PHARMACOLOGICAL REVIEWS
Vol. 25, No. 1

Printed in U.S.A.

Biochemical Formation and Pharmacological, Toxicological, and Pathological Properties of Hydroxylamines and Hydroxamic Acids

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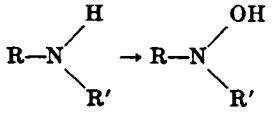
I. Introduction

AROMATIC and aliphatic N-hydroxy compounds are chemically and metabolically quite reactive. Relatively few such chemicals occur naturally in the environment. However, N-hydroxylation, especially of aromatic amines, does proceed readily as part of the now established metabolic biochemical hydroxylations which are so well developed in mammalian systems (224, 332, 439, 471, 489). Quantitatively, N-hydroxylation is usually a minor reaction, but significantly it leads to pharmacologically and pathologically very important metabolic intermediates, as will be discussed. Basically, the reaction involves substitution of one of the hydrogens of an amine with the hydroxy group.



Like many hydroxylated metabolites, N-hydroxy derivatives are subject to further metabolic conversions, such as conjugation with glucuronic, sulfuric, and perhaps phosphoric acid, and also to a reduction back to the parent amine.

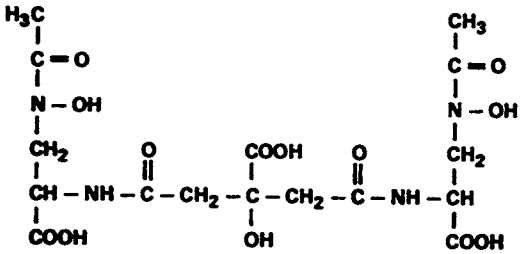
Whereas most hydroxylations, like those on carbon, lead to "detoxified" readily excreted metabolites (160, 360), N-hydroxylation develops metabolites which are often reactive. By virtue of this fact they can exert harmful effects *in vivo* through combination as such, or after further reactions, with essential life-supporting molecular species.

Biochemical N-hydroxylation has been alluded to by some pioneers in biochemical pharmacology; Heubner (168) first suggested in 1913 that methemoglobin formation due to certain aromatic amines like *p*-chloroaniline might be due to an N-hydroxy metabolite. However, detailed and specific research in this area is only about 15 years old. A reinvestigation by Kiese of methemoglobin formation by aromatic amines (221), utilizing newly developed analytical techniques (167), led to the discovery that this event could be related to the formation of arylhydroxylamines or nitroso compounds in the animals treated with aromatic amines (222). Furthermore, investigation of the metabolites responsible for the carcinogenicity of certain aromatic amine derivatives, more specifically N-2-fluorenylacetamide (or 2-acetylaminofluorene), revealed that a key metabolite was the N-hydroxy derivative, in the form of N-2-fluorenylacetohydroxamic acid (83).

Several reviews of both the effect of N-hydroxy derivatives of amines in methemoglobin formation and in the metabolic activation of aromatic amine carcinogens are available (223, 332, 439, 485, 489). The purpose of the current paper is to present salient recent developments in both areas and to develop the biochemical and pharmacological perspective of this important reaction, resulting more often than not in adverse effects (447). Eventually an understanding of the controlling factors in N-hydroxylation may permit either the total prevention or a reduction of untoward consequences.

A. Natural Products Containing N-Hydroxy Derivatives

A number of products of bacterial metabolism contain the N-hydroxy group such as muta-aspergillic acid (345), nocardamin



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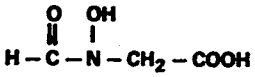
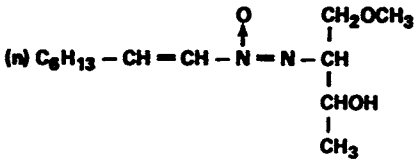


HADACIDIN



ELAIOMYCIN

FIG. 1. Structures of some naturally occurring N-hydroxy or N-oxy compounds.

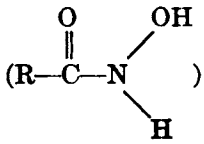
(413), mycelianamide (42, 359), aerobactin (132), mycobactin (400, 401), and hadacin (210) (fig. 1). For reviews on hydroxamic acids of natural origin see Thompson *et al.* (428) and Emery (99). In addition, there are several N-oxides: iodenine and related structures (78, 130), elaiomycin (410), a number of products related to alkyl-4-oxy-quinoline N-oxides (81), pulcherriminic acid (79), aspergillic acid and derivatives (96), and a number of cyclic and acyclic antibiotics described by Ochiai (355). Also, naturally occurring aliphatic nitro compounds are converted easily into N-oxy acetates with acetic anhydride (409).

B. Formation of N-Hydroxy Derivatives of Aromatic Amines

There are three main procedures leading to arylhydroxylamines and derivatives, by chemical synthesis, by biochemical oxidation of arylamines and derivatives *in vivo*, mostly by mammalian systems, and by biochemical reduction of nitro compounds by mammalian and microbiological systems.

II. Chemical Synthesis and Reactions of N-Hydroxy Compounds

Methods, both general and specific, for the synthesis of hydroxylamines are presented in various reference works (3, 314, 492, 517) and in the continuing series by Theilheimer (427). Some of the uses of the parent hydroxylamine or of substituted hydroxylamines in organic syntheses are given in Fieser and Fieser (119, 120). A partial review of arylhydroxylamines has been made by Utzinger (456), and hydroxamic acids



have been discussed by Yale (504). Majumdar (290) discusses chemicals related to N-benzoylphenylhydroxylamine which are used mainly as analytical reagents for various metals. The reviews in Houben-Weyl (517) are very comprehensive and cover the various methods of preparing aliphatic and aromatic hydroxylamines, some of their reactions, and the products so obtained.

A. Aliphatic Hydroxylamines

Aliphatic hydroxylamines can be prepared by controlled reduction of nitro- or nitrosoalkanes or of oximes. Zinc dust with ammonium chloride (492), electrochemical methods (220, 492), controlled catalytic reduction (3), amalgams (62), or ammonium sulfide may be employed. A more recent method uses diborane to reduce oximes or nitro salts to hydroxylamines (117, 118).

Controlled oxidation of amines in which the nitrogen is attached to a tertiary carbon can be employed to form some hydroxylamines with Caro's acid (314, 492). However, in a patent, it was recently claimed that a good yield of N-cyclohexylhydroxylamine was obtained from cyclohexylamine, using 30% hydrogen peroxide as the oxidizing agent (217).

Alkylation of hydroxylamine has been found useful in some cases (181). Furthermore, the addition of hydroxylamine to an unsaturated conjugated system affords substituted hydroxylamines (408, 492). However, addition of substituted hydroxylamines to such a conjugated system may not always be straightforward. Woodman *et al.* (502) showed that phenylhydroxylamine, when condensed with hydroxymethylenedesoxybenzoin, did not yield the expected vinylhydroxylamine but benzoylphenylacetanilide instead.

B. Aromatic Hydroxylamines

Arylhydroxylamines generally are likewise furnished by the controlled reduction of aromatic nitro compounds. Zinc dust and water with ammonium chloride, aluminum amalgam, ammonium or sodium sulfide, phenylhydrazine, or partial catalytic reduction have all proved useful. However, not all of these agents will give the desired arylhydroxylamines since some of the reactions are quite specific and attention must be paid to the exact experimental technique. Thus, practically all of these methods furnish good yields of phenylhydroxylamine from nitrobenzene. Ammonium sulfide gives an almost quantitative yield of 1-naphthylhydroxylamine (517) but at best a 60 % yield of 2-fluorenylhydroxylamine (276) and about 45 % of N-hydroxy-2-anthrylamine (386). Aluminum amalgam furnished a manageable amount of 2-naphthylhydroxylamine from 2-nitronaphthalene (58) or of 4-hydroxylaminobiphenyl (453), but it failed to give more than a trace of 2-fluorenylhydroxylamine from 2-nitrofluorene (83, 466).

Phenylhydrazine, although affording excellent yields of hydroxylamines from nitroanthraquinones or nitropyridine N-oxides (356), did not reduce 2-nitrofluorene or 2-nitrobiphenyl (466). In certain cases, as with purines or quinoline N-oxides, an activated ring chlorine can react with hydroxylamine to furnish a heterocyclic N-hydroxylamine (357). In the quinoline N-oxide series, the partial reduction of the nitro group to hydroxylamino was also accomplished with sodium borohydride (419).

The N-acyl derivatives of various arylhydroxylamines have been prepared by Miller and Miller and their associates (5, 83, 334, 387) by controlled catalytic hydrogenation of the nitro compounds on palladium-charcoal in the presence of triethylamine and an acyl anhydride. The procedure generally yielded the N,O-diacyl derivative which was hydrolyzed to the N-acyl compound by dilute ammonium hydroxide. Similar methods were employed by Yost and Gutmann (516). The yields for this general procedure varied from about 2 to 36 %, depending on the structure of the individual compounds. N-Acylation of various phenylhydroxylamines was readily accomplished by the acyl chloride in an ether-solid sodium bicarbonate mixture (397).

C. Reactions of Arylhydroxylamines

Arylhydroxylamines can be reduced further to the amines or oxidized by mild agents such as ferric ammonium sulfate (276, 515) or potassium ferricyanide (453) to the corresponding nitroso compounds. Other useful oxidizing agents for this purpose are diethyl azodicarboxylate (63, 423) or in some cases nickel peroxide (344). On the other hand, stronger oxidizers such as lead tetraacetate or potassium permanganate can very rapidly deacylate and oxidize the N-acyl derivatives of arylhydroxylamines to nitroso derivatives (31, 495). Biochemically, horseradish peroxidase and hydrogen peroxide were capable of forming 2-nitrosofluorene and N-acetoxy-2-fluorenylacetamide from N-hydroxy-2-fluorenylacetamide. The reaction was presumed to go through a nitroxide radical which dismutated to the products (28, 30).

Arylhydroxylamines couple with nitroso compounds to form azoxybenzenes. This reaction often occurs during oxidation of the hydroxylamines to nitroso compounds (116, 344). Since the nitroso compounds are readily formed from arylhydroxylamines, even during storage, or by a disproportionation reaction (390),

$$2\ RNHOH \rightarrow RNO + RNH_2 + H_2O$$

the azoxy derivatives may often contaminate samples of arylhydroxylamines. Reaction of hydroxylamines with aldehydes, ketones, or ketone derivatives furnishes nitrones (for reviews see 159, 381 and 405) which in turn can be oxidized to nitroxides (11). An unusual nitrone was found from 4-nitrobenzylpyridinium bromide and phenylhydroxylamine (376). The nitro group was reduced to nitroso with subsequent elimination of pyridine and couplings to yield nitrones of azoxy- and azobenzenes. Irving (184) reported that azoxyfluorene was formed by incubation of N-hydroxy-2-fluorenylacetamide with rabbit liver microsomes, presumably through deacetylation, partial oxidation, and condensation. However, a communication by Yost (515) casts doubt on the identity of the compound reported by Irving as 2,2′-azoxyfluorene and suggests that 2,2′-azofluorene may be involved. An azoxybenzene derivative was also the main product from incubation of *p*-thioanisidine with rabbit liver microsomes. The primary step was N-oxidation to 4-methylmercaptophenylhydroxylamine, followed by condensation with the subsequently formed nitroso derivative (384).

In acid solution arylhydroxylamines undergo the Bamberger rearrangement. Accordingly, in concentrated sulfuric acid, phenylhydroxylamine gives a good yield of *p*-aminophenol. In dilute hydrochloric acid, or in methanol, ethanol, or aniline the corresponding *o*- and *p*-substituted chloroanilines, or anisidines, phenetidines, and semidines are formed, besides varying amounts of by-products as azobenzene, azoxybenzene and nitrosobenzene (21–23, 56, 82). In absolute ethanol phenylhydroxylamine also reacted with sulfur dioxide and rearranged to yield largely *o*- and *p*-aminobenzenesulfonic acid, aniline, phenylsulfamic acid, and very small amounts of *m*-aminobenzenesulfonic acid (98). Although no mechanism was proposed for this latter reaction, the results are reminiscent of the Bamberger rearrangement.

$$ArNHOH + HX \rightarrow \underset{X(o,\ p)}{Ar{-}NH_2}$$

In our studies on the metabolism of the arylhydroxamic acid N-hydroxy-2-fluorenylacetamide we have found that some of the products of such a rearrangement can appear as metabolic artifacts. This especially occurred when hydrolysis with dilute hydrochloric acid was used to free certain conjugates of the arylhydroxamic acid. On the other hand, Stöhrer and Brown (412) thought that 8-chloro- and 8-methylmercaptoxanthine were urinary metabolites of 3-hydroxyxanthine, a purine N-oxide. They theorized that an activated intermediate, like 3-acetoxyxanthine, reacted with intracellular chloride ion or methionine to yield these metabolites since identical products were obtained *in vitro*. The mechanism of this reaction has been investigated by Birdsall *et al.* (43, 44).

