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N-ACETOXY-*N*-ACETYLAMINOARENES AND NITROSOARENES

ONE-ELECTRON NON-ENZYMATIC AND ENZYMATIC OXIDATION PRODUCTS OF VARIOUS CARCINOGENIC AROMATIC ACETHYDROXAMIC ACIDS

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SUMMARY

A number of carcinogenic aromatic acethydroxamic acids (*e.g.* *N*-hydroxy-*N*-acetyl derivatives of 2-aminofluorene, 3-aminofluorene, 4-aminostilbene, 1-aminonaphthalene, 2-aminonaphthalene, 2-aminophenanthrene, and 4-aminobiphenyl) are readily oxidized by alkaline $Fe(CN)_6^{3-}$ or Ag_2O. The free nitroxide radicals thus formed dismutate in organic solution according to second order kinetics to yield the corresponding *N*-acetoxy-*N*-acetylaminoarenes and nitrosoarenes. The structures of the latter products were established by mass and infrared spectrum analyses. Evidence was obtained for a similar one-electron oxidation of these acethydroxamic acids with horseradish peroxidase and H_2O_2 at pH 7. One-electron oxidation of *N*-hydroxy-2-acetylaminofluorene was also demonstrated with lactoperoxidase and human myeloperoxidase. The possible relevance of a similar peroxidative attack *in vivo* to the carcinogenic activities of some aromatic amines and amides is discussed.

INTRODUCTION

Most, if not all, chemical carcinogens appear to have strong electrophillic derivatives as their ultimate carcinogenic and reactive forms, and the reactions of these strong electrophiles with one or more nucleophilic targets in the cells are presumed to be key steps in the carcinogenic processes they induce[1]. In the case of 2-acetylaminofluorene (2-AAF) its hepatocarcinogenic activity in the male rat appears to depend primarily on its *N*-hydroxylation and on the subsequent sulfuric acid esterification of the *N*-hydroxy derivative[1-4]. However, the sulfotransferase activities for *N*-hydroxy-2-AAF in the other rat tissues studied appear to be very low as com-

Abbreviations: 2-AAF, 2-acetylaminofluorene; 3-AAF, 3-acetyl-aminofluorene; K_D, disintegration constant for the free nitroxide radicals ($A_{max}^{-1} \cdot min^{-1}$); Ar, aryl.

pared to that for rat liver, and it is possible that other electrophilic derivatives may be more important than the sulfuric acid ester in carcinogenesis in extrahepatic tissues[2,5]. In the latter connection it is noteworthy that a number of aromatic amides which have strong carcinogenic activity in some extrahepatic tissues and whose *N*-hydroxy derivatives are strong carcinogens at the site of subcutaneous injection, have little or no carcinogenic activity for rat liver[6–11]. These hydroxamic acids were poorer substrates for sulfuric acid esterification by liver sulfotransferases than *N*-hydroxy-2-AAF[2].

The possible roles of other esters of *N*-hydroxy-2-AAF and of the glucuronides of *N*-hydroxy-2-AAF and of *N*-hydroxy-2-aminofluorene in carcinogenesis by the fluorene compounds have received consideration[2,12–18]. Another activation mechanism of potential importance is the one-electron oxidation of the hydroxamic acids as studied with *N*-hydroxy-2-AAF by Bartsch and co-workers[19,20] and with *N*-hydroxy-4-acetylaminobiphenyl by Forrester *et al.*[21]. In extending the latter studies we have found a similar rank order for the velocities of the dismutation of the free nitroxide radicals derived from the one-electron oxidation of a number of carcinogenic hydroxamic acids and their carcinogenic activities in the subcutaneous tissue of the rat. The data on these hydroxamic acids are reported herein.

MATERIALS AND METHODS

Spectroscopic methods

Mass spectra were determined with a Varian CH-7 mass spectrometer; perfluorokerosene (Penninsular ChemResearch, Gainesville, Fla.) was used as an internal standard for calibration of the mass units. Infrared spectra were recorded with a Beckman IR-10 spectrophotometer. A Beckman DB spectrophotometer, equipped with a Sargent SR recorder, was used for determination of ultraviolet and visible spectra; absorbance was determined with a Zeiss PMQ-IV spectrophotometer.

Chromatography

Plates for preparative and analytical thin-layer chromatography were prepared from silica gel PF_{254} and HF_{254} (Merck), respectively. The chromatograms were developed in an equilibrated atmosphere with dichloromethane, (System A) dichloromethane–acetone (85:5, v/v), or (System B) dichloromethane–cyclohexane (1:1, v/v). The products on the chromatograms were located by visualization under ultraviolet light (254 nm) and/or by spraying with vanillin-H_2SO_4 reagent[22] followed by heating of the plates at 110 °C for about 15 min.

