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METABOLIC REARRANGEMENT OF FLUORENONE OXIME BY RAT LIVER HOMOGENATES

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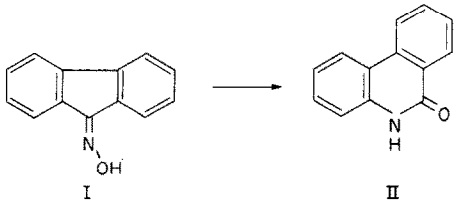
Abstract—Fluorenone oxime is enzymatically converted to phenanthridinone by liver homogenates. The reaction is identical to the chemical Beckman rearrangement, occurring with oximes in the presence of an acid catalyst. The oxime-amide rearrangement (OAR) enzymes are located in the mitochondrial and microsomal fractions of liver. A substance present in the nuclear sediment appears to inhibit the reaction. These enzymes do not require oxygen for activity, but the reactions are greatly facilitated by the presence of an NADPH cofactor. The reaction is not affected by a CO atmosphere, indicating that cytochrome P-450 is not involved in the reaction. Microsomal enzyme activity can be stimulated 3-fold by prior administration of phenobarbital to animals.

Oximes are formed as products of the metabolic oxidation of primary aliphatic amines [1, 2]. These compounds have been considered metabolically inert. However, they are known to undergo facile hydrolysis to ketones [3]. In addition, we have previously shown [4, 5] that, under strictly anaerobic conditions, an oxime reductase is active that catalyzes the conversion of the oxime to the corresponding *N*-hydroxylamine and primary amine. Reduction is mediated in several animal species by the cytochrome P-450 system localized in liver microsomes. More recently, *in vivo* experiments with rats [6] revealed that fluorenone oxime, I, was converted to phenanthridinone (PA), II, which was subsequently excreted in the urine. This metabolic reaction is analogous to the chemical Beckman rearrangement[7]. To help characterize this metabolic conversion, an *in vitro* study of the reaction has been carried out in the rat to determine: (a) if the process is enzymatic and occurs *in vitro*, (b) if the reaction is mediated by hepatic enzymes, (c) the subcellular distribution of enzyme activity, and (d) the cofactor requirement for the enzyme system.



I II

Scheme 1.

MATERIALS AND METHODS

Apparatus. High pressure liquid chromatography (h.p.l.c.) was performed on a component system consisting of a Waters model 6000-A solvent delivery system, a model U-6K septumless injector and a model 440 dual channel absorbance detector operated at 254 nm. Mass spectrometry was done with a Varian CH-5 mass spectrometer, with ionizing voltage maintained at 70 eV. Centrifugation procedures were accomplished with a Beckman model J-21C centrifuge and a Beckman model LS-50 ultracentrifuge. Metabolic incubations were carried out in a Dubnoff metabolic incubator, maintained at 37°.

Reagents. Fluorenone oxime and phenanthridinone were synthesized as previously described [8]. Methanol (ChromAR), chloroform and phenanthridinone were purchased from Mallinckrodt Inc. (St. Louis, MO). Dichloromethane was obtained from Baker Chemical Co. (Phillipsburg, NJ). *N,N*-dimethylformamide was purchased from Fisher Co. (Fairtown, NJ).

Enzyme preparation. Male Sprague–Dawley rats weighing 200–250 g were killed by decapitation. Livers were immediately excised, washed once with ice-cold 1.15% KCl-0.02 M Tris–HCl (pH 7.4) buffer, weighed and homogenized with 5 vol. of cold buffer using a glass-Teflon homogenizer driven at 2000 rev/min by a portable drill. Subcellular fractions were obtained in the following way: the nuclear fraction represents washed (i.e. the sediment resuspended in buffer was homogenized and then recentrifuged) material sedimented at 600 *g* in 30 min in a refrigerated Beckman model J-21C centrifuge; the mitochrondrial fraction is the washed material obtained at 9000 *g* in a similar way; and the microsomes represent washed material sedimented at 105,000 *g* in 1.5 hr in a Beckman model LS-50 ultracentrifuge. All preparations were washed by resuspending sedimented material in buffer equal in volume to the original suspension, followed by recentrifugation. All subcellular fractions were reconstituted to the original volume of the supernatant fraction from which they were obtained.

Protein content was determined by the method of Lowry *et al.* [9]. Bovine serum albumin was used as the protein standard.