The model reactions of certain arylhydroxylamines with thiols to yield mercapturic acid derivatives were investigated by Boyland *et al.* (56, 57). It was postulated that the reactivity of aromatic hydroxylamines with nucleophilic centers may explain the toxicity and carcinogenicity of these compounds.

Within the past few years, hydroxylamine derivatives have found increased utility as reagents for peptide synthesis (139, 342). Also Neunhoeffer (352) has reported finding N-hydroxy peptides derived largely from arginine, lysine, aspartic acid, and histidine both in spontaneous tumors from various mammals and in chemical- or virus-induced tumors. The possibility that N-alkyl-N-acylhydroxylamines may serve as specific acyl transfer agents in biological events was alluded to by Neunhoeffer and Gottschlich (353). Additionally the O-acyl esters of N-acyl-N-arylhydroxylamines appear to be active intermediates in the reactions of carcinogenic amines with various tissue nucleophiles such as DNA, RNA, guanosine, tyrosine, tryptophan, methionine, and cysteine (322, 331). These

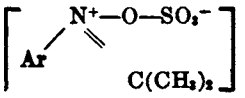
reactions will be discussed elsewhere in this article.

The synthesis of such acyl esters of N-hydroxy-2-fluorenylacetamide was readily accomplished by treating the sodium salt of the amide with an acyl anhydride or chloride (279) or by reacting the amide with the anhydride in pyridine (152), analogous to schemes devised by Bamberger (19) or Horner and Steppan (173).

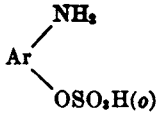
The decomposition rates of several N-acetoxy-N-arylacetamides derived from stilbene, phenanthrene, naphthalene, fluorene, or biphenyl in aqueous solution, in citrate buffer, or in solutions containing methionine or guanosine were determined by Scribner *et al.* (387). The compounds which decomposed most slowly also reacted less with methionine or guanosine. However, there was no correlation between the decomposition rate of the acetoxy derivatives and the carcinogenic activity of the parent N-hydroxy compounds.

The glucuronide of N-hydroxy-N-2-fluorenylacetamide could not be made synthetically by Irving (185) since it proved too labile to alkali. However, the triacetyl methyl ester glucuronide of the acetohydroxamic acid could either be synthesized or isolated from the urine of rabbits fed 2-fluorenylacetamide. The true glucuronide (or sodium salt) could only be prepared biosynthetically (169). Further studies with this compound (196, 317) showed that it reacted with amino acids and nucleic acids to a much lower extent than the O-acyl derivatives of N-hydroxy-2-fluorenylacetamide. Furthermore, the sodium or copper salts of the triacetyl methyl ester glucuronide did not cause tumors, casting doubt on the role of glucuronides in the development of neoplasia. However, the O-glucuronide of the N-2-fluorenylhydroxylamine was made in good yield from either the biosynthetically formed sodium (N-acetyl-N-2-fluorenylhydroxylamine-β-D-glucoside) uronate or its triacetyl methyl ester with sodium methoxide in methanol (192). The fluorenylhydroxylamine glucuronide reacted readily with DNA or RNA *in vitro* and with guanosine 5′-monophosphate but not with the 5′-monophosphates from uridine, cytidine, or adenosine.

The synthesis of the O-sulfate ester of N-hydroxy-2-fluorenylacetamide might also be feasible by reacting the sodium salt with chlorosulfonic acid, sulfuric acid in dimethylformamide (172), or triethylamine-sulfur trioxide complex (162). Mechanistically, the O-sulfates of acylarylhydroxylamines appear to be active intermediates in the carcinogenic process, as discussed elsewhere in this review. In view of this implication, a recent report merits attention. Manson (293) investigated the formation of *o*-aminoaryl hydrogen sulfates from arylhydroxylamines and pyridine-SO_3 in acetone. He concluded that the intermediate aryl N-sulfohydroxylamine (which could be obtained from pyridine-SO_3 in benzene) formed an intermediate aryl N-isopropylidene-N,O-sulfate with the acetone.



Rearrangement with loss of the isopropylidene group led to the *o*-aminoaryl hydrogen sulfate.



Whether naturally occurring carbonyl compounds might thus be involved in the metabolism of aromatic amines, as proposed by Manson, remains an intriguing possibility.

III. Analysis of N-Hydroxy Compounds

One of the most sensitive tests for arylhydroxylamines is furnished by the reaction with sodium pentacyanoamino ferrate which generally forms red-violet colored complexes (115). However, nitrosobenzene and its analogues interfere since they react in a similar fashion. Thus this sensitive, rapid test cannot be employed if both arylhydroxylamino and nitroso compounds are present.

However, arylhydroxylamines react with aldehydes (*p*-dimethylaminobenzaldehyde or salicylaldehyde) to yield nitrones which can be estimated spectrophotometrically. Although this procedure is less sensitive, it can be of value when interfering materials are present (58). Boyland and Nery (58) have compared the sensitivities of three tests for arylhydroxylamines, *i.e.*, salicylaldehyde, potassium ferrocyanide, and sodium pentacyanoamino ferrate at various time intervals and pH values. However, Brill and Radomski (64) reported that in the presence of magnesium ion, N-2-naphthylhydroxylamine reacted quantitatively with pentacyanoamino ferrate without interference from other metabolites, as was not the case when magnesium was omitted.

Weil (462) developed several chromatographic systems for separation of disubstituted hydroxylamines and the corresponding nitroxides. Iodine or potassium permanganate sprays were used for detection of the compounds.

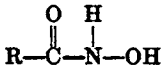
For analysis of the actual content of 2-fluorenylhydroxylamine in an organic preparation, reduction with excess titanous chloride followed by back-titration with ferric ion was employed by Poirier *et al.* (365) according to a technique developed by Horner and Steppan (173).

Although N-acylarylhydroxylamines react only very slowly with aldehydes in the presence of acid to form nitrones (83), they can be oxidized readily by agents such as lead tetraacetate (31), silver oxide (124), or potassium permanganate (495) to the corresponding nitroso compounds. In turn, these can be easily estimated by complex formation with pentacyanoamino ferrate. Similarly, oxidation of the arylhydroxylamines to nitroso derivatives and determination as such was employed by Uehleke and Nestel (453) in studies on 4-aminobiphenyl.

A different method for quantitation of nitrosobenzene and its analogues formed in the blood during metabolism of aniline was developed by Herr and Kiese (167), based on the observation of Bamberger (20) that nitrosobenzene could be diazotized. The nitroso compounds formed were selectively extracted into carbon tetrachloride, diazotized, and coupled with N-(1-naphthyl)-ethylenediamine to form a dye which was determined spectrophotometrically.

These analytical methods can often be advantageously employed for alkylhydroxylamines or N-hydroxycarbamates. Thus Boyland and Nery (59) found pentacyanoamino ferrate to be a useful reagent for estimation of various alkyl-N-hydroxy compounds.

Although aryl N-hydroxy compounds usually form colored complexes with metals, some of these color reactions are not always suitable for analytical determinations. However, for compounds of the following type, complex formation is quite useful (123, 290).



Overall, it appears that the reaction with pentacyanoamino ferrate affords the best derivative for such purposes.

Another method of great utility is to form the trimethylsilyl ethers of the N-hydroxy compound such as N-hydroxy-2-fluorenylacetamide by reaction with any of several silylating agents as bis(trimethylsilyl)acetamide (187). These silyl ethers are stable and can be determined quantitatively by gas-liquid chromatography. However, it has been observed in our laboratory (141) that if any ring-hydroxylated derivative of N-2-fluorenylacetamide is present, reaction with bis(trimethylsilyl)acetamide led to two peaks on gas-liquid chromatography from each ring-hydroxylated metabolite. Presumably, bis(trimethylsilyl)acetamide reacted both with the phenolic hydroxyl group and perhaps the amide hydrogen of the acetamido moiety. The use of N-trimethylsilylimidazole (Trisilyl Z) as a silylating agent obviated the formation of double peaks but readily yielded the trimethylsilyl ethers for quantitative determi-

nations. Metzler and Neumann (311) made an extensive study on the separation of the ring and N-hydroxylated metabolites of 4-aminostilbene and 4-aminobibenzyl by gas-liquid chromatography. N-Trimethylsilylacetamide was employed as a silylating agent; of the many hydroxylated materials, only 3-hydroxy-4-aminostilbene gave two peaks after treatment with N-trimethylsilylacetamide.

Due to the appreciable volatility of alkyl carbamates and the corresponding N-hydroxy compounds, even the acetyl, as well as the trimethylsilyl, derivatives could be employed for gas chromatographic determinations of these materials (348).

Gas-liquid chromatography was also employed by Radomski and Brill (373) to determine the total N-oxidation products of 1- or 2-naphthylamine or 4-biphenylamine in dog urine. The procedure was based on oxidation of any N-hydroxylamine with ferricyanide to the more volatile nitroso compound which was then extracted and measured by gas chromatography. Interestingly, the N-1- or-2-naphthylhydroxylamines appeared to be converted to the nitroso derivatives on the gas chromatographic column.

The antiinflammatory drug Bufexamac (*p*-butoxyphenylacethydroxamic acid) was determined in plasma or urine readily by silylation with bis(trimethylsilyl)acetamide prior to injection into the gas chromatograph (379).

IV. Formation of N-hydroxy Derivatives by Biochemical Oxidation-N-Hydroxylation in Vivo

A. Metabolites in Urine

1. Evidence for N-hydroxylated metabolites in urine. Definitive indication that some aromatic amines like aniline are hydroxylated not only on the ring, which had been known for a long time, but also on the nitrogen under *in vivo* conditions was adduced first in the laboratories of Kiese and Uehleke by detecting the corresponding hydroxylamino and nitroso derivatives in the blood (166, 222, 233). The excretion of this type of novel metabolite in urine was discovered quite independently by Cramer *et al.* (83) shortly thereafter, in connection with studies on the overall metabolism of the carcinogen N-2-fluorenylacetamide. This finding aroused considerable interest among workers engaged in carcinogenesis research because of the demonstration that the N-hydroxylated metabolite was probably involved in the carcinogenic process, in contrast to the ring-hydroxylated metabolites which were inactive. Other investigators concerned with the metabolism of arylamines began examining urine for this class of metabolite. Environmental conditions and factors controlling the production and excretion of N-hydroxy derivatives were also investigated.

von Jagow *et al.* (206) studied the excretion of N-hydroxylated metabolites of a number of arylamines in the urine of rabbits, dogs, and guinea pigs. After intravenous injection of as much as 200 mg per kg of aniline into rabbits no phenylhydroxylamine was detected in urine. However, increasing amounts of arylhydroxylamines, measured as the corresponding nitroso derivatives, were observed with *p*-chloraniline, *p*-ethylaniline, 2-fluorenamine, and *m*-aminopropiophenone; the largest amounts were seen with 4-aminobiphenyl, 20% of the dose, and with *p*-aminopropiophenone, 31%. Importantly, in most cases the bulk of the N-hydroxy metabolites was found in the first 4 hr. With *p*-aminopropiophenone the percentage of dose excreted as N-hydroxy compound was largely independent of dose over a range from 1 mg per kg to 40 mg per kg. After 2-fluorenamine the urine of rabbits also contained the N-acetyl-N-hydroxy derivative as the glucuronide which was hydrolyzed by β-glucuronidase. The urine of guinea pig also gave evidence of N-hydroxylated metabolites, from 0.5% of dose after 2-fluorenamine, 3.2% with *m*-aminopropiophenone, 4.7% with 4-aminobiphenyl, and 15% with *p*-aminopropiophenone, again collected soon after treatment. Curiously, dogs exhibited the lowest

yields of N-hydroxy products with these amines. The data refer to excretion of free compounds, except with *p*-aminopropiophenone, where an additional amount of N-hydroxy derivative was liberated after incubating the urine in an inert atmosphere at pH 4.5. Addition of β-glucuronidase failed to release more compound. The interpretation was that the materials liberated by incubation probably were not O-but N-glucosiduronic acids, presumed unstable at pH 4.5. Another possibility is that mammalian β-glucuronidase, known to be excreted in urine, was sufficiently active under the conditions of the incubation to release O-glucuronides.