Chemicals

N-Hydroxy-2-AAF[23,24] (m.p. 151 °C), *N*-acetoxy-2-AAF[25] (m.p. 110 °C), *N*-hydroxy-4-acetylaminobiphenyl[10] (m.p. 150 °C), *N*-acetoxy-4-acetylaminobiphenyl[26] (m.p. 118 °C), *N*-hydroxy-4-acetylaminostilbene[27] (m.p. 200 °C), *N*-acetoxy-4-acetylaminostilbene[27] (m.p. 107–108 °C), *N*-hydroxy-2-acetylaminophenanthrene[11] (m.p. 197 °C), *N*-acetoxy-2-acetylaminophenanthrene[11] (m.p. 119 °C), *N*-hydroxy-1-acetylaminonaphtalene[26] (m.p. 128 °C), *N*-acetoxy-1-acetylaminonaphthalene[26] (52–54 °C), *N*-hydroxy-2-1-acetylaminonaphthalene[28] (m.p. 85 °C), and *N*-acetoxy-2-1-acetylaminonaphthalene[26] (49–50 °C) were prepared in this laboratory by published methods.

N-Hydroxy-3-AAF (m.p. 135 °C) was prepared from 3-nitro-9-fluorenone (Aldrich Chemical Co., Milwaukee, Wisc.) by the procedure of Yost and Gutmann[29], except that *m*-chloroperbenzoic acid (Aldrich Chemical Co.) was used for the oxidation of 3-aminofluorene to 3-nitrofluorene (m.p. 106 °C)[30]. *N*-Acetoxy-3-AAF was prepared from *N*-hydroxy-3-AAF by the procedure used for the 2-isomer[25]; the product was recrystallized twice from acetone–water. M.p. 105 °C (lit.[31] 104–105 °C); yield, 77%. Infrared spectrum (KBr): $\lambda_{N-O-C=O}$, 1790 cm^{-1}; $\lambda_{N-C=O}$, 1695 cm^{-1}. 3-Methylmercapto-2-AAF was generously supplied by Dr T. Lloyd Fletcher, University of Washington Medical School, Seattle, Wash.

The following chemicals were purchased from the sources indicated: Ag_2O (D. F. Goldsmith Chemical and Metal Corp., Evanston, Ill.) K_3 ($Fe(CN)_6$) (Allied Chemicals, Morristown, N. J.), H_2O_2 (30%, Merck and Co., Rahway, N.J.), *N*-acetyl-DL-methionine (California Biochemicals, Los Angeles, Calif.), methyl-3-(methylthio)-propionate (Aldrich Chemical Co.), horseradish peroxidase (EC 1.11.1.7; Type II, approx. 135 purpurogallin units per mg solids, Sigma Chemical Co., St. Louis, Mo.), lactoperoxidase (EC 1.11.1.7; approx. 40 purpurogallin units per mg solids, Sigma Chemical Co.) human myeloperoxidase (EC 1.11.1.7; RZ = 0.80, generously provided by Dr J. Schultz, Papanicolaou Cancer Research Institute, Miami, Fla.). L-[*Me*-^{3}H]-methionine (100 mCi/mmole, Amersham-Searle Corp., Arlington Heights, Ill.).

Procedures for one-electron oxidations of N-arylhydroxamic acids

Method A. The *N*-hydroxy-*N*-acetylaminoarene (0.15 mmole dissolved in 15 or 30 ml of benzene) was mixed on a vortex stirrer with 1.5 ml of oxidant (10 ml of 0.5 M $K_3(Fe(CN)_6)$ and 1 ml of 1 M NaOH for a predetermined time. The organic phase was immediately separated with Whatman siliconized paper, washed with a small volume of water, dried over anhydrous $MgSO_4$, and used for measurements. For the experiments in which the reaction products were isolated, the $MgSO_4$ was removed after 1 h and the filtrate was left under N_2 at room temperature for 24 h before the solvent was removed under reduced pressure.

Method B. The *N*-hydroxy-*N*-acetylaminoarene (0.1 mmole dissolved in 20 ml of benzene) and 0.1 mmole of Ag_2O (ground in a mortar) were shaken for 5 h under N_2, the solution was filtered, and the solvent was evaporated under reduced pressure.

For the preparative isolation of the reaction products from either Method A or Method B the residue, dissolved in dichloromethane, was applied to silica gel PF_{254} plates which were then developed repeatedly with dichloromethane until the nitroso compound had moved one-half to two-thirds the distance from the spotting line to the solvent front. The yellow-green zone of nitroso compound was then scraped off, and the plate was redeveloped in System A. The zone of *N*-acetoxy-*N*-acylaminoarene (R_F about 0.3–0.4), visualized under ultraviolet light and identified by comparison with the authentic reference compound, was then scraped from the plate. Each of these products was eluted from the silica with dichloromethane, the solution was passed through a fritted glass filter, the solvent was removed, and the product was assayed gravimetrically. Under these conditions the compounds generally crystallized spontaneously, and all of the peaks in the M^+ to M^+-60 region of the mass spectrum analyses for each compound were those expected for the compound under study.