Enzymatic assay. Incubation mixtures contained enzyme equivalent to 100 mg liver, an NADPH-

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generating system [NADP (1 μmole), glucose 6-phosphate (20 μmoles), glucose 6-phosphate dehydrogenase (1 unit) and $MgCl_2$ (100 μmoles)], fluorenone oxime substrate (0.5 μmole) and 1.15% KCl–0.02 M Tris–HCl (pH 7.4) buffer adjusted to a final volume of 5 ml. Incubations were carried out in a Dubnoff metabolic incubator in air for 15 min at 37°. To study the influence of atmosphere on enzyme reactivity, mixtures were evacuated and gassed with either air (4:1, N_2–O_2) or a 4:1 CO–O_2 mixture in modified Thunberg tubes. The reaction was initiated by adding substrate from the side arm. Incubations were carried out as described above. For metabolite identification and characterization, the quantities of all the ingredients in the incubation mixtures were increased 15-fold over those previously stated and incubation times were extended to 30 min.

Analytical procedure. The reactions were stopped by adding 10 ml chloroform to the incubation vessels and cooling them in the ice bath. Mixtures were then shaken for 20 min and centrifuged; the organic layers were then separated and evaporated to dryness under nitrogen. Phenanthrene (20.8 nmoles) was added to each residue as an internal standard; this mixture was redissolved in 200 μl of *N,N*-dimethylformamide. These samples were injected, in 10 μl volumes, onto the liquid chromatographic column.

Chromatography. Chromatography was carried out by reverse phase chromatography, using a μBondapak C_{18} column (4 mm o.d. × 30 cm) (Waters Assoc., Inc., Milford, MA) operating at 2.0 ml/min with methanol–dichloromethane–water (60:5:35) as the mobile phase. These conditions generated retention volumes of phenanthridinone, fluorenone oxime and phenanthrene (internal standard) of 7.1, 10.4 and 26.7 ml respectively. For quantitative analysis, a standard curve was prepared from a series of liver homogenate samples to which twelve different concentrations (8.6×10^{-8} to 1.45×10^{-4} M) of phenanthridinone and a constant amount (1.04×10^{-4} M) of phenanthrene (internal standard) had been added. These samples were carried through the analysis scheme and subjected to h.p.l.c. Peak height ratios (phenanthridinone: internal standard) were then plotted vs known phenanthridinone concentrations to generate the standard curve which was used in subsequent analyses of metabolic mixtures (containing unknown levels of II).

RESULTS

Analytical methodology. Fluorenone oxime and its potential metabolite, phenanthridinone (PA), were monitored by high pressure liquid chromatography. As shown in Fig. 1, the two species of interest were separated from one another and other extracted materials, with near-baseline resolution. The high molar absorptivities of the species permitted their spectrophotometric detection (at 254 nm) in the chromatographic eluent to levels of 5×10^{-8} M. For quantitative analysis, the efficiency of chloroform extraction of phenanthridinone was determined by analysis of liver homogenate (600 *g* supernatant fraction) samples spiked with known amounts of analyte. An extraction efficiency of 85 ± 2.5 per cent was found with chloroform. Quantitation of II was based on comparison of peak height ratio (phenanthridinone: internal standard) with a standard curve. The curve prepared by analyzing liver homogenate samples containing varying amounts of analytes in the range 8.6×10^{-8} to 1.45×10^{-4} M was linear as determined by least squares fit of the data to a straight line. The line $y = 0.050x - 0.005$ was generated with a zero order correlation of +0.999.