Mesidine or 2,4,6-trimethylaniline, hepatotoxic and a weak carcinogen, is metabolized chiefly by oxidation of the *o*- or *p*-methyl groups. However, N-hydroxymesidine was also detected by careful chromatographic resolution of urines of rats given mesidine. This metabolite was postulated as an intermediate in the ultimate formation of 2,6-dimethylhydroquinone, also a metabolite of mesidine (265).

N-Hydroxylation was not confined to amines or amides. Rats fed 2,4,6-trimethylacetophenone imine excreted the corresponding oxime in the urine (361). Its mode of formation remains to be clarified.

Uehleke (444) showed that dogs pretreated with phenobarbital and given an oral dose of *p*-phenetidine excreted less of the unchanged drug and more of N-oxidation products measured as *p*-nitrosophenetol in urine compared to controls without phenobarbital. Burns and Conney (73) found an N-hydroxy derivative of phenacetin in the urine of dogs or men given phenacetin. Uehleke (444), examining the urinary excretion of nitrosophenetol, found less of this product after phenacetin than after similar intake of phenetidine, but his method could not detect an N-acetyl-N-hydroxy compound. In rats given *p*-phenetidine only a small amount, approximately 0.1% of the dose, of 4-nitrosophenetol was recovered. Uehleke believes that there might also be glucosiduronic acids which cannot be properly quantitated because any liberated 4-hydroxylaminophenetol might undergo decomposition if attempts were made to hydrolyze the glucuronide enzymatically. Since it has been established that bacterial β-glucuronidase is not inhibited by pentacyanoamino ferrate, it might be useful to reexamine the excretion of conjugates by performing the incubations in the presence of this fairly specific reagent.

The urinary excretion of the N-hydroxy derivative of 4-acetylaminobiphenyl (or 4-phenylacetanilide), mostly as glucuronide, and conditions affecting the amount of this metabolite were reported by Miller *et al.* (334). Young rats eliminated more metabolite than older rats, whereas those pretreated with N-2-fluorenylacetamide for 10 weeks, leading to liver damage and regeneration, also excreted larger amounts. However, urinary excretion of the N-hydroxyacetylaminobiphenyl did not increase during feeding (fig. 2). In rats, virtually the same metabolites were noted when 4-aminobiphenyl was administered, attesting to the facile acetylation of arylamines in this species. On the other hand, as expected, this did not occur in dogs and only weak evidence for the occurrence of 4-hydroxylaminobiphenyl could be adduced. Even with dogs pretreated with pantothenate and riboflavin no acetylation was detected. Nonetheless, N-hydroxylation did take place, for dogs given 4-acetylaminobiphenyl excreted the N-hydroxy derivative.

After four doses of 30 mg per kg of 4-aminobiphenyl, approximately 0.6% was characterized in the urine of a rhesus monkey as N-hydroxy-4-acetylaminobiphenyl, but no evidence of an N-hydroxy derivative could be gathered after similar dosing with 4-nitrobiphenyl (102).

Andersen *et al.* (5), Baldwin and associates (15, 17, 18), and Metzler and Neumann (312) showed that, upon injection of the highly toxic and carcinogenic *trans*-4-stilbenacetamide, 4-stilbenamine, or 4-dimethylaminostilbene, rats excreted an N-

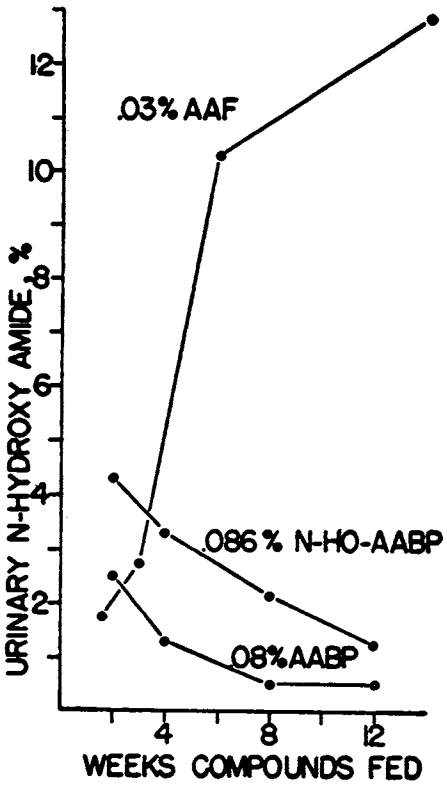


FIG. 2. The urinary excretion of N-hydroxy-4-acetylaminobiphenyl (N-HO-AABP) when it or 4-acetylaminobiphenyl (AABP) was fed to rats.

The data for 2-acetylaminofluorene (AAF) are given for comparison. Each point represents the average excretion for 24 hours for four adult male rats. Data are corrected for the differences in molecular weights (334).

hydroxy derivative chiefly as a glucuronide. Although N,N-dimethyl-4-stilbenamine and N-4(4'-fluorostilbene)acetamide yielded an N-hydroxy amine, 4-acetylaminobibenzyl did not. Again, the N-hydroxylated derivatives were more carcinogenic than the parent compounds. Inasmuch as stilbenamines yield mostly tumors of the ear duct in rats and appear to have relatively little effect on the liver, it might be expected that during chronic feeding relatively little increase in the urinary excretion of the N-hydroxy derivative would occur unless this material also affected the bacterial flora in the gut, an as yet unknown point. As an additional correlation between N-hydroxylation and biological effect, the growth of the Walker 256 tumor is inhibited by this type of compound. The N-hydroxy derivative was more active than the parent compounds.

2-Naphthylamine, formerly an important industrial dyestuff intermediate, is no longer manufactured on a large scale in most countries because of its recognized carcinogenicity in animals and man (50). It has elicited the interest of pharmacologists for a long time inasmuch as a metabolite rather than the amine itself was suspected as the actual carcinogen. For some time *o*-hydroxylation found favor (76). It was only after the N-hydroxylation of N-2-fluorenylacetamide was established that the corresponding hydroxylamino and N-acetylhydroxamic acids were discovered in the urine of several species given 2-naphthylamine. Boyland and Manson (54, 55) detected 2-naphthylhydroxylamine and 2-nitrosonaphthalene in urine of dogs but not of guinea pigs, hamsters, rabbits, or rats, nor did they find the N-acetyl derivative. Just as seen with the arylamines studied by Kiese's group, naphthylhydroxylamine was found only in freshly collected urine shortly after administration of the amine. It was demonstrated that the hydroxylamine was unstable in urine over a 24-hr period. After feeding 2-acetylaminonaphthalene the N-hydroxy derivative was found, conjugated with glucuronic acid, in the urine of dogs but not in the urine of rats, guinea pigs, hamsters, or rabbits. On the other hand, administration of the N-hydroxy derivative of 2-acetylaminonaphthalene led to the excretion in urine of rats, guinea pigs, and rabbits of the corresponding glucosiduronic acid.

Enomoto *et al.* (102) thought that chromatograms of urines of a rhesus monkey given four doses of 30 mg per kg of 2-naphthylamine or 2-acetylaminonaphthalene exhibited faint zones of color with a mobility like that of authentic N-hydroxy-2-acetylaminonaphthalene after the relatively specific acidic *p*-dimethylaminobenzaldehyde reagent.

Uehleke and Brill (450) also reported

that female beagle dogs given 2-naphthylamine orally had in their urine naphthylhydroxylamine, measured as 2-nitrosonaphthalene. Furthermore, dogs pretreated with six doses of phenobarbital excreted much larger amounts of the N-oxidation products, and incidentally, also had higher methemoglobinemia (445). In this connection, Uehleke (442) also demonstrated resorption of aromatic amines such as aniline, 2-naphthylamine, and 4-aminobiphenyl as well as phenylhydroxylamine and 2-naphthylhydroxylamine after instillation into the urinary bladder of dogs, by measuring their blood levels or the occurrence of methemoglobinemia. Thus, these carcinogens as well as other drugs inserted into this organ may not only exert a local effect but could also act systemically (67, 189).

Brill and Radomski (65) adduced evidence that dogs given 1-naphthylamine excreted the hydroxylamino derivative. However, Uehleke (445) indicated that with the technique of Uehleke and Brill (450), as noted, dogs pretreated with phenobarbital before one dose of 1-naphthylamine did not always excrete 1-naphthylhydroxylamine. Uehleke (445) likewise could not find the N-hydroxy derivative or the nitroso compound after incubation of 1-naphthylamine with microsomes from livers of rabbits pretreated with phenobarbital, a system with powerful capability to convert aromatic amines to the N-hydroxy derivatives. The discrepancy may reside in a problem of dosage. Thus, Radomski and Brill (372, 373) found that administration of large doses of 1- or 2-naphthylamine yielded detectable amounts of the corresponding hydroxylamines in the urine of dogs (fig. 3). On the other hand, administration of the amines at lower levels, which under chronic conditions led to cancer with the 2 isomer, gave rise to identifiable amounts of 2-naphthylhydroxylamine but not of the 1 isomer. Poirier *et al.* (365) also demonstrated a dose-related effect. Whereas dogs fed 500 mg of 2-naphthylacetamide had approximately 0.1% of

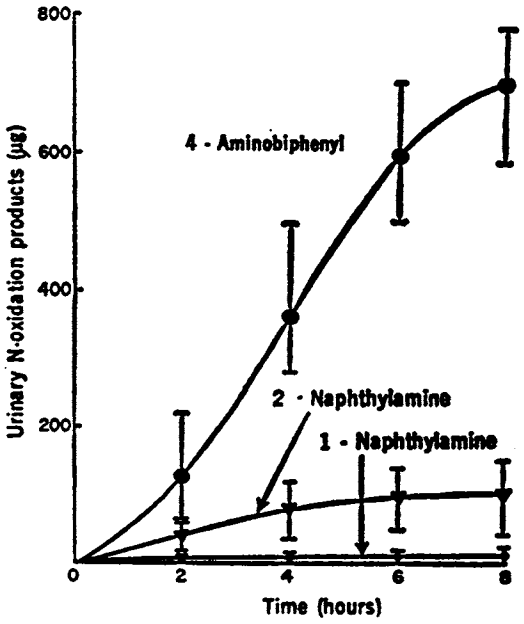


FIG. 3. Mean cumulative excretion of total N-oxidation products of four beagle dogs given single doses (5 mg/kg) of 1-naphthylamine, 2-naphthylamine, or 4-aminobiphenyl; ranges are indicated (372).

the N-hydroxy derivative in urine, none was detected when 131 mg were administered.

After a single dose of N-2-fluorenylacetamide rats excrete only small amounts of the N-hydroxy derivative in urine, chiefly as the glucosiduronic acid. Most of the metabolites are ring-hydroxylated compounds and their conjugates (468, 485). On the other hand, there are species differences in respect to the quantity of N-hydroxy metabolite. Of all animals surveyed the rabbit yields the largest amount of the glucosiduronic acid of N-hydroxy-N-2-fluorenylacetamide in urine (182). In part, this may be due to the fact that with a variety of drugs (497), but in particular with the glucuronide of N-hydroxy-2-fluorenylacetamide, the rabbit excretes much less in the bile and more directly *via* the renal pathway (200) (see table 1). Thus, in the rabbit, there is less opportunity for conversion of the metabolite by gut bacteria and modification by the enterohepatic cycle (142, 473, 495).