Determination of the disintegration constants of nitroxide radicals formed on one-electron oxidation of N-arylhydroxamic acids

The disintegration of the nitroxide radicals was measured by the decrease in absorbance at the λ_{max} as a function of time, generally at 1-min intervals over a 30-min period, on the assumption that the amount of light absorbed at the λ_{max} was a linear function of the radical concentration. The velocity constants for the disintegrations (K_D) were determined from the slopes of the straight lines obtained on plotting A_{max}^{-1} *vs* time[19].

Oxidation of N-arylhydroxamic acids with horseradish peroxidase and H_2O_2 as evidenced by reaction of the products with methionine

The reaction of esters of *N*-arylacethydroxamic acids with [*Me*-^{3}H]methionine to yield sulfonium derivatives which decompose to benzene-soluble [*Me*-^{3}H]methylmercaptoarylamides has served as a convenient means of detecting and assaying these compounds[2,26]. This reaction was used as an indicator of the formation of *N*-acetoxy-*N*-acetylaminoarenes during the horseradish peroxidase–H_2O_2-mediated oxidation of *N*-arylacethydroxamic acids. The following ingredients, added in the order in which they are listed, were incubated in a total volume of 1 ml in stoppered vials for 20 h at 37 °C under N_2: 0.8 ml 0.067 M Sorensen buffer (pH 7.0); 0.08 mg horseradish peroxidase; 0.5 μmole of hydroxamic acid dissolved in 0.1 ml of 2-methoxyethanol; 48.6 mμmoles of L-[*Me*-^{3}H]methionine (spec. act., 17.7 mCi/mmole); and 1.29 μmoles of H_2O_2. After the incubation the assay mixture was heated at 90 °C for 45 min; this ensures the decomposition of the sulfonium derivatives and, with the more stable esters, it also facilitates the reaction with methionine[2,26]. The mixture was then cooled in ice, brought to pH 9–10 by addition of 11 M KOH, and extracted with 7 ml of 30% benzene in *n*-hexane. The organic phases were washed 3 times with 1-ml aliquots of water and dried over anhydrous $MgSO_4$. Aliquots were evaporated to dryness and dissolved in a toluene-based scintillation fluid (Liquifluor, Pilot Chemicals, Watertown, Mass.) for determination of radioactivity in a Packard Tri-Carb scintillation counter. In control experiments identical incubations were carried out, except that 0.25 μmole of the *N*-acetoxy-*N*-acetylaminoarenes were substituted for the *N*-arylhydroxamic acids and the horseradish peroxidase and H_2O_2 were omitted.

In other experiments 4.2 μmoles of *N*-hydroxy-2-AAF and 167 μmoles of *N*-acetyl-DL-methionine or methyl-3-(methylthio)propionate were incubated with 10 ml of the incubation mixture listed above for 15 min at 37 °C. After extraction and decomposition of the sulfonium derivative as described above, an aliquot of the organic phase was evaporated to dryness and dissolved in CS_2 for gas–liquid chromatography under the conditions used previously for determination of *o*-methylmercapto-AAF, except that a hydrogen flame detector replaced the ^{90}Sr detector[2]. 3-Methylmercapto-2-AAF was used for standardization of the gas chromatograph. However, the data are recorded as yields of *o*-methylmercapto-2-AAF, since both 1- and 3-methylmercapto-2-AAF are formed on reaction of esters of *N*-hydroxy-2-AAF with methionine[2] and since these isomers co-chromatograph under the conditions of our gas chromatography.

Comparative oxidation of N-hydroxy-2-AAF by various peroxidases

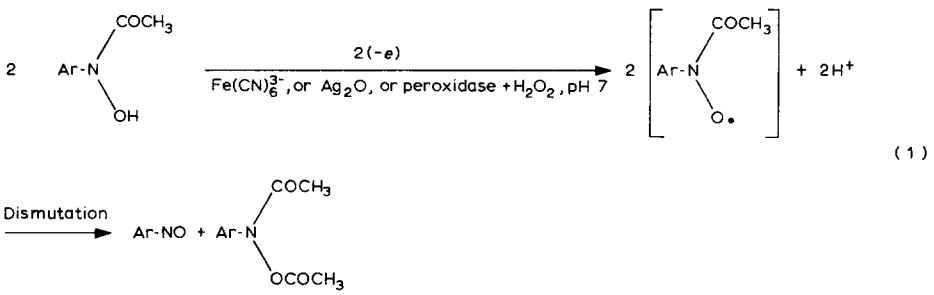
The following ingredients were incubated in a total volume of 1 ml for 60 min