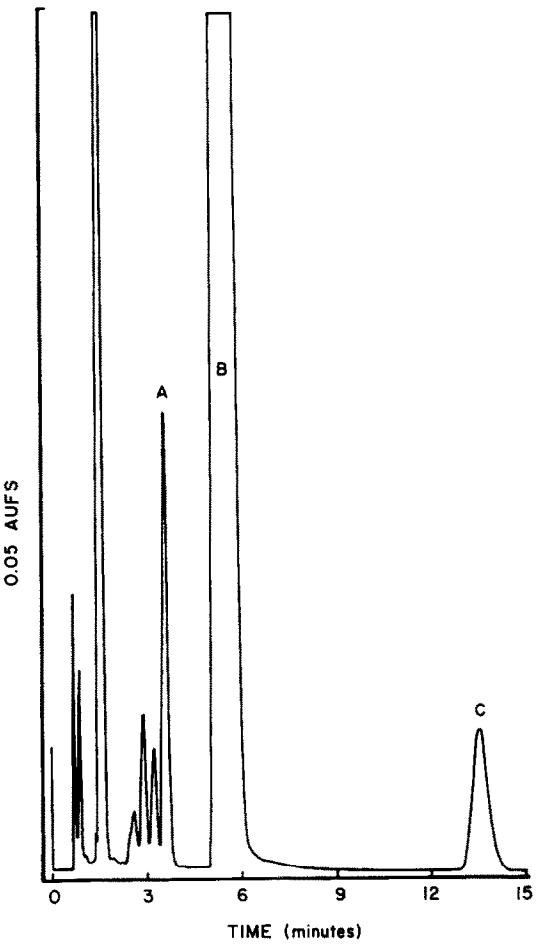


Fig. 1. Chromatograph of a mixture of phenanthridinone (A), fluorenone oxime (B) and phenanthrene (C) (internal standard) separated by reverse-phase chromatography using methanol–dichloromethane–water (60:5:35) as the mobile phase. AUFS = absorbance units full scale.

Enzymatic nature of reaction. The conversion of fluorenone oxime to phenanthridinone was studied in liver homogenates. Table 1 shows that, in buffer alone and in boiled whole liver homogenate, the substrate is not converted to II. In the presence of a viable (non-boiled) hepatic suspension, however, phenanthridinone is slowly formed. The rate of product formation can be significantly enhanced by the addition of an NADPH-generating system to the mixture. Phenanthridinone formation was linearly dependent on incubation time (to 15 min) and enzyme concentration (to levels present in 125 mg liver). The regression lines for time and enzyme concentration dependencies were $y = 4.54x - 2.66$ (correlation coefficient = 0.99) and $y =$

Table 1. Intracellular distribution of oxime-amide rearrangement (OAR) enzymes in rat liver homogenates*

Preparation	Phenanthridinone formed† (nmoles/100 mg liver/15 min)	(nmoles/mg protein/15 min)
Buffer	0.00	0.00
Buffer, NADPH‡	0.00	0.00
Buffer, whole homogenate	<1.00	<0.04
Buffer, whole homogenate, NADPH	3.81 ± 0.97	0.17 ± 0.04
Buffer, boiled whole homogenate, NADPH§	0.00	0.00
Nuclear fraction‖	1.10 ± 0.24	0.10 ± 0.01
Mitochondrial fraction‖	1.71 ± 0.67	0.86 ± 0.34
Microsomal fraction‖	2.35 ± 0.13	1.48 ± 0.14
600 *g* Supernatant fraction‖	6.79 ± 1.27	0.49 ± 0.11
9000 *g* Supernatant fraction‖	3.32 ± 0.92	0.29 ± 0.07
105,000 *G* Supernatant fraction‖	1.00 ± 0.03	0.13 ± 0.01

* All incubations contained protein equivalent to 100 mg liver.

† Results are the mean ±S. D. of triplicate determinations of five experiments.

‡ NADPH-generating system consisting of NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and Mg^{2+}.

§ Homogenate was boiled for 5 min and rehomogenized before use.

‖ Contains buffer and NADPH.

0.53x − 2.68 (correlation coefficient = 0.99) respectively. These results demonstrate that the conversion of I to II occurs in liver homogenates and that the reaction is enzyme controlled. The enzymes responsible for the conversion will be designated as oxime-amide rearrangement (OAR) enzymes.

Intracellular localization of responsible enzyme. Table 1 indicates that the enzyme system responsible for the transformation of I to II is located primarily in the microsomal fraction of liver homogenates. The apparent OAR activity in the 9000 *g* sediment may reflect enzymic activity in mitochrondria or may arise from contamination of mitochrondria by microsomes resulting from co-sedimentation of these fractions. OAR activity was reduced 30 per cent by repeated washings (four) of the 9000 *g* sediment with buffer (similar treatment of the microsomal pellet had no effect on OAR activity) and by a similar amount when mitochondria were prepared in sucrose, suggesting that microsomal contamination contributes to the activity in the 9000 *g* sediment. The residual activity in the sediment, however, suggests that OAR enzymes may be present in mitochrondria, although no definitive statement can be made with regard to the activity. Comparing the relative enzymic activity in the whole homogenate and the 600 *g* supernatant fraction, phenanthridinone is shown to be formed twice as fast in the absence of nuclear sediment (removed by centrifugation) as in the crude homogenate. To determine if a substance is present in nuclear fraction that inhibits oxime rearrangement, the 600 *g* supernatant fraction was recombined with the nuclear fraction (Table 2). The resulting suspension showed OAR activity similar to whole homogenate, indicating that the apparent enhanced enzymatic activity of the 600 *g* supernatant fraction (compared with crude homogenate) may be due to the presence of an inhibitor in the nuclear fraction. Furthermore,