In addition to rats and rabbits, dogs,

TABLE 1

*Biliary and urinary excretion of metabolites of N-2-fluorenylacetamide and N-hydroxy-2-fluorenylacetamide in cholecystectomized rabbits with bile duct cannulae**

Compound Injected	Percentage of Dose Excreted in 24 hr as			
	N-Hydroxy-2-fluorenylacetamide glucuronide		7-Hydroxy-2-fluorenylacetamide glucuronide	
	Bile	Urine	Bile	Urine
N-2-Fluorenylacetamide	Trace	5.9 ± 0.54	2.1 ± 0.99	14.1 ± 1.72
N-Hydroxy-2-fluorenylacetamide	Trace	12.2 ± 1.56	0	1.6 ± 0.21

* Data from ref. 200.

hamsters, cats, mice, mastomys, monkeys, and men have been shown to excrete the glucuronide of N-hydroxy-N-2-fluorenylacetamide after single doses of the amide, thus demonstrating their capability of performing this reaction (table 2).

Of particular interest is the excretion of the N-hydroxylated metabolite by human beings (475). Five patients given identical tracer doses of isotopic N-2-fluorenylacetamide had differing quantities of the N-hydroxy derivative in their urine as the glucuronide. The other major metabolite was the 7-hydroxy derivative. Thus, the picture in man was like that in rhesus monkey (102) and rabbit (182). Man also acted like these two species, insofar as the major portion of the dose was excreted in urine, and only small amounts in the stools, suggesting that with these compounds man has relatively little biliary excretion in contrast to dog (365) or rat. Therefore, if human beings were ever exposed to sufficiently large amounts of such carcinogens, they would likely exhibit most tumors in the liver or the urogenic pathway. The variability in the quantities of N-hydroxy-N-2-fluorenylacetamide produced by the five individuals tested may find a parallel in the *in vitro* hydroxylation with microsome fractions in which Enomoto and Sato (105) found similar quantitative differences. Such variability was also encountered by Belman *et al.* (35) and Troll and Belman (429) who examined the excretion of N-hydroxy derivatives by men given large doses of several aromatic amines and derivatives. Free N-hydroxy-N-acetyl derivatives and the glucuronic acid conjugates were determined by a method based on the formation of copper chelates in a chloroform extract of the urinary metabolites, a method not as specific as the chromatographic procedures used by Weisburger *et al.* (475). Benzidine exhibited the highest total excretion of such metabolites, amounting to 2% (range 0.24 to 6.1) of a dose of 200 mg per person. Lower levels were found with 3,3′-dichlorobenzidine, 1-naphthylamine, 2-acetylaminonaphthalene, acetanilide, 2-naphthylamine, and lowest with phenacetin. The individuality in N-hydroxylation in different persons may account for their own peculiar sensitivity or resistance to such carcinogens or agents as regards their biological, pharmacological, or toxicological response generally.

By specific chromatographic techniques on 24-hr samples of urine, only trace amounts of N-hydroxy-N-2-fluorenylacetamide, or none, were observed in a strain of steppe lemmings (480), guinea pigs (324) or rainbow trout (277) administered N-2-fluorenylacetamide. However, Kiese *et al.* (232) and Kiese and Wiedemann (236) reported the occurrence in guinea pig urine of 2-fluorenylhydroxylamine, identified as the nitroso compound, after large doses of 2-fluorenamine. The amount found was small, and it was present mostly in the first few hours after administration of the amine. Also it seemed important to collect the urine freshly at low temperature and at pH 4.5, where

TABLE 2

N-Hydroxylation of N-2-fluorenylacetamide in several species

Species	Carcinogenic Effect of N-2-Fluorenylacetamide	Reference	Percentage of Dose Excreted in Urine as N-Hydroxy Derivative	Reference	N-Hydroxylation *in Vitro* by Liver	Reference
Cat	+		1.5	479	+	184
Chicken	+				+	184
Dog	+	324, 468	5.2	365	+	184
Guinea pig	−		0	324	0	184, 271
Hamster	+		5	324	+	184, 271
			15–20	478		
Human	?		4–14	475	+	105
					0	184
Mastomys			2.3	509		
Monkey	?	97	0.6–2.7	97, 102		
Mouse	+	488	1.8–2.3	324, 328	+	184, 271
Rabbit	+		13–20	182	+	48, 183, 184, 271
Rainbow trout	− (Minimal)	158	Not detected	277	Not detected	277
Rat	+	488	0.3–15*	328	±	184, 271
Steppe lemming	−	45	Trace	480		

* After chronic intake.

the hydroxylamino derivatives are most stable. After injection of N-2-fluorenylacetamide into guinea pigs, Kiese and Wiedemann (235) saw no evidence for excretion of the N-hydroxy derivative as such or as glucuronide (as also reported by other investigators). Again a small portion of the dose was present early after the injection as the corresponding hydroxylamine. Kiese and Renner (231) as well as Irving (184) reported that guinea pig liver possesses substantial capability to deacetylate N-2-fluorenylacetamide or the N-hydroxy derivative, possibly accounting for the excretion of the hydroxylamine rather than the N-acetyl derivative.

After administration of 2-fluorenylhydroxylamine to dogs, no known acetylated metabolites were detected in urine by Poirier *et al.* (365), in contrast to the situation in rats (481). The former group also established that 2-fluorenamine failed to yield any of the N-acetylated metabolites, in contrast to rats, and furthermore by isotope techniques confirmed that arylamines cannot be acetylated by dogs which are deficient in acyltransferase. The acetyl derivative, N-2-fluorenylacetamide, did undergo N-hydroxylation.

Just as blocking the *p*-position in aniline with chlorine leads to more extensive N-hydroxylation (212, 233), the substitution of fluorine in the 7-position in N-2-fluorenylacetamide, one of the major ring positions subject to hydroxylation, also leads to increased N-hydroxylation and consequently enhanced carcinogenicity in rats (316). Likewise, N-2-phenanthrylacetamide was converted to an N-hydroxy derivative which was more carcinogenic than the parent compound.

2. *Modifying factors.* With most aromatic amines for which N-hydroxylation was demonstrated, there have been relatively few additional studies on environmental or other factors controlling or modifying N-hydroxylation, except possibly for tests on the effect of enzyme inducers such as phenobarbital or methylcholanthrene, or *in vitro* studies with select inhibitors, already described in part.

However, in respect to the urinary ex-

cretion of N-hydroxylated metabolites, a major effort was exerted with the carcinogen N-2-fluorenylacetamide and related compounds to relate N-hydroxylation to the biological effect. As mentioned previously, a single dose of N-2-fluorenylacetamide results in the urinary excretion of only small amounts of N-hydroxylated derivative. However, upon continuing intake of the carcinogen, Miller *et al.* (328) found progressively larger amounts as glucuronic acid conjugates in urine. Interestingly, in rats the simultaneous administration of 3-methylcholanthrene failed to yield this increase; the amount of N-hydroxy derivative remained low, whereas some of the ring-hydroxylated, particularly the 3- and 5-hydroxy, derivatives were increased appreciably (fig. 4). This same picture also prevailed when 2-fluorenamine as well as the more carcinogenic N-(7-fluoro-2-fluorenyl)-acetamide were fed to rats. In both cases methylcholanthrene prevented the increased excretion of N-hydroxy derivative. The slightly carcinogenic N-2-fluorenylformamide gave only low levels of N-hydroxy derivative, with or without additional 3-methylcholanthrene.

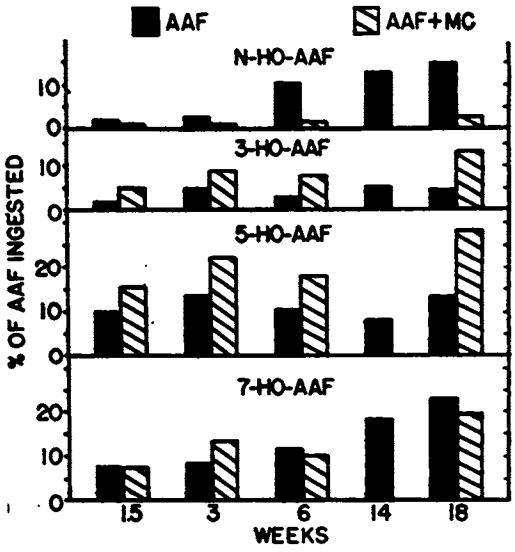


FIG. 4. Urinary excretion of the hydroxy derivatives of N-2-fluorenylacetamide (AAF) during feeding the carcinogen, with or without the addition of 3-methylcholanthrene.

Each figure represents the average excretion for 24 hr for four rats (328).

In contrast to the situation seen with N-2-fluorenylacetamide in rats, decreasing levels were found when rats were fed 4-acetylaminobiphenyl or even the N-hydroxy derivative itself (334) (see fig. 2). Moreover, the urinary level of N-hydroxy derivative was not affected by the joint administration of dietary methylcholanthrene which has such a pronounced influence with N-2-fluorenylacetamide, as discussed previously. Confirming these metabolic data, the addition of methylcholanthrene has only slight modifying action on the carcinogenicity of 4-acetylaminobiphenyl and its N-hydroxy derivative, in contrast to the sharp depression with the fluorene carcinogen.

Rabbits also excreted more N-hydroxy metabolite upon chronic intake of N-2-fluorenylacetamide, with a peak between days 3 and 23 (182). On the other hand, although mice eliminated appreciable amounts of the N-hydroxy compound after a single dose, the amounts did not increase much upon continuing intake nor were they influenced greatly by the presence or absence of dietary methylcholanthrene. Of considerable interest is the fact that, whereas hamsters excreted an appreciable fraction of a dose of N-2-fluorenylacetamide as the glucuronide conjugate of the N-hydroxy derivative (478), administration of methylcholanthrene increased quite considerably the amount of N-hydroxy derivative found in urine according to Lotlikar *et al.* (271) and Enomoto *et al.* (104). These workers also found that continued feeding gave increasing levels of the N-hydroxy derivative at several time intervals over a 20-week period and somewhat lower levels thereafter. During the first 12 weeks simultaneous intake of methylcholanthrene also increased the excretion of the N-hydroxy derivative. Methylcholanthrene or N-2-fluorenylacetamide pretreatment of hamsters increased appreciably the *in vitro* yield of N-hydroxy metabolite with microsomes or a 9000 $\times$ *g* supernatant of

hamster liver. As may be expected, therefore, in contrast to the situation with rats, the joint administration of methylcholanthrene and N-2-fluorenylacetamide increased the yield of tumors in hamsters (104). The mechanism of the differential effect of methylcholanthrene in rats, hamsters, or other species is not clear.

Chronic phenobarbital intake (503) or injection for 7 days (301) increased the excretion of hydroxylated metabolites of N-2-fluorenylacetamide but particularly of the N-hydroxy compound. Microsomes obtained from rats given phenobarbital for 7 days yielded more than twice the amount of N-hydroxy derivative *in vitro*. Nonetheless, we found that the amount of label from [9-^{14}C]N-2-fluorenylacetamide in the liver or bound to DNA, RNA, or proteins of phenobarbital-treated rats was about 50% that of the control rats. The key effect appears to be induction of glucuronyltransferase, increasing the excretion in urine of glucosiduronic acids, particularly that of N-hydroxy-N-2-fluorenylacetamide. Sulfate esters, in contrast, are lower. In possible conformity, Wyatt and Cramer (503) and Peraino *et al.* (362) reported that the phenobarbital treatment diminished the carcinogenicity of the agent. Curiously, phenobarbital following carcinogen increased carcinogenicity (362). An inhibitory effect on the carcinogenicity of both N-2-fluorenylacetamide and N-hydroxy-2-fluorenylacetamide was also found with the antioxidant butylated hydroxytoluene. Biochemical studies confirmed that, as with phenobarbital, much larger amounts of the N-hydroxy derivative were excreted more rapidly as the glucuronide (455).

The sizable production of the N-hydroxy derivative of N-2-fluorenylacetamide in rats pretreated with this agent led to the question whether N- and ring-hydroxylation involved the N-acetyl compound as such or whether prior hydrolysis to the amine took place (328). Indeed, in connection with studies of the metabolism of N-2-naphthylacetamide, it had been thought that the 6-hydroxy derivative arose from hydroxylation of the acetylamino derivative but that the 1-hydroxy derivative was derived from hydroxylation of the amine (496, p. 462). By determining the dilution of the label from acetyl-labeled N-2-fluorenylacetamide in the hydroxylated derivatives found in urine of rats prefed unlabeled compound, it was concluded that the major portion of the hydroxylated derivatives resulted from the direct hydroxylation of the amide. Thus, the urinary N-2-fluorenylacetamide and the N-, 3-, 5-, and 7-hydroxy derivatives retained half or more of the radioactive acetyl group. The 1-hydroxy compound had only one-third, possibly resulting somewhat more from the hydroxylation of the amine. However, it was also shown that the *o*-hydroxy derivatives may in part stem from an isomerization of the N-hydroxy derivative, as discussed elsewhere in this review.