at 37 °C under N_2: 0.7 ml 0.067 M Sorensen buffer (pH 6.8); 25 μmoles *N*-acetyl-DL-methionine, 0.45 mg lactoperoxidase, 0.42 mg myeloperoxidase; or 0.49 mg horseradish peroxidase; 1 μmole *N*-hydroxy-2-AAF dissolved in 0.1 ml of 2-methoxyethanol; and 1.29 μmoles of H_2O_2. Each of the peroxidases was also incubated with all of the above ingredients except H_2O_2. After extraction and decomposition of the sulfonium derivatives, an aliquot of the organic phase was analyzed for *o*-methylmercapto-AAF as described above.

RESULTS

Formation of free nitroxide radicals by one-electron oxidants

Previous studies, which included ESR measurements, indicated that oxidation of *N*-hydroxy-2-AAF with alkaline $Fe(CN)_6^{3-}$ yielded nitroxide free radicals which disintegrated via a bimolecular reaction to yield *N*-acetoxy-2-AAF and 2-nitrosofluorene[19]. The data reported here indicate that this series of reactions is characteristic of a number of *N*-arylhydroxamic acids:



Thus, as in the case of *N*-hydroxy-2-AAF, treatment of a number of *N*-arylhydroxamic acids with alkaline $Fe(CN)_6^{3-}$ or Ag_2O resulted in the formation of deeply colored solutions with λ_{max} of 490–670 nm; the intensities of absorption at these wavelengths gradually decreased ater removal of the oxidant. The disintegrations were generally bimolecular, since, except for *N*-hydroxy-4-acetylaminostilbene and *N*-hydroxy-3-AAF, straight lines were obtained when the reciprocal of the absorbance at the λ_{max} (A_{max}^{-1}) was plotted as a function of time after removal of the alkaline $Fe(CN)_6^{3-}$ (Fig. 1). The velocity constants for the second-order disintegrations ($A_{max}^{-1} \cdot min^{-1}$) for the nitroxide radicals are thus represented by the slopes of the lines. The reasons for the deviations of the disintegrations of the radicals from *N*-hydroxy-4-acetylaminostilbene and *N*-hydroxy-3-AAF from an uncomplicated bimolecular mechanism are not clear. However, the one-electron oxidations of N-hydroxy-4-acetylaminostilbene and *N*-hydroxy-3-AAF also differed from the oxidations of the other hydroxamic acids in that appreciable amounts of products other than the nitrosoarenes and *N*-acetylaminoarenes were found at the end of the decomposition period.

Products formed by one-electron oxidation of N-arylacethydroxamic acids.

The major products isolated from the one-electron oxidations of the *N*-hydroxy-*N*-acetylaminoarenes with silver oxide or alkaline $Fe(CN)_6^{3-}$ were the corresponding *N*-acetoxy-*N*-acetylaminoarenes and nitrosoarenes (Table I). The identities of these

TABLE I

YIELDS AND CHROMATOGRAPHIC PROPERTIES OF MAJOR REACTION PRODUCTS FORMED BY ONE-ELECTRON OXIDATION OF *N*-HYDROXY-*N*-ACETYLAMINOARENES BY ALKALINE $Fe(CN)_6^{3-}$ OR Ag_2O

The reaction conditions and isolation procedures are given in the Materials and Methods and in the legend to Fig. 1. Yields are calculated as percent of theory according to Eq 1.

Hydroxamic acid	*N-Acetoxy-N-acetylaminoarene*				*Nitrosoarene*			
	Yield (%) with		*R_F in System A**	*Color (vanillin–H_2SO_4 reagent)*	*Yield (%) with*		*R_F in System B*	*Color (vanillin–H_2SO_4 reagent)*
	$Fe(CN)_6^{3-}$	*Ag_2O*			*$Fe(CN)_6^{3-}$*	*Ag_2O*		
N-Hydroxy-3-AAF**	14	14	0.40	Light brown	10	11	0.37	Yellow-green
N-Hydroxy-AAS**	4	9	0.44	Red-brown	19	19	0.37	Yellow-brown
N-Hydroxy-1-AAN	Not detectable	30	0.38	Pale violet	31	17	0.38	Light-brown
N-Hydroxy-AAP	26	42	0.42	Green-brown	61	43	0.41	Brown
N-Hydroxy-2-AAF	46	61	0.37	Green-brown	82	44***	0.37	Yellow-green
N-Hydroxy-AABP	20	67	0.44	Brown	62	59	0.40	Red
N-Hydroxy-2-AAN	15	57	0.39	Blue-yellow	40	48	0.42	Yellow-brown

Abbreviations: AAF, acetylaminofluorene; AAS, 4-acetylaminostilbene; AAN, acetylamino naphthalene; AAP, 2-acetylaminophenanthrene; AABP, 4-acetylaminobiphenyl.