Table 2. Effect of nuclear fraction on the rate of oxime rearrangement*

Preparation	Phenanthridinone formed (nmoles/100 mg liver/15 min)
Whole homogenate	5.05 ± 0.21
Nuclear fraction	1.38 ± 0.36
600 *g* Supernatant fraction	6.25 ± 0.11
600 *g* Supernatant + nuclear fraction	4.17 ± 0.59
600 *g* Supernatant + boiled nuclear fraction†	4.95 ± 0.43

* Incubations were carried out as described under Materials and Methods.

† Nuclear fraction was boiled for 5 min and rehomogenized in buffer before use.

boiling the nuclear material prior to recombination of fractions yielded a suspension with the same activity as the crude homogenate, suggesting that the inhibitor is heat stable. The pH of the 600 *g* supernatant fraction was identical to the pH of the crude homogenate so that the observed difference in activity of the whole homogenate and the 600 *g* supernatant fraction does not reflect a variation in pH environment of the two fractions and pH dependence of the enzyme systems. Recovery of II from the 600 *g* supernatant fraction and from the crude homogenate was 85 per cent. Thus, the difference in activity does not reflect variations in efficiency of extraction of metabolite from the two suspensions. Furthermore, inhibition of OAR activity by the nuclear fraction does not appear to arise from an effect on NADPH generation. Similar OAR behavior was observed when either NADPH or an NADPH generating system was used as a cofactor. Thus, the difference in activity seems to be due either to the presence of an inhibitor substance localized in the nuclear frac-

Table 3. Influence of atmosphere on OAR activity

Atmosphere	Phenanthridinone formed* (nmoles/100 mg liver/15 min)
Air	4.06 ± 0.16
N_2/O_2 (4:1)	3.94 ± 0.26
CO/O_2 (4:1)	3.82 ± 0.48
N_2	4.20 ± 0.62

* Incubations were carried out with 600 *g* supernatant fraction equivalent to 100 mg liver.

tion or arises from removal of available substrate by binding to nuclei. The latter possibility could not be evaluated with the available methodology.

Effect of CO atmosphere on phenanthridinone formation. Carbon monoxide inhibits NADPH-dependent mixed function oxidases by competitively inhibiting complex formation of O_2 cytochrome P-450 [10]. Since the reaction under investigation was not affected by the presence of a CO atmosphere (Table 3), the involvement of cytochrome P-450 in the process is doubtful. This is not unexpected since the metabolic reaction does not involve participation by molecular oxygen, i.e. reaction proceeds as rapidly in a nitrogen atmosphere as it does in air.

Cofactor requirements. As previously noted, II is formed very slowly from I, in the absence of cofactor. As shown in Table 4, NADP and NAD significantly facilitate the conversion in the 600 *g* supernatant fraction, but the reaction proceeds most efficiently in the presence of NADPH. In isolated mitochrondria and microsomes, however, NADPH is the only effective cofactor, i.e. NADP and NAD do not efficiently promote the rearrangement. NADH is inactive as a cofactor for this reaction.

Since chemically analogous Beckman rearrangements are catalyzed by acid or initial esterification of the oximino hydroxyl group [7], the effects of a number of endogenous esterifying cofactors on the enzyme-catalyzed conversion of I to II were studied. However, compounds capable of phosphorylation (adenosine triphosphate, ATP), sulfate ester formation (3'-phosphoadenosine-5'-phosphosulfate, PAPS), carboxylic ester formation (acetyl CoA) or acetal formation (uridine diphosphoglucuronic acid, UDPGA) did not facilitate the reaction occurring in the 600 *g* supernatant fraction of liver homogenates. These results suggest that the role of the enzyme is not esterification of the oximino hydroxyl group.