Increased levels of urinary N-hydroxy derivatives of N-2-fluorenylacetamide were also noted in rats with liver damage caused by a variety of agents such as thioacetamide, ethionine, 3′-methyl-4-dimethylaminoazobenzene, or even N-2-fluorenylacetamide (294), or after partial hepatectomy, after protein starvation followed by refeeding of high protein diet, after ingestion of thermally oxidized oils (which also increased the carcinogenicity (415), and also more in young compared to older rats. Ring-hydroxylation, especially at the 7-position, was also higher after these treatments but not as much and not invariably. Margreth *et al.* (294) noted relatively little increase after repeated small doses of carbon tetrachloride, but Weisburger and Weisburger (486) noted a doubling of the level of N-hydroxy derivative after a large dose of carbon tetrachloride. Simultaneous administration of carbon tetrachloride and N-2-fluorenylacetamide induced liver tumors faster than carcinogen alone (483, 488).

The increasing levels of N-hydroxylated metabolites of N-2-fluorenylacetamide excreted in urine upon continuing intake may

in part be due to progressive liver damage induced by this agent itself.

Also supporting this concept is the fact that continuing ingestion of other arylamines which do not exert a direct hepatotoxic effect, such as 4-acetylaminobiphenyl, failed to increase but rather decreased levels of N-hydroxy metabolites in urine. However, in addition the action of the bacterial flora in the gut needs to be considered. The effect of 4-acetylaminobiphenyl on bacterial flora is not known. However, N-2-fluorenylacetamide and the N-hydroxy derivative are bacteriostatic. Therefore, during continuing intake of the carcinogen there were sizable alterations in the bacterial flora in the lower intestine (495). Inasmuch as the bacterial flora participate to a considerable extent in the metabolism of the carcinogen, specifically with respect to hydrolysis of the glucosiduronic acid and reduction of the N-hydroxy derivative (see fig. 5), it has been postulated that these changes also influence the excretory pattern of metabolites in urine, as well as the circulating levels of metabolites available to target tissues. With the noncarcinogenic acetanilide there were changes in the pattern of urinary metabolites and binding to DNA, RNA, or protein of rats following chronic ingestion *versus* a single dose. However, no evidence for urinary excretion of N-hydroxyacetanilide was found even after continued feeding (144).

8-Hydroxyquinoline moderated the carcinogenicity of N-2-fluorenylacetamide (512) probably because of lowered N-hydroxylation (212). This finding deserves further investigation in light of the possible interference of this chelating agent with hemoprotein synthesis, and hence perhaps cytochrome P-450 levels. The development of enzyme activity for the N-hydroxylation of *p*-chloroaniline with increasing age of fetal and newborn rats follows that of P-450, thus implying some connection (454). Other data indicate, however, that N-hydroxylation may be P-450 independent (304, 448).

Hormonal factors have sizable effects in modifying the carcinogenicity of agents like N-2-fluorenylacetamide (467, 486). For this reason a number of studies were undertaken to relate such effects to the metabolism of the carcinogen and more particularly on the levels of N-hydroxylated derivatives. Lotlikar *et al.* (270) noted decreased levels of N-hydroxy-N-2-fluorenylacetamide in urine of adrenalectomized, hypophysectomized, young rats given N-2-fluorenylacetamide or diacetamide. Thyroidectomy had less effect. Although ligation of the bile duct of rats caused a 2-fold increase in urinary excretion of the N-hydroxy derivative after a dose of N-2-fluorenylacetamide, adrenalectomized-ligated rats excreted levels only slightly higher than did controls (272). Replacement therapy with cortisone, deoxycorticosterone, or adrenocorticotropic hor-

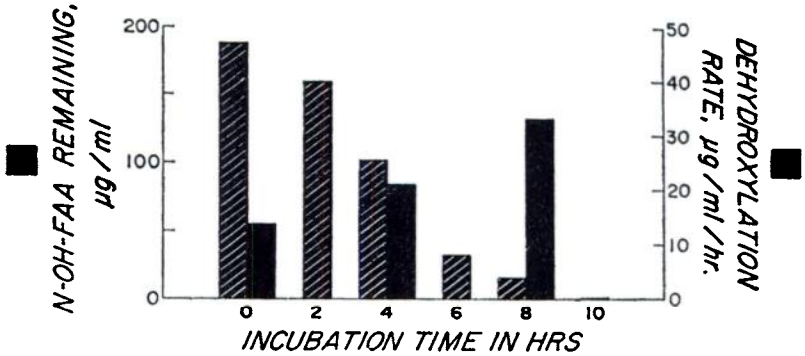


FIG. 5. Dehydroxylation of N-hydroxy-N-2-fluorenylacetamide (N-OH-FAA) by buffered filtrate of rat cecal contents at 30°. Substrate was reintroduced at 0, 4, and 8 hr after ether extraction for measurement of 1-hr rate changes.

Note the progressive increase in 1-hr rate of dehydroxylation as exposure time increases (495).

mone partially restored the excretion of N-hydroxy derivative. Weisburger *et al.* (476) also noted that the increasing levels of urinary N-hydroxy-N-2-fluorenylacetamide seen when N-2-fluorenylacetamide was fed to normal animals did not occur or took place to a decreased extent with hypophysectomized rats. The effect of these hormonal manipulations which reduce the carcinogenicity of N-2-fluorenylacetamide may be mediated by lower conversion to or maintenance of inadequate levels of the N-hydroxy derivative. As discussed elsewhere in this review, the ultimate activation step, sulfate ester formation (332), is also controlled by hormonal factors.

On the basis of the observation by Firminger and associates (358, 371) that the administration of relatively large amounts (2% in the diet) of chloramphenicol inhibited the carcinogenicity of N-2-fluorenyldiacetamide, the conversion of this agent to urinary N-hydroxy-N-2-fluorenylacetamide was examined. After intake over a 6-week period of carcinogen or carcinogen plus chloramphenicol, the latter group excreted 133% of the glucosiduronic acid of the N-hydroxy derivative compared to the controls fed carcinogen alone (484). More recently it was found that in rats prefed chloramphenicol alone for 4 weeks and given a single dose of carcinogen there were increased levels of N-hydroxy derivative as in untreated controls. In this case the changes may have been due to a dual effect on liver and on gut bacterial flora (143, 303).

As a sequel to these studies the effect of acetanilide and related anilines on the toxicity, carcinogenicity, and metabolism of N-2-fluorenylacetamide was determined (145, 507, 508). Thus, after feeding 0.8% acetanilide to rats, a single dose of N-2-fluorenylacetamide yielded a slightly increased excretion of N-hydroxy-N-2-fluorenylacetamide glucuronide in urine compared to untreated controls. However, after consuming a mixture of 0.8% acetanilide and 0.02% of the carcinogen, much lower levels of the N-hydroxy derivative were found in urine, compared to rats fed N-2-fluorenylacetamide alone (see fig. 6). It seems, therefore, that acetanilide given chronically may have a dual effect. It can increase N-hydroxylation of a small amount of other amides such as N-2-fluorenylacetamide. Under chronic conditions it protects the liver from extensive damage, thus reducing the extent of N-hydroxylation of the carcinogen, and it possibly competes successfully against limited amounts of another aromatic amine. Administration of the mixture of N-2-fluorenylacetamide and acetanilide decreased the toxicity of the carcinogen and delayed considerably the expression of the carcinogenic effect (508), as did several related toluidines and aminobenzoic acids (507). Relevant to the protective effect may be the discovery that aniline exerts a severe inhibitory effect on an acetyltransferase involved in converting an N-acetyl to a reactive O-acetyl arylamine (29, 241). Acetanilide and its metabolite 4-hydroxyacetanilide also inhibited mammary tumors in female rats from either N-2-fluorenylacetamide or the N-hydroxy derivative. Although a protective effect on the liver was also seen in mice and hamsters fed the carcinogens, forestomach and bladder tumors were not inhibited (490).

3. *N-Hydroxylation of aminoazo dyes.* A number of aminoazo dyes has been extensively studied because of their carcinogenic properties. One of the oldest known carcinogenic azo dyes, *o*-aminoazotoluene, presumably undergoes N-hydroxylation just as has been described for the classic arylamine derivatives. However, many compounds derived from *p*-aminoazobenzene are carcinogenic only if they bear at least one N-methyl group. An exception is the *o*-methoxy derivative of 4-aminoazobenzene, which is mainly active in extrahepatic tissues. Most of the dyes, typified by N,N-dimethyl-4-aminoazobenzene (or N,N-dimethyl-*p*-phenylazoaniline) affect chiefly rat liver. A number of earlier hypotheses on the activation of such carcinogens involved oxidation of the N-methyl group to hydroxymethyl (320), but a N,N-dialkyl-N-oxide has also been proposed (424). The N-oxide is as

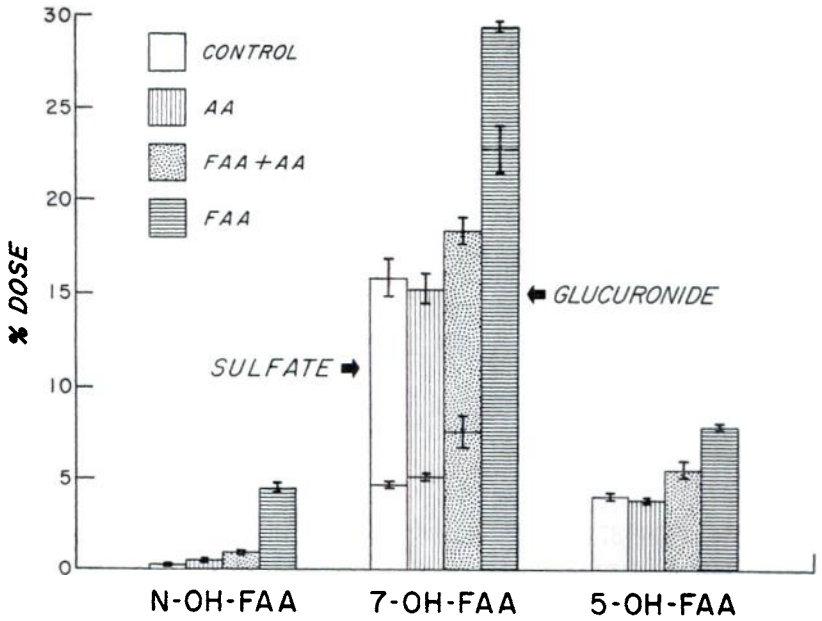


FIG. 6. Main ether-soluble metabolites, as determined by paper chromatography, in groups of rats fed 0.8% acetanilide (AA), 0.8% acetanilide + 0.02% N-2-fluorenylacetamide (FAA + AA), 0.02% N-2-fluorenylacetamide (FAA), or control diet (control) for 6 weeks, followed by a single dose of isotopic FAA.

N-Hydroxy-N-2-fluorenylacetamide (N-OH-FAA) and 5-hydroxy-2-fluorenylacetamide are excreted largely as glucuronides. 7-Hydroxy-2-fluorenylacetamide (7-OH-FAA) is present both as a glucuronide and as a sulfate (145).

carcinogenic as but not more than the N,N-dimethylamino compound itself.

Injection of N,N-dimethyl-4-aminoazobenzene or the monomethyl dye into rats led to excretion of small amounts as an N-acetyl-N-hydroxy derivative (382). Slightly larger amounts were noted after giving the N-acetylazo dye but not much more when the N-acetyl-N-hydroxy compound itself was injected, indicating that this metabolite is itself quickly converted to other materials. Mice or hamsters given the N-acetyl compound eliminated larger quantities of the N-hydroxy compounds than rats, paralleling their capability in this respect with the fluorene compounds. However, the N-acetyl or the N-acetyl-N-hydroxy metabolites of the azo dyes were not carcinogenic in rats. Therefore, N-hydroxylation with the simultaneous loss of the N-methyl groups does not constitute a metabolic activation pathway with this class of liver carcinogens. As discussed elsewhere, an unstable N-hydroxy-N-methyl azo dye is the likely carcinogenic intermediate. Just as with the fluorenamine derivatives, administration of a large molar excess of acetanilide inhibits the carcinogenicity of azo dyes (369, 506).