* The R_F values coincide with those of the reference compounds.

** These hydroxamic acids gave rise to other major by-products which were not identified.

*** 0.1 mmole *N*-hydroxy-2-AAF was shaken with 1 mmole Ag_2O for 10 min.

TABLE II

CHARACTERIZATION BY MASS AND INFRARED SPECTRAL ANALYSES OF THE *N*-ACETOXY-*N*-ACETYLAMINOARENES AND NITROSOARENES FORMED FROM THE ONE-ELECTRON OXIDATIONS OF *N*-HYDROXY-*N*-ACETYLAMINOARENES

The probable fragmentation pattern was based on the mass spectrum of *N*-acetoxy-2-AAF where the fragmentation pattern was established by metastable ions[19].

Aryl group	*N-Acetoxy-N-acetylaminoarene*				*Nitrosoarene*	
	Carbonyl absorption (cm^{-1})		*Mass spectrum*		*Mass spectrum*	
	N-Acetoxy	*N-Acetyl*	*m/e*	*Probable fragment*	*m/e*	*Probable fragment*
3-Fluorenyl	1790	1695	281 M^+		195 M^+	
			239 M^+-42	(ketene)	165 M^+-30	(nitroso)
			197 $M^+-42-42$	(2 ketenes)		
4-Stilbenyl	1790	1690	295 M^+		209 M^+	
			252 M^+-42-1	(ketene)	179 M^+-30	(nitroso)
			235 M^+-59-1	(acetoxy)		
1-Naphthyl	1790	1690	243 M^+		157 M^+	
			201 M^+-42	(ketene)	127 M^+-30	(nitroso)
			183 M^+-59-1	(acetoxy)		
2-Phenanthryl	1790	1690	293 M^+		207 M^+	
			251 M^+-42	(ketene)	177 M^+-30	(nitroso)
			233 M^+-59-1	(acetoxy)		
4-Biphenylyl	1790	1680	269 M^+		183 M^+	
			227 M^+-42	(ketene)	153 M^+-30	(nitroso)
			210 M^+-59	(acetoxy)		
2-Naphthyl	1800	1690	243 M^+		157 M^+	
			201 M^+-42	(ketene)	127 M^+-30	(nitroso)
			185 M^+-59+1	(acetoxy)		

oxidation products were ascertained by infrared and mass spectrum analyses (Table II). The infrared spectra of the *N*-acetoxy-*N*-acetylaminoarenes exhibited carbonyl absorption bands at 1790–1800 cm^{-1} and 1680–1690 cm^{-1}. The mass spectra of the *N*-acetoxy-*N*-acetylaminoarenes were identical to those of the authentic compounds

and were characterized by the presence of the parent ion (M^+) and major fragment ions which arose through the loss of ketene (M^+-42) and/or of acetoxy groups (M^+-59). The structures of the nitrosoarenes were deduced primarily from their mass spectra, each of which showed the peak of the parent ion and a characteristic fragment ion (M^+-30) formed by loss of the nitroso group[32].

The yields of the *N*-acetoxy-*N*-acetylaminoarenes were 4–67% and of the nitrosoarenes 10–82% under the conditions described in the MATERIALS AND METHODS section and in Table I. These yields were calculated on the assumption that the oxidations yielded nitroxide free radicals and that two of these nitroxide radicals dismutated to yield 1 mole of *N,O*-diacylate and 1 mole of nitrosoarene (Eqn 1). The greater yields of the nitroso derivatives, as compared to those of the *N,O*-diacylates, when the oxidations were carried out with alkaline $Fe(CN)_6^{3-}$, is in accord with the greater lability of the latter compounds under alkaline conditions. On the other hand, the continuous generation of the free radical during the Ag_2O oxidation probably increased the probability of loss of the nitroso derivative through reaction with the free radical[21]. While no *N*-acetoxy-1-acetylaminonaphthalene could be isolated from oxidations with alkaline $Fe(CN)_6^{3-}$ under the standard conditions, it was isolated in 30% yield when the oxidation was carried out with Ag_2O in benzene (Table I).

The oxidation of N-arylacethydroxamic acids by various peroxidases and H_2O_2

Bartsch and Hecker[20] showed that *N*-hydroxy-2-AAF was oxidized at neutral pH in a horseradish peroxidase–H_2O_2 system to yield a nitroxide radical and that the nitroxide radical dismutated under these conditions to yield *N*-acetoxy-2-AAF and 2-nitrosofluorene. These products were each isolated from the enzyme-containing reaction mixture, and the former compound was also trapped by its reaction with guanosine to yield *N*-(guanosin-8-yl)2-AAF.