Enzyme induction and inhibition studies. The ability of phenobarbital and SKF-525A to affect the rate of conversion of I to II was also investigated. Table 5 indicates that daily administration of phenobarbital (80 ml/kg) to rats for 3 days prior to their being killed increased the enzymic activity for this rearrangement by 40 per cent in the 600 *g* supernatant fraction and increased the microsomal OAR activity 3-fold. Mitochondrial OAR activity was only increased by a factor of 1.4 by phenobarbital pretreatment. SKF-525A had no inhibitory effect on OAR activity, when added *in vitro* to incubation mixtures (10 μM/incubation) or when administered intraperitoneally (85 mg/kg) to rats for 3 days prior to their death. In fact, animals pretreated with SKF-525A showed modest enhancement of microsomal OAR activity.

Metabolite identification. The substrate was incubated with liver homogenates (600 *g* supernatant

Table 4. Cofactor requirements for OAR activity

Fraction	Cofactor	Phenanthridinone formed* (nmoles/100 mg liver/15 min)
Buffer	NADPH†	0.00
	NADH†	0.00
	NADP†	0.00
	NAD†	0.00
600 *g* Supernatant fraction	None	0.85 ± 0.16
	NADPH†	6.15 ± 0.14
	NADH†	0.86 ± 0.12
	NADP†	2.38 ± 0.11
	NAD†	2.38 ± 0.16
	ATP‡	6.27 ± 0.44
	PAPS‡	4.97 ± 0.26
	UDPGA‡	5.82 ± 0.33
	Acetyl CoA‡	6.77 ± 0.04
Mitochondria	None	0.32 ± 0.02
	NADPH†	2.98 ± 0.09
	NADP†	0.18 ± 0.02
	NAD†	0.10 ± 0.00
Microsomes	None	0.04 ± 0.07
	NADPH†	2.28 ± 0.15
	NADP†	0.19 ± 0.09
	NAD†	0.00

* Each value represents the mean ±S. D. of triplicate determinatioons.
† Present at concentrations of 1 μmole/incubation.
‡ Incubation contained NADPH and secondary cofactors at concentrations of 1.5 μmoles/incubation.

Table 5. Induction and inhibition of OAR activity*

Liver fraction	Modifier	Phenanthridinone formed (nmoles/100 mg liver/15 min)
600 *g* Supernatant fraction	Control†	6.04 ± 0.47
	Phenobarbital‡	9.58 ± 0.09
	SKF-525A§	5.97 ± 1.32
	SKF-525A‖	6.27 ± 0.66
Mitochondria	Control†	2.79 ± 0.06
	Phenobarbital‡	4.00 ± 0.49
	SKF-525A§	2.88 ± 0.25
	SKF-525A‖	2.34 ± 0.16
Microsomes	Control†	2.02 ± 0.19
	Phenobarbital‡	5.49 ± 0.49
	SKF-525A§	3.88 ± 0.64
	SKF-525A‖	1.99 ± 0.49

* Experiments were run with a minimum of three animals and all incubations were run in triplicate.

† Control animals received 0.5 ml of isotonic NaCl, i.p., for 3 days prior to killing.

‡ Animals received Na phenobarbital (80 mg/kg) dissolved in isotonic NaCl solution, i.p., daily for 3 days prior to killing (24 hr after last dose, animals were killed).

§ Animals received SKF-525A (85 mg/kg) dissolved in isotonic NaCl solution, i.p., daily for 3 days prior to killing (24 hr after last dose, animals were killed).

‖ SKF-525A was added directly to the incubation mixture to give a final concentration of 10 μM/incubation.

fraction) and carried through the previously described analysis scheme. The peak corresponding to the metabolite, eluting at 7.1 ml, was collected and submitted for mass spectral analysis. The electron impact (70 eV) mass spectrum showed a base peak at m/e 195 corresponding to the molecular ion and a fragmentation pattern identical to authentic II, which had been synthesized from I. The spectrum of the metabolite is tabulated in Table 6, and is considerably different than the spectrum for the corresponding oxime isomer (I).