4. *N-Hydroxylation of urethan.* After the discovery of the importance of N-hydroxylation for the carcinogenicity and biochemical reactivity of certain aromatic amines, this concept was extended to urethan or ethyl carbamate. Tests with N-hydroxyurethan showed this compound to be as carcinogenic, or perhaps slightly less active, than urethan itself (36, 328). However, the situation is not clear, as reviewed by Mirvish (335). Metabolism studies, after injection of the N-hydroxy compound, led to the conclusion that urethan was the more prevalent product. On the other hand, N-hydroxyurethan, but not urethan itself, possesses mutagenic activity and biochemical reactivity and leads to methemoglobin formation (350).

Thus, the N-hydroxy compound and the N-acetoxy derivative reacted with nucleic acids and proteins. In this case the base attacked was chiefly cytosine (347). The salient reaction may be the conversion of urethan to an active intermediate, perhaps the N-hydroxy derivative, which could give an activated ethoxy carbonyl leaving group capable of interacting with molecular targets in the cell (51, 60). Such reactions are also postulated for certain of the carcinogenic pyrrolizidine alkaloids (84, 85, 307, 354, 385).

B. Metabolites in Bile

Excretion of metabolites of N-2-fluorenylacetamide in bile in sizable amounts was first reported by Weisburger *et al.* (469), and Irving *et al.* (200) have determined the biliary metabolites present as a function of time. The bile of rats injected with a single dose of N-2-fluorenylacetamide contained a fair proportion of the dose as the glucuronide of the N-hydroxy derivative. In contrast, under the same conditions only a small fraction of the dose was excreted in the urine as this conjugate. Likewise, after injection of the N-hydroxy derivative virtually all of the metabolite in the bile was the glucuronide of the N-hydroxy derivative. Furthermore, subcutaneous injection of the glucosiduronic acid of N-hydroxy-2-fluorenylacetamide resulted in the excretion in bile of rats of almost all of the injected compound, but in the urine only 64% was present as this material. After oral intake of the glucuronide, an even lower proportion of the total metabolites in urine was in the form of the compound administered (198). Adult male or female rats excreted about 30% of a dose of N-hydroxy-4-acetylaminobiphenyl in bile in 24 hr as the glucosiduronic acid. Under similar experimental conditions only about 20% of the analogous fluorenyl conjugate was found (193).

The metabolism and biliary excretion of N-2-fluorenylacetamide in rats was stimulated by pretreatment with benzo(a)pyrene but inhibited by piperonyl butoxide. However, neither agent influenced the biliary excretion of intravenously injected N-hydroxy-2-fluorenylacetamide (259). Thus, metabolism to a hydroxylated product was a key reaction modified by enzyme inducers or inhibitors, and this in turn controlled the biliary secretion of metabolites.

In the rabbit, injection of either N-2-fluorenylacetamide or the N-hydroxy derivative led to the excretion in bile of only small amounts of metabolites, in contrast to the rat. Williams *et al.* (497) have demonstrated that biliary excretion of drug metabolites in rats is in part a function of the molecular size and shape of the drug (398). The larger the molecule the more is excreted, often as glucuronic acid conjugates (315, 398). On the other hand, not many comparative studies have been performed in the rabbit.

von Jagow *et al.* (206) found 0.6% of a dose of 40 mg per kg of *p*-aminopropiophenone as a glucuronic acid conjugate of the hydroxylamino derivative, and only a small amount as free compound, in the bile of rabbits. The bulk again was excreted in the 1st hr. On the other hand no N-hydroxy derivative was seen after aniline, *p*-ethylaniline, or *p*-chloroaniline. Also, quite unexpectedly, considering the low urinary excretion of N-hydroxylated metabolites in urine, no N-hydroxy derivative was seen in the bile of dogs after *p*-chloroaniline or *p*-aminopropiophenone. Thus, one would surmise that the major portion of many drugs would not undergo biliary excretion in rabbits and rather would be eliminated from the liver *via* the blood, subsequent to which step renal filtration would transfer metabolites to the urine. This species difference may account for the fact that agents like N-2-fluorenylacetamide and the N-hydroxy derivative yield more cancer in the urogenic system in the rabbit, as compared to what holds in most strains of rat.

C. Metabolites in Blood

The pioneering discoveries of N-hydroxy compounds derived from aromatic amines and circulating in blood were made by Kiese and his associates and by Uehleke

who have published comprehensive reviews on this subject (224, 439).

Hustedt and Kiese (178) injected aniline intravenously into cats and dogs. About 20 min later they found the maximal amount of nitrosobenzene in the blood of cats which decreased relatively rapidly, reaching low levels 6 hr later. In dogs the concentration of nitrosobenzene dropped somewhat less rapidly. Likewise, after injection of acetanilide into cats or of N-acetylphenylhydroxylamine into dogs or cats nitrosobenzene was also noted in the blood. These tests provided the first instance of an oxidation of an aromatic amine or an aromatic amine derivative on the nitrogen. One comment on these results is based on the fact that the nitroso derivative was readily observed shortly after injection of an amine but became undetectable in a relatively short period. Also, the pattern cited reflected the sum of phenylhydroxylamine plus nitrosobenzene for the analytical technique of Herr and Kiese (167) used did not discriminate between these N-oxidation products.

Heringlake *et al.* (166) extended these results to a study of the fate of 2-naphthylamine in dogs and cats. Utilizing a spectrophotometric assay method 2-nitrosonaphthalene was found in the blood shortly after an injection of large doses of the amine.

Nitrosobenzene was also detected in a perfusate containing aniline and red cells, not only through isolated livers but also lungs of cats (233). Baader *et al.* (12) reported that the level of amines and of nitrosobenzene derivatives in the blood of dogs injected with aniline or substituted anilines increased somewhat more rapidly with 3-month- than with 8-month-old animals and also decreased more quickly in the younger group. Methemoglobin concentrations in the blood of dogs incubated with phenacetin or with phenetidine were a function of age, but here older dogs exhibited higher levels. In part, this finding may be related to the amount of N-hydroxy derivative formed, but the rate of elimination of the amine or the N-oxidation products seems to be more important. Thus, Bayerl and Kiese (32) noted that the blood level of nitrosobenzene rose more rapidly in dogs than in rabbits after intravenous infusion of phenylhydroxylamine, reflecting a higher rate of elimination of these compounds from the blood of rabbits, chiefly by reduction to aniline (234). Kiese and Renner (230) demonstrated unambiguously the presence of *p*-chloronitrosobenzene in the blood of dogs injected with *p*-chloroaniline with several analytical techniques. Kiese (223) extended these results to a number of aniline derivatives. As with aniline itself the highest concentration of nitrosotoluene was seen shortly after intravenous injection into dogs of *m*-toluidine. The *p* isomer yielded lower amounts per ml of blood, but with the *o*-, *m*-, and *p*-chloroanilines the para isomer gave the highest levels of nitroso derivative. Because *o*-chloroaniline was not extractable from the carbon tetrachloride solution by acid as required by the procedure of Herr and Kiese (167), the presence of oxidation products in blood could be detected only indirectly by methemoglobin formation, which was highest with *p*- and lowest with the *o*-chloroaniline. Likewise, the determination of nitroso derivatives was not performed with *p*-aminopropio- and butyrophenones, isomeric acetylaminophenylallyl ethers, or N-(β-hydroxyethyl)aniline. The N-oxidation of these arylamines was also estimated indirectly by the presence of oxidized hemoglobin. *p*-Aminopropiophenone was an excellent inducer of methemoglobinemia (140), but the ortho and meta derivatives had very low potency. Perhaps more sensitive analytical techniques, such as gas chromatography and mass spectrometry, would be helpful to detect and quantitate N-oxidation products of some of the arylamines for which the existing colorimetric techniques failed (126).

The procedure of detecting the presence of arylhydroxylamines or nitrosobenzene derivatives in blood by evaluating the

amount of methemoglobin present requires a word of caution. Indeed, it must be noted that some aromatic amines, even those subject to N-hydroxylation at an extensive rate, are not highly active in leading to methemoglobin formation. For example, 4-(2-methoxyethoxy)-3-acetylaniline and *m*-aminopropiophenone, even though readily hydroxylated, are relatively sluggish in methemoglobin formation in the dog again because under *in vivo* conditions facile reduction to the amines may occur (228).

von Jagow *et al.* (206) studied the blood levels of nitroso compounds after administration of *p*-ethylaniline, 2-fluorenamine, and 4-aminobiphenyl to dogs and the same compounds plus *p*-aminopropiophenone in rabbits. Shortly after the intravenous injection of the amines the corresponding nitroso compounds accumulated in blood. The rate of formation and maximum levels of nitroso compounds attained varied depending on the compounds and the species (figs. 7 and 8).

The N-oxidation of the carcinogen 4-aminobiphenyl, which had been an important industrial chemical, was examined in detail by Uehleke and Nestel (453). The nitroso derivative was readily detected, although relatively low levels were present, in the blood of cats given large amounts of this amine. By similar techniques, Uehleke (444) discovered *p*-nitrosophenetol in the blood of dogs fed the analgesic drug *p*-phenetidine. Increased levels were noted in dogs pretreated with phenobarbital.

A detailed study deals with the metabolites of the carcinogen N-2-fluorenylacetamide and the N-hydroxy derivative in the blood of rats (482). In addition to the presence of small amounts of free N-hydroxylation products, evidence was produced that the blood contained glucuronic acid conjugates of the N-hydroxy metabolite, possibly in some loose combination with plasma proteins. Bahl and Gutmann (13) and Deckers *et al.* (92, 93) noted that albumin and α-globulins were labeled after administration of the ^{14}C-labeled N-hydroxy compound. The red cells contained lower amounts of the conjugate and significantly also of the free N-hydroxy derivative than the plasma. Rapid reduction of N-hydroxy-2-fluorenylacetamide to 2-fluorenylacetamide was observed in the red cell. Such reductive processes were described with many N-oxy derivatives by Kiese and associates (225, 229) and by Uehleke (444, 453). Radioactivity from the labeled carcinogen was bound to plasma proteins as well as proteins obtained after lysis of the red cells. Earlier Jackson and Thompson

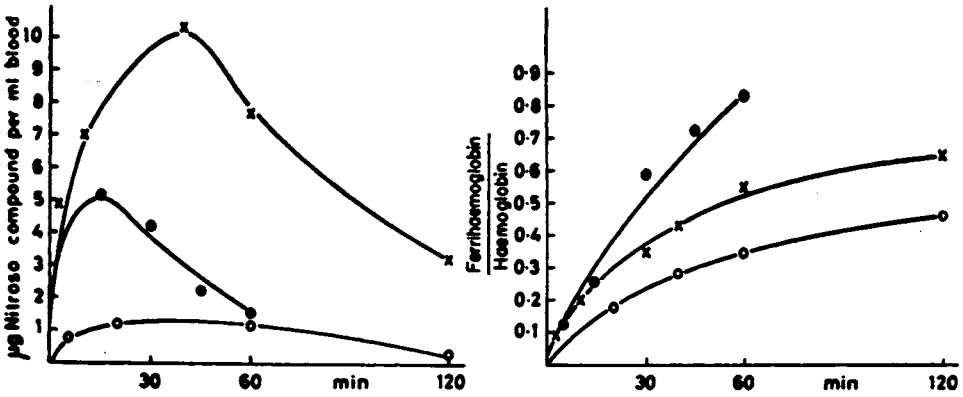


FIG. 7. Concentration of N-hydroxy derivatives and nitroso analogues (determined as nitroso compounds) and concentration of ferrihemoglobin in the blood of dogs after the intravenous injection of aromatic amines (206).

×, *p*-ethylaniline, 75 mg/kg, five experiments; ○, 2-aminofluorene, 100 mg/kg, one experiment; ●, 4-aminobiphenyl, 80 mg/kg, one experiment.

