of either microsomal or cytosol fractions with acetyl CoA (an acetylating substrate), uridinediphosphoglucuronic acid (a glucuronidation precursor), or adenosine triphosphate (a phosphorylating reagent) failed to facilitate the conversion of I to II.

The sulfation reaction was studied in greater detail because of its participation in the analogous enzymatic conversion of arylhydroxylamines to electrophilic intermediates (13). However, reaction of I (at levels of 10^{-4} *M*) with cytosol and/or microsomal homogenates to produce



Scheme II—Postulated mechanism for enzymatic transformation of fluorenone oxime to phenanthridinone.

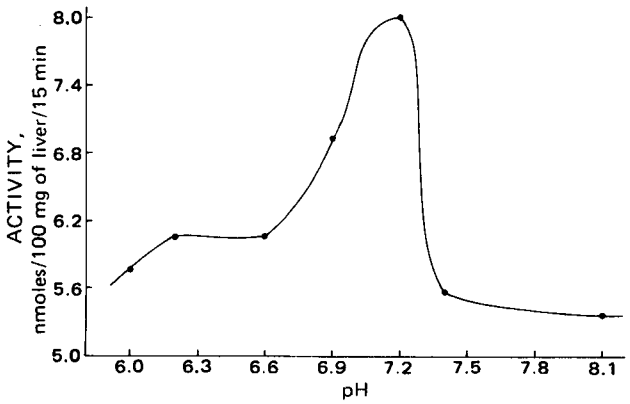


Figure 2—*Hepatic microsomal oxime-amide rearrangement enzyme activity toward fluorenone oxime determined as a function of pH. Each point is the average of six determinations.*

II was not stimulated by the addition of either inorganic sulfate (6.5×10^{-4} *M*) or adenosine triphosphate plus sulfate. Furthermore, the reaction was not inhibited by *p*-nitrophenol (1×10^{-4} *M*), a known consumer of sulfate (14) (and, therefore, a potential competitive inhibitor of the oxime-amide rearrangement system), or 3′,5′-adenosine diphosphate, another known sulfation inhibitor (14). These results suggest that enzymatic conversion of I to II does not involve oxime substrate esterification prior to rearrangement. The reaction mechanism remains a mystery but is being studied.

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Stabilization of 5-Azacytidine by Nucleophilic Addition of Bisulfite Ion

DULAL C. CHATTERJI ˣ **and JOSEPH F. GALLELLI**

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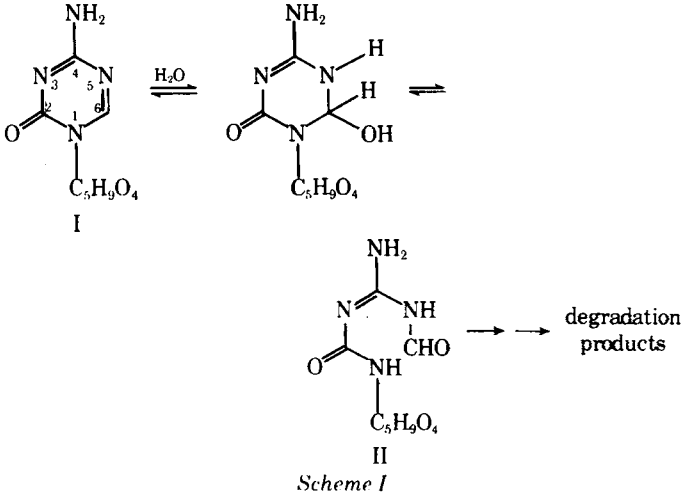
Abstract □ 5-Azacytidine (I) stability was increased approximately 10-fold over its stability in water or lactated Ringer injection by the addition of excess sodium bisulfite and the maintenance of pH ~2.5. The increased stability in the presence of bisulfite at pH 2.5 was attributed to the addition of bisulfite across the 5–6 protonated imine bond of I, which prevented the hydrolytic attack on this labile double bond. However, above pH 4, bisulfite increased I degradation. At higher pH, the compound was no longer protonated and bisulfite did not form the stable addition product. The addition compound quickly decomposed above pH 6 to give back the parent compound and, thus, acted as a I prodrug. The intact drug remaining was assayed by high-pressure liquid chromatography (HPLC), and the reversibility of the bisulfite–I addition product above pH 6 was demonstrated by UV spectrophotometry and HPLC. The potential utility of the bisulfite–I addition product as a I prodrug in parenteral and possible oral dosage forms is discussed.

Keyphrases □ Azacytidine—prodrugs, sodium bisulfite, stabilization in aqueous solutions, effect of pH □ Sodium bisulfite—stabilization of azacytidine in aqueous solutions, effect of pH, prodrugs □ Prodrugs—azacytidine, stabilization by sodium bisulfite □ Antineoplastic agents—azacytidine, prodrugs, sodium bisulfite complex

The use of 5-azacytidine (I) in acute myelogenous leukemia (1–3) is often limited by severe and sometimes dose-limiting nausea and vomiting (4, 5). Although the GI toxicity can be controlled effectively by administering the drug as a slow infusion (6, 7), extreme drug instability (8–10) poses a serious problem. Even when I is infused in lactated Ringer injection, which provides optimum stability conditions for the drug at pH 6.4, ~10% of I is lost in 2 hr at room temperature (8).

BACKGROUND

Systematic kinetic studies on the I hydrolysis (8–10) revealed that hydrolysis proceeds by the classical acid-catalyzed hydration of the 5–6 imine double bond (Scheme I), followed by deamination to yield the formyl derivative, *N*-(formylamidino)-*N*-β-D-ribofuranosylurea (II).



Scheme I

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