In vivo *studies.* A series of rats were given fluorenone oxime (600 mg/kg) intraperitoneally and urine was collected over sodium fluoride for 48 hr. The urine samples were evaporated to dryness and the residue extracted with chloroform–methanol (9:1). The extracts were evaporated to dryness and analyzed by h.p.l.c. A peak was observed with $V_R = 7.1$ ml; the peak was collected and submitted to mass spectral analysis. The spectrum was identical to that shown in Table 6, indicating that oxime rearrangement occurs, *in vivo* as well as in liver homogenates, to form the amide, II.

Table 6. Mass spectrum of fluorenone oxime metabolite*

Peak (m/e)	% Relative abundance of base peak
195	100
194	14
168	3
167	15
166	13
140	6
139	10
63	3

* Sample was obtained from incubation of fluorenone oxime with 600 *g* supernatant fraction (NADPH). Incubation mixture was analyzed as described under Materials and Methods. The h.p.l.c. solvent eluting 7.1 min after injection was collected and submitted for mass spectral analysis.

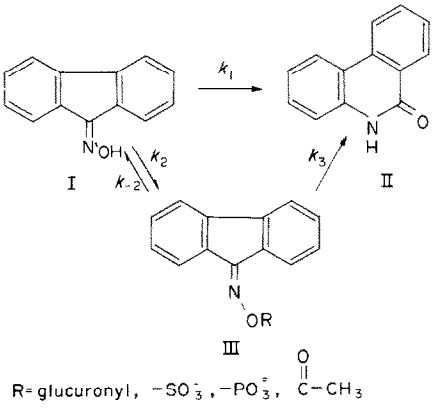
DISCUSSION

These experiments have shown that fluorenone oxime undergoes enzymic rearrangement to form phenanthridinone. The primary enzyme system responsible for the conversion, located in liver microsomes, does not require O_2 and was not affected by a CO atmosphere, suggesting that it is cytochrome P-450-independent. Biotransformation was accelerated by NADPH. Although NADP and NAD showed modest effectiveness as a cofactor with crude homogenates, they were unable to facilitate oxime rearrangement with isolated mitochondrial or microsomal preparations. NADP may be consumed in the crude preparations and converted to NADPH which then serves as cofactor for isomerization. Similar cofactor reduction may be less feasible in "purified" subcellular fractions.

The activity in the 600 *g* supernatant fraction was significantly greater than that observed in whole liver homogenate. This difference is apparently due to the presence of an inhibitor of the rearrangement reaction in nuclear sediment or may arise from binding of substrate to the nuclei.

The role of the enzyme in catalyzing the rearrangement has not yet been elucidated The chemically analogous Beckman rearrangement is carried out in the presence of an acid catalyst or esterifying reagent (acetic anhydride, PCl_5, $POCl_3$) [7]. These compounds facilitate the loss of the oximino hydroxyl group, producing a transient imonium cation, which undergoes carbon migration and upon hydration yields an amide. It was postulated, therefore, that the enzyme was responsible for esterifying the oxime, thus promoting rearrangement. Enzymatic esterification of *N*-hydroxy

compounds has been well documented [11]. Our studies with common esterifying cofactors, however, have failed to demonstrate their role in the rearrangement, suggesting that OAR enzymes do not simply act by esterification of the oxime, facilitating cleavage of the N–O bond, prompting concomitant rearrangement. These results remain inconclusive with respect to the role of an esterifying cofactor in the reaction under study, since liver homogenates contain hydrolases (some are normally present, others are released from cell bodies destroyed during homogenation), which could hydrolyse any esterified oxime as it forms before it can proceed to form product, II.



Scheme 2.

If $k_{-2} \geq k_3$ or $k_2 \leq k_{-2}$, breakdown of any ester, III, that forms may not lead to formation of the rearrangement product, II, but rather to regeneration of oxime, I. Alternatively, the enzyme may serve as a general or specific acid catalyst. The chemical role of the enzyme in facilitating oxime isomerization is receiving additional attention. Beckman rearrangements carried out in chemical systems are generally concerted reactions [12]; however, regardless of whether carbon atom migration occurs in one step or in two discrete stages, a transient cationic species is generated during the process. The ability of this species to react with nucleic acids or other tissue nucleophiles has yet to be determined. Such results may provide information regarding the potential toxicity and carcinogenicity of oximes and their metabolic precursors, primary aliphatic amines. It is interesting to note in this regard that hydroxyl loss from structurally analogous aryl hydroxylamines and aryl hydroxamic acids produces intermediates that apparently are responsible for the pathogenic effects induced by such compounds [13].

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