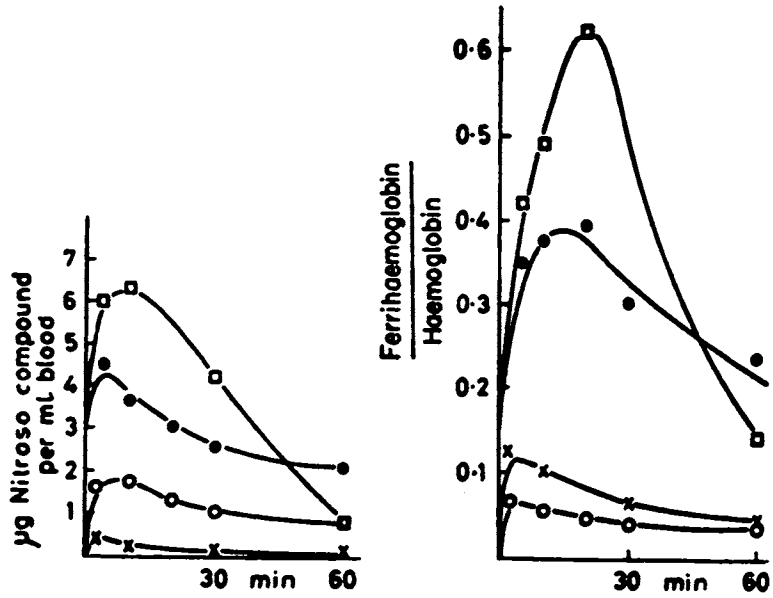


FIG. 8. Concentration of N-hydroxy derivatives and nitroso analogues (determined as nitroso compounds) and concentration of ferrihemoglobin in the blood of rabbits after the intravenous injection of some aromatic amines (206).

×, *p*-ethylaniline, 50 mg/kg, one experiment; ○, 2-aminofluorene, 100 mg/kg, four experiments; ●, 4-aminobiphenyl, 100 mg/kg, two experiments; □, *p*-aminopropiophenone, 30 mg/kg, four experiments.

(205) noted that label from isotopic *p*-iodophenylhydroxylamine was combined with red cell components. Uehleke and associates thought also that oxidative processes in blood, and particularly the red cell, led to the disappearance of arylhydroxylamine derivatives from the circulation. Beutler (38) recently summarized drug-induced anemias.

D. Methemoglobin Production by N-Hydroxy Compounds

It has been long known that aniline or nitrosobenzene cause methemoglobin formation *in vivo*, particularly under conditions of chronic exposure. However, the underlying mechanism did not become clear until the concept was developed that a major reason for the methemoglobinemia with these compounds was their biochemical conversion to N-hydroxy derivatives (224, 439). Methemoglobin formation is a function of the specific compound administered, of the species, and of environmental conditions modulating the main variants. In turn, explanations for differences in methemoglobin formation reside in the relative susceptibility of the compounds administered to conversion to N-hydroxy derivatives. Methemoglobin formation will be higher when there is higher production of arylhydroxylamines, although aminophenols or quinones can also lead to methemoglobin. Furthermore, an important consideration is the relative rate of the subsequent metabolism of arylhydroxylamines. For example, conjugation with glucuronic acid withdraws a compound from the pool of effective material which may be either the free compound or a more reactive ester form. While in some cases methemoglobin formation has been seen with nitrosoaryl derivatives, present evidence suggests that the nitroso compounds are active chiefly after reduction to the hydroxylamines.

The cat appears to be most susceptible to methemoglobin formation, possibly because of a deficient glucuronyltransferase system, so that more of the arylhydroxylamine circulates in reactive forms. The dog is likewise

fairly susceptible, perhaps because of its well known low acetylation capability. The rabbit, the rat, and the mouse yield lower methemoglobin titers as a result of their capacity to reduce methemoglobin. The response of man appears to be variable (389). Methemoglobin formation is often described in clinical case reports on accidental exposure to nitrobenzene or arylamine derivatives or analogues.

Under select experimental conditions some arylhydroxylamines are better methemoglobin formers than others. In part, such differences may be due to detoxification reactions to which these compounds are subject prior to reaching the center of methemoglobin production. Such reactions could be conjugation with glucuronic acid, acetylation, or reduction to the arylamines. We have noted that erythrocytes readily convert N-hydroxy-N-2-fluorenylacetamide to N-2-fluorenylacetamide. Also, in contrast to aniline, with acetanilide itself, there appears to be a self-limiting effect on methemoglobin formation, although in this case the mechanism has been ascribed to a depletion of the peroxide pool, thus giving methemoglobin indirectly.

Nitrosobenzene was demonstrated in the blood of cats shortly after intravenous injection of aniline or of acetanilide. Coincidentally there was considerable methemoglobinemia, more with acetanilide than with aniline (178). Phenylhydroxylamine, but much less so N-acetylphenylhydroxylamine (phenylacetohydroxamic acid) formed methemoglobin *in vitro* with red cells of ox, man, or dog in the presence of glucose and air. In cats and in dogs similar levels of nitrosobenzene and of methemoglobin formation were observed after injection of phenylhydroxylamine or N-acetylphenylhydroxylamine. This indicates that the acetyl group is readily removed in both species, although the reaction is somewhat slower in dogs.

Addition of a homogenate of liver increased enormously the rate of methemoglobin formation with phenylhydroxylamine or with the acetyl derivative. The reason may be that reductive enzymes enhanced the rate of arylhydroxylamine formation from the nitroso derivative. Under these conditions the cyclic oxidation-reduction systems giving rise to oxidized hemoglobin were facilitated (234).

m-Toluidine was converted to the nitroso derivative as was the *p* isomer (223). However, whereas methemoglobin formation after *o*- and *m*-toluidine was like that of aniline, the *p* isomer gave very little increase (fig. 9). Likewise, *p*-chloroaniline

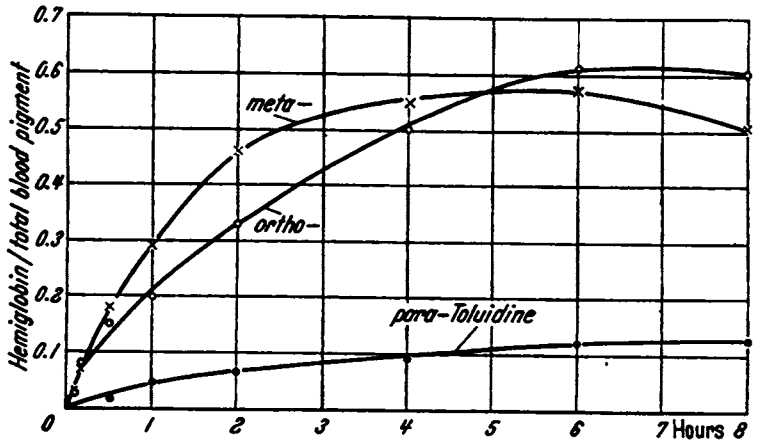


FIG. 9. Concentration of hemoglobin in the blood of dogs after the intravenous injection of 111.1 mg of toluidine hydrochloride per kg.

The symbols indicate the means of experiments on four dogs (223). O——O, *o*-toluidine; ●——●, *p*-toluidine; ×——×, *m*-toluidine.

gave appreciable levels of *p*-chloronitrosobenzene but relatively low titers of methemoglobin. On the other hand, whereas *p*-aminopropiophenone or the corresponding butyro compound led to considerable methemoglobinemia, no N-oxidation products could be detected in blood. Curiously, *o*- and *m*-aminopropio- or butyrophenones yielded only low methemoglobin values. Therefore, N-oxidation does not always parallel methemoglobin formation (228).

In dogs phenacetin and *p*-phenetidine yielded higher levels of methemoglobin compared to *p*-hydroxyacetanilide. The effect with these drugs and also with aniline was more pronounced in older dogs than in young animals. *p*-Nitrosophenetol was observed in the blood of dogs injected with phenetidine (12). 4-Aminobiphenyl, given orally to dogs, was most effective in developing methemoglobinemia, whereas an equivalent dose of 2-naphthylamine was much less active. 1-Naphthylamine produced only negligible levels of methemoglobin (373).

Following numerous reports (68, 69, 73, 227, 370) dealing with the mechanism of the iatrogenic effects seen with the important drugs phenacetin and *p*-phenetidine, Uehleke (444) documented the presence of N-hydroxylated metabolites in blood and urine of dogs given phenetidine. Pretreatment with phenobarbital increased the extent of the reaction. Although rats exhibited methemoglobinemia, they excreted little N-hydroxy metabolite in urine. N-Hydroxylation also occurred with liver or kidney microsomes from rabbits, and this reaction likewise was higher if the rabbits were injected previously with phenobarbital. Confirming Kiese *et al.* (234) with nitrosobenzene, the observation was made that *p*-nitrosophenetol catalyzes the rapid oxidation of hemoglobin in ox erythrocytes in the presence of microsomes or of a soluble fraction of liver with NADPH. In 5 min 1 molecule of nitrosophenetol led to the formation of 44 molecules of methemoglobin. Uehleke (444, 446) related the effects of phenacetin and phenetidine on the kidneys in sensitive subjects to the production of antigenic substances in this organ, by combination of reactive N-oxidized metabolites with macromolecules.

The presence of 2-nitrosonaphthalene was detected in the blood of dogs and cats after an injection of large amounts of 2-naphthylamine (166). This amine was less effective in methemoglobin formation than phenylhydroxylamine, perhaps because of the greater degree of secondary reactions with the red cells. 4-Nitrosobiphenyl and methemoglobin were noted in the blood of cats injected with large doses of 4-aminobiphenyl (453). Under *in vitro* conditions 4-nitrosobiphenyl led to methemoglobin formation in red cells of ox and cat, but again to a lesser degree than nitrosobenzene. Miller *et al.* (334) described the rapid onset of methemoglobinemia after intraperitoneal injection into rats of N-hydroxy-4-acetylaminobiphenyl, but not after 4-acetylaminobiphenyl. Lower levels of methemoglobin were noted upon continuing intake of the N-hydroxy compound.

It was reported recently that of certain substituted anilines, methemoglobin formation was most rapid with N-hydroxymesidine, a metabolite actually demonstrated in the urine of rats given mesidine Another metabolite of mesidine, 2,6-dimethylbenzoquinone, postulated as arising from the N-hydroxy derivatives, also exhibited appreciable methemoglobin-forming ability. Mesidine and pseudocumidine gave lower levels of methemoglobin after a lag period, probably because of delayed formation of N-hydroxy metabolites. The 2,4-, 2,5-, and 2,6-xylidines had decreasing potency in forming methemoglobin (265).

McLean *et al.* (309) examined methemoglobin formation in cats injected intravenously with a large number of substituted aniline derivatives, including the 2-, 3-, 4-methyl and ethyl derivatives, a number of dimethyl analogues as well as the fluoro-trifluoromethyl-, chloro-, bromo-, and polyhalogenated anilines, the isomeric methoxy

and ethoxy and hydroxymethyl compounds. In part the level of methemoglobinemia, which usually peaked at 1 to 3 hr after administration of the amine, was related to the rate of N-hydroxylation of the compounds. However, there were additional considerations such as the relative ease of conversion between nitroso- and hydroxylamine derivative and the reactivity of these compounds in the hemoglobin-methemoglobin equilibrium. With most of these compounds the effect was dose-related. The activity of aniline was enhanced by substitution at the 4-carbon except with methyl or methoxy which presumably gave rise to the less potent aminobenzoic acid or other derivatives. Ortho or meta substitution made little difference or decreased potency, whereas polysubstitution usually reduced the effect. SKF-525A failed to alter the methemoglobin level after aniline, suggesting further that enzymic N-hydroxylation is distinct from ring-hydroxylation of aromatic molecules. McLean *et al.* (309) did not feel that steric effects around the amino group were important. Nonetheless, whereas 2,4,6-trimethylaniline is hepatotoxic and possibly carcinogenic, the N-acetyl derivative is not, presumably due to steric effects involving N-hydroxylation (472). Prilocaine was found to be a potent methemoglobin former in man (170). There were important individual variations, but the mean peak values were dose-related. Methylene blue, but not ascorbic acid, prevented methemoglobinemia or reduced the level of oxidized hemoglobin.