Esters of the aromatic acethydroxamic acids also react with methionine and related methylmercapto compounds to yield sulfonium derivatives which decompose with the formation of mercapto aromatic amides[2,25,26,33]. From the amounts of *o*-methylmercapto-2-AAF formed on incubation of *N*-hydroxy-2-AAF with H_2O_2 and the peroxidase it is evident that horseradish peroxidase, lactoperoxidase, and human myeloperoxidases each catalyze the one-electron oxidation of *N*-hydroxy-2-AAF (Table III). Under the conditions of these experiments 17–35% of the *N*-acetoxy-2-AAF which could theoretically be formed from *N*-hydroxy-2-AAF according to Eqn 1 was converted to the *o*-methylmercapto amide in the presence of a 25 M excess of *N*-acetyl-DL-methionine as the nucleophilic reagent.

The yield of the methylmercapto amides can also be estimated from the amount of benzene–hexane-soluble tritium obtained by reaction of esters of the hydroxamic acids with [*Me*-^{3}H]methionine[2,26]; this procedure is advantageous in those cases where the methylmercapto amides are not available for reference standards for gas chromatography. When various aryl acethydroxamic acids were incubated for 20 h with the horseradish peroxidase–H_2O_2 system at neutrality in the presence of limiting amounts of [*Me*-^{3}H]methionine, the yields of benzene–hexane-soluble tritium calculated as methylmercapto derivatives, ranged from 0.1% with *N*-hydroxy-3-AAF to 12% with *N*-hydroxy-2-AAF as substrate (Table IV). As shown in Table IV and in previous studies[26,31], the reactivities of the acetic acid esters of these hydroxamic acids are strongly dependent on the nature of the aryl group. Thus, the formation

TABLE III

THE ACTIVITIES OF VARIOUS PEROXIDASES FOR THE ONE-ELECTRON OXIDATION OF *N*-HYDROXY-2-AAF

The amount of one-electron oxidation was determined from the amount of *o*-methylmercapto-2-AAF formed during the oxidation of *N*-hydroxy-2-AAF in the presence of *N*-acetyl-DL-methionine. The details of the procedure are described in the Materials and Methods. When H_2O_2 was omitted, each of the peroxidases gave rise to less than 0.3 μg of o-methylmercapto-2-AAF per assay.

Peroxidase	*o-Methylmercapto-2-AAF formed*	
	μg/assay	*% of theory**
Lactoperoxidase	39	35
Myeloperoxidase (human)	28	25
Horseradish peroxidase	20	17

* For this calculation it was assumed that each mole of *N*-hydroxy-2-AAF oxidized yielded 0.5 mole of *N*-acetoxy-AAF.

TABLE IV

COMPARATIVE FORMATION FROM [*Me*-^{3}H] METHIONINE OF BENZENE-SOLUBLE TRITIUM DERIVATIVES WITH THE HORSERADISH PEROXIDASE-MEDIATED OXIDATION OF *N*-HYDROXY-*N*-ACETYLAMINOARENES OR FROM *N*-ACETOXY-*N*-ACETYLAMINOARENES

Oxidation of *N*-hydroxy-*N*-acetylaminoarenes was carried out with horseradish peroxidase and H_2O_2 at pH 7.0, as described in the Materials and Methods. % reaction was calculated on the basis of the methionine, which was the limiting reactant.

Aryl group	*Benzene-soluble ^{3}H-labelled derivatives (% reaction)*	
	*Via oxidation of N-hydroxy-N-acetyl-aminoarenes**	*From N-acetoxy-N-acetylaminoarenes*
2-Fluorenyl	12.4	12.0
2-Naphthyl	2.5	2.2
1-Naphthyl	1.4	0.55
4-Biphenylyl	0.98	0.95
2-Phenanthryl	0.85	1.2
4-Stilbenyl	0.16	0.14
3-Fluorenyl	0.13	0.12

* For this calculation it was assumed that each of the *N*-hydroxy-*N*-acetylaminoarenes was oxidized and the free radical dismutated according to Eqn 1 and that the ^{3}H-labelled derivative was obtained by reaction of the *N*-acetoxy derivative with methionine, as studied in detail for esters of *N*-hydroxy-AAF[2,25,26]. All values were corrected by subtraction of 0.035%, the apparent reaction obtained when all of the ingredients except an aryl derivative were incubated and treated as described in the Materials and Methods.

of benzene–hexane-soluble tritium derivatives from the aryl hydroxamic acids in the horseradish peroxidase–H_2O_2 system indicates that esters of the hydroxamic acids were formed, but the relative yields of the methylmercapto derivatives from different aryl acethydroxamic acids would not be proportional to the amounts of the esters which were formed. In fact, the similarities between the amounts of the methylmercapto derivatives obtained from each of the hydroxamic acids in the presence of the horseradish peroxidase–H_2O_2 system and from its acetic acid ester suggest that the various hydroxamic acids were generally similar in their susceptibilities to oxidation by the peroxidase system. The amounts of benzene–hexane-soluble tritium formed from *N*-hydroxy-3-AAF and *N*-hydroxy-4-acetylaminostilbene or their esters were so low under these conditions, however, as to preclude any conclusions about the peroxidase oxidation of these hydroxamic acids.

In studies with *N*-hydroxy-2-AAF the formation of *o*-methylmercapto-AAF,

as assayed by gas chromatography of the product[2], was shown to be dependent on the addition of both a peroxidase and H_2O_2 and to be similar whether L-methionine, *N*-acetyl-DL-methionine, or methyl-3-(methylthio)propionate was the nucleophilic reagent. The latter finding is contrary to the suggestion of Scribner *et al.*[26] that an unesterified carboxy group might be important in the reaction of *N*-acetoxy-2-AAF with a methylthio derivative.

DISCUSSION

The earlier observations[19–21] on the facile oxidation of *N*-hydroxy-2-AAF and of *N*-hydroxy-4-acetylaminobiphenyl to *N*-acetoxy-*N*-acetylaminoarenes and nitrosoarenes have been extended in this paper to a number of other carcinogenic aromatic acethydroxamic acids. The reaction mechanism proposed by Bartsch *et al.*[19] for the oxidation of *N*-hydroxy-2-AAF is a one-electron oxidation of the acethydroxamic acid to a free nitroxide radical and the subsequent dismutation of 2 molecules of the free radical to 1 molecule of *N*-acetoxy-*N*-acetylaminoarene and 1 molecule of nitrosoarene (Eqn 1). This reaction mechanism appears to be generally applicable to the aromatic acethydroxamic acids studied and is supported by the isolation in most cases of major amounts of the corresponding *N*-acetoxy-*N*-acetylaminoarene and nitroso-

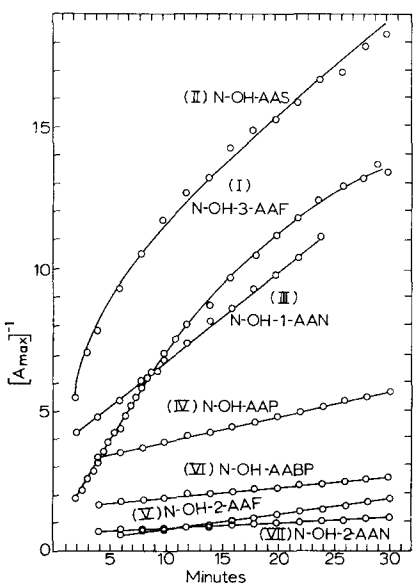


Fig. 1. Disintegration in benzene solution as a function of time of nitroxide radicals formed from various *N*-arylhydroxamic acids by one-electron oxidation with alkaline $Fe(CN)_6^{3-}$. Experimental procedures are given in the Methods and Materials section. The specific conditions under which the data in the graphs were obtained are listed in parentheses in the following order: molarity of the hydroxamic acid solution; reaction time (s) with $Fe(CN)_6^{3-}$; time-dependent absorption maximum (nm); and disintegration constant K_D ($A_{max}^{-1} \cdot min^{-1}$). I, *N*-hydroxy-3-AAF (0.01 M; 15; 490 (inflection); 0.6 within the first 10 min); II, *N*-hydroxy-4-acetylaminostilbene (0.005 M; 15; 590, 660*; approx. 0.3 after 10 min); III, *N*-hydroxy-1-acetylaminonaphthalene (0.01 M; 15, 540 (inflection); 0.3); IV, *N*-hydroxy-2-acetylaminophenanthrene (0.005 M; 60; 670; 0.08); V, *N*-hydroxy-2-AAF (0.005 M; 60; 538; 0.06); VI, *N*-hydroxy-4-acetylaminobiphenyl (0.01 M; 60; 660; 0.04); VII, *N*-hydroxy-2-acetylaminonaphthalene (0.01 M; 60; 540; 0.02).
* Used for estimation of K_D.

arene (Table I). The linearity, in most cases, of the reciprocal of the absorbance at the absorption maximum of the free radical as a function of the time of decay is also consistent with this mechanism (Fig. 1). Only the dismutations of the free radicals from *N*-hydroxy-4-acetylaminostilbene and *N*-hydroxy-3-AAF exhibited deviations from second-order reaction kinetics, and these compounds showed linear responses during the first minutes. The deviations from linearity were paralleled by the recovery of smaller amounts of the two major products and increased amounts of unidentified products, which, on the basis of the study of Forrester *et al.*[21], may have included the corresponding nitro and amide derivatives.

The oxidations were studied primarily in non-aqueous solution with alkaline $Fe(CN)_6^{3-}$ or Ag_2O as the one-electron oxidant. The yields of the *N*-acetoxy-*N*-acetylaminoarenes and nitrosoarenes were functions of the oxidant, the reaction conditions, and the stabilities of the products. Thus, the yields of the products listed in Table I should not be used for quantitative comparisons between the various arenes or for correlations with the biological activities of the parent hydroxamic acids.

On the other hand, it is possible to compare the stabilities of the various free nitroxide radicals in benzene solution, as evidenced by their disintegration constants (Fig. 1). As noted in Table V the stabilities of these radicals are roughly in an inverse order to the carcinogenicities of the parent hydroxamic acids in the subcutaneous tissue of the rat. *N*-Hydroxy-1-acetylaminonaphthalene is an exception, since its free radical dismutated rapidly, while the hydroxamic acid did not induce sarcomas in rats under the conditions studied (J. D. Scribner, J. A. Miller and E. C. Miller, unpublished). *N*-Hydroxy-3-AAF has not been tested for carcinogenicity in the subcutaneous tissue of the rat, but it is a potent mammary carcinogen when administered intraperitoneally[34].

As reported previously for *N*-hydroxy-2-AAF[19] and as determined in this paper for other aromatic acethydroxamic acids, the same type of one-electron oxidation to a free nitroxide radical and the dismutation to *N*-acetoxy-*N*-acetylaminoarenes and nitrosoarenes can be catalyzed in an aqueous system at pH 7 with horseradish peroxidase and H_2O_2. Two mammalian peroxidases, lactoperoxidase and human myeloperoxidase, were also efficient in catalyzing the one-electron oxidation of *N*-

TABLE V

COMPARISON OF THE DISINTEGRATION CONSTANTS OF THE FREE NITROXIDE RADICALS DERIVED FROM AROMATIC ACETHYDROXAMIC ACIDS AND THE CARCINOGENICITIES OF THE HYDROXAMIC ACIDS FOR THE SUBCUTANEOUS TISSUE OF THE RAT

N-Hydroxy-N-acetylaminoarene	*Disintegration constant of free nitroxide radical**	*Carcinogenic activity*** *(subcutaneous tissue)*
N-Hydroxy-3-AAF	0.6***	Not tested
N-Hydroxy-AAS	0.3***	++++
N-Hydroxy-1-AAN	0.3	o
N-Hydroxy-AAP	0.08	++++
N-Hydroxy-2-AAF	0.06	+++
N-Hydroxy-AABP	0.04	+
N-Hydroxy-2-AAN	0.02	+

Abbreviations: see Table I.

* In benzene solution.

** Ref. 9 and unpublished data.

*** Approximate values at 10 min; plots are non-linear.

hydroxy-2-AAF. Therefore, although preliminary attempts to demonstrate a similar process with rat liver preparations have not succeeded, it is reasonable to expect that peroxidative attack *in vivo* could also yield the free nitroxide radical. Peroxidase activity has been demonstrated in a number of mammalian tissues[35,36]. The binding of estrogens, possibly *via* radical intermediates, can be mediated with either horseradish or a uterine peroxidase[37,38], and a possibly similar type of reactive species has been generated by the action of hepatic microsomal oxygenases on estrone[39].

The meaning of the approximate apparent inverse relationship between the stabilities of the free nitroxide radicals and carcinogenesis by the aromatic acethydroxamic acids is obscure. It may only be a reflection of structural parameters contributed by the various aryl residues which affect the stability of the nitroxide radical and carcinogenic activity independently. On the other hand, the apparently central role of electrophilic reagents as ultimate carcinogenic forms of nearly all chemical carcinogens[1] suggests that the free nitroxide radical, if formed *in vivo*, could be important in the induction of neoplasia at some sites or under some conditions. Thus, the free radical would itself be an electrophilic reagent which might react with critical tissue nucleophiles. Furthermore, its dismutation gives rise to two electrophilic reagents. As a class *N*-acetoxy-*N*-acetylaminoarenes are each somewhat more carcinogenic at the site of subcutaneous injection in the rat than their corresponding hydroxamic acids[9]. With the exception of *N*-acetoxy-3-AAF[31], these esters have all shown reactivity with nucleophilic compounds at neutrality, although there are large quantitative differences[26] (Table III). 2-Nitrosofluorene is carcinogenic for the subcutaneous tissue, mammary gland, and forestomach of the rat[34,40,41], and it has shown potent activity as a frameshift mutagen for certain strains of *Salmonella typhimurium*[42]. Its electrophilicity is demonstrated by its facile reaction with reduced glutathione[43], although its reaction with the other tissue nucleophiles studied has been very slight compared to that of *N*-acetoxy-2-AAF[43,44].

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