Methemoglobin formation by aniline derivatives was affected by other agents. Anesthesized cats exhibited lower methemoglobin levels than unanesthesized cats treated with certain aromatic amines (308) because the anesthetic agents used induced enzymes which activated C- rather than N-hydroxylation. Therefore, a given dose of arylamine yielded lower levels of arylhydroxylamines which in turn formed less methemoglobin. Heinz body formation appeared to be based on a different mechanism from methemoglobin production. Ascorbic acid injected into rats at the same time as aniline reduced the maximal methemoglobin levels somewhat but increased significantly Heinz body formation (286). *In vitro* ascorbic acid also failed to affect methemoglobin formation by phenylhydroxylamine (46).

Methemoglobin levels depended not only on the rate of synthesis but also on the rate of reduction. In part these processes are controlled by systems involved in glucose metabolism which indirectly affect the levels of cofactors, NADPH and ATP, required for methemoglobin formation (71, 72, 451). For example, in pigs methemoglobin formation was low because of a poor rate of glucose utilization, whereas in the cow the limiting factor was a deficient enzyme activity controlled by depressed NADPH and ATP levels (459). There are coupled cyclic processes whereby arylnitroso and hydroxylamino derivatives in the presence of oxygen evoke methemoglobin formation in the presence of an energy source. Thus, under suitable conditions, a small amount of arylhydroxylamine can give sizable amounts of methemoglobin. Occasionally methemoglobinemia can be an important adverse reaction in susceptible patients administered drugs containing aromatic amine groups which can transform into arylhydroxylamines (171). In addition to the direct demonstration of N-hydroxylation in man (35, 475), the occurrence of methemoglobinemia provides highly suggestive evidence for the production and presence of arylhydroxylamine derivatives in man exposed to the amines.

Methemoglobin formation is often a good index of the production of N-hydroxy derivatives of amines under *in vitro* conditions (437, 438). N-Oxidation of sulfanilamide or of dapsone (4,4′-diaminodiphenylsulfone) was also followed by the development of methemoglobin in added bovine red cells (426). Dapsone was about twice as active as aniline (171, 425). By incubation with a microsomal fraction of rat liver (203),

dapsone was converted to a hydroxylamino derivative which was a potent methemoglobin former. This latter reaction was even faster in the presence of glutathione or of NADPH (245). A series of related *p*-substituted diphenylsulfones were all less active. In dogs, the 4,4′-dihydroxylaminodiphenylsulfone was a most active generator of methemoglobin, as might be expected (500). The *in vitro* N-oxidation mediated by mucosa from pig bladder of *p*-chloroaniline, 4-aminobiphenyl, 2-naphthylamine, aniline, and 2-fluorenamine decreased in that order as measured by methemoglobin formation in bovine red cells. This system was a simple detector for N-oxidation of phenetidine with rabbit liver microsomes (440, 444).

Aliphatic N-hydroxy derivatives, typified by ethyl N-hydroxycarbamate (or N-hydroxyurethan) also led to methemoglobin formation in air but not in an inert atmosphere (350). The reaction proceeded only in the presence of the N-hydroxy compound, but the concentration of the chemical remained constant. Even so, the rate of methemoglobin formation was proportional to the concentration of ethyl N-hydroxycarbamate. A number of related N-hydroxycarbamates, like the methyl or the n-propyl esters had similar catalytic properties, but hydroxyurea did not. Incubation of human or dog hemoglobin with urethan failed to give methemoglobin unless mouse or rat liver slices were also present, perhaps indirect evidence for the formation of the N-hydroxy derivative under these conditions.

V. N-Hydroxylation in Vitro

This subject has been discussed extensively in several recent reviews (10, 187, 448, 489). Hence only a brief summary of the salient features of N-hydroxylation of aromatic amines *in vitro* is presented here. Like other biochemical hydroxylation reactions, N-hydroxylation of aromatic amines and derivatives is mediated by a complex enzyme system located on and bound to the endoplasmic reticulum of cells. When cells are broken by homogenization techniques, the enzymes are found mainly in the microsomal fraction. They require the presence of NADPH, or less effectively a NADPH-generating system, and exhibit an optimum reaction rate about pH 7.5 to 8 in a buffer system such as Tris or phosphate. *In vitro* hydroxylations are usually linear with time for 30 min or less.

Enzyme systems performing N-hydroxylation are also highly inducible. Of all inducers, phenobarbital pretreatment appears best. Next are DDT and similar chlorinated hydrocarbons, and least effective as inducers are polycyclic aromatic hydrocarbons like 3-methylcholanthrene. Phenobarbital yields the highest levels of N-hydroxylation enzymes in hamsters and rabbits but is somewhat less effective in other rodents like mice.

The enzyme system performing N-hydroxylation of aromatic amines appears to be somewhat different from other such oxidation reactions in a number of respects.

A. Species

Relatively few arylamines or derivatives have been studied in many species. However, typically microsomal fractions of livers from hamsters or rabbits are most active; those of dogs, chickens, pigs, mice, rats, cats, and oxen are decreasingly active (54, 87). With some substrates, but not with others, liver microsomes from guinea pig exhibited some activity (87, 232, 271, 446), and more research on this point is desirable. Microsomes from human liver appear to be very variable, depending on as yet unclear factors (table 2).

B. Tissue

Depending on species, liver is usually the most effective tissue, but some activity is also associated with microsomes of kidney, lung, bladder, and small intestine (435, 443).

C. Stability and Modifiers

The enzyme system is fairly stable when microsomal fractions are stored at low temperature. Usually the reaction is inde-

pendent of carbon monoxide, SKF-525A, or 2,4-dichlorophenol. However, 8-hydroxyquinoline, iproniazid, and metyrapon (2-methyl - 1,2 - di(3 - pyridyl) - 1 - propanone) (449) are strong inhibitors, whereas cyclohexane is less effective (452). α,α-Dipyridyl, penicillamine, cupric chloride, or *p*-chloromercuribenzoate have small inhibitory capability (6, 211, 213–215, 436, 437). Of all the enzyme inducers studied, phenobarbital, especially after repeated administration, stimulates N-hydroxylation very considerably, mostly with rabbit liver and to some extent with rat, dog, and cat liver (256, 257, 441, 444). Although pretreatment of rats with 3-methylcholanthrene also stimulated N-hydroxylation of 2-fluorenylacetamide by liver microsomes, ring-hydroxylation was increased to a greater extent (268). However the situation was reversed in hamsters where N-hydroxylation was greater (304). Toxic agents, for example, thioacetamide, can decrease significantly the various enzyme levels and the cytochromes P-450 and b_5 (24).

D. Structure Requirements

N-Hydroxylation is highly stereospecific. With the same microsome preparation from a given species and tissue the structure of the arylamine substrate largely determines the quantitative aspects of the reaction. Further substitution of the arylamine by a group in a para or equivalent position makes a better substrate than an ortho substituted amine (441, 448). Bulky ortho substituents as in 2,6-dimethylacetanilide, decreased the reactivity, while on the other hand, a blocked para position reducing ring-hydroxylation at that point increased N-hydroxylation as in *p*-chloroaniline or 7-fluoro-N-2-fluorenylacetamide (184). N-Hydroxylation has in common with other biochemical oxidation reactions the fact that modifying treatments may also specifically affect N-hydroxylation. However, differential effects are apparent which are discussed in detail elsewhere (134, 489). Not all cytochrome systems, for example, may be involved directly to the same extent in N- and C-hydroxylation, as are for example, cytochrome b_5 or the ethyl isocyanide-reactive compounds 430 or 434. There is even some controversy whether cytochrome P-450 is part of the chain leading to N-hydroxylation (257).

We feel that the new concept that these membrane-bound enzyme systems effecting a variety of biochemical oxidations, including *N*-hydroxylations, might be constituted by a series of isozyme-like systems deserves further investigation. Certainly in the field of the soluble enzymes. like lactic dehydrogenase, aldolase, and pyruvic kinase, enzyme systems with varying kinetic and substrate specificities have been demonstrated. In view of the progress in solubilizing some portions of the membrane-bound oxidative enzyme systems (128, 282–284), the possible existence of such isozymes can be investigated. Such studies are potentially rewarding, especially for those systems relating to N-hydroxylation of aromatic amines, which is an important activation reaction leading mainly to products with adverse effects in animal and human systems. Precise knowledge of the mechanisms controlling these reactions may lead to practical ways of controlling their deleterious actions.

VI. Formation of N-Hydroxy Derivatives by Biochemical Reduction

N-Oxidation is one important method of production of N-hydroxy derivatives of aromatic amines. Other ways of obtaining them are briefly discussed, especially those corresponding to arylamines. This subject area has been reviewed in detail elsewhere (133, 489).

It had been known for a long time that exposure of animals or man to nitrobenzene derivatives was followed by disturbances of the hematopoietic system similar to those seen with the aromatic amines. It was thought that the reason was a reduction of the nitro compounds to the amines. However, newer views indicate that the active entity is actually an arylhydroxylamine.

Experimental evidence demonstrated that nitro compounds are reduced stepwise to arylamines *via* nitroso and hydroxylamino derivatives. Judged by a biological or pathological effect, the nitro analogues of the carcinogenic aromatic amines are also carcinogens and often exhibit about the same organotropism and effects as the amines when administered under similar conditions. In addition, and typically, the nitro compounds induce tumors of the forestomach upon feeding to rodents, suggesting that reduction to active intermediates like the hydroxylamino derivatives can occur in the stomach. In addition, reduction of aromatic nitro compounds by microorganisms in the gut has also been demonstrated (383).

Many studies on the mechanism of the reduction were performed with rat liver. It was first believed that reductase activity converting nitrobenzene derivatives to arylamines was present in the soluble fraction of a rat liver homogenate. However, subsequent tests documented that the reduction involved at least a two-step sequence, involving both a microsomal and a soluble fraction.

Because of the ease of analysis many experiments utilize *p*-nitrobenzoic acid. However, this substrate has a low pK_a and is fully ionized under most conditions. Membrane transport and similar features may develop an impression of lower enzyme activities with this material than with less polar nitro compounds, typified by drugs, carcinogens, and the like.

With rat liver microsomes and under anaerobic conditions, NADPH acts as a cofactor, but the reaction is stimulated by flavine mononucleotide and is inhibited severely by oxygen. When the cofactor is NADH, little reduction of nitro compound is seen with microsomes but when the hydroxylamino compound is the substrate, the arylamine is formed. In the latter reduction, flavine mononucleotide exerts relatively little effect.

Investigation of the rate of reduction of nitroaryl compounds by the 10,000 × *g* supernatant fraction from rat liver indicated that this conversion was much slower than the further reduction of the corresponding hydroxylamino derivatives to the arylamines. Thus, with these substrates, it is difficult to demonstrate the presence of the intermediary arylhydroxylamines (368). In contrast, with 4-nitroquinoline-1-oxide, the situation is the reverse, and the hydroxylamino compound accumulates. The microsomal reduction to both hydroxyamino and amino derivatives is enhanced by phenobarbital or methylcholanthrene pretreatment in the NADPH-microsome but not the NADH-microsome mediated reaction. Reduction by the supernatant fraction with either cofactor was not stimulated.

The carcinogen 4-nitroquinoline-1-oxide is reduced also to a hydroxylamino derivative, the probable active carcinogenic intermediate, which in turn goes on to the amine, as is true with model compounds such as nitrobenzoic acid. Further, an enzyme with the properties of liver "DT-diaphorase," present mainly in soluble fractions (110), reduces this compound. An excellent summary of chemical, biophysical, metabolic, and various biological studies with 4-nitroquinoline-1-oxide and related compounds, including the hydroxyaminoquinoline-1-oxides, is now available (101).

Not much is known on the biochemical reduction of aliphatic nitro compounds but it would seem logical that such chemicals undergo reduction by steps analogous to those seen with the aryl compounds. Also, these biochemical reactions may be related to the well known inorganic nitrogen cycle between nitrate, nitrite, hydroxylamine, and ammonia best studied in microbiologic systems.

VII. Formation and Reactions of Tertiary Amine and Heterocyclic N-Oxides

The synthesis, chemical, physical, and pharmacological properties of aromatic N-oxides, with emphasis on heterocyclic

N-oxides, is a field in which Ochiai (355) and collaborators have pioneered. Also, the biochemistry of naturally occurring N-oxides, the pharmacology and toxicology of N-oxides, the formation of such compounds during drug metabolism, and the various processes involved in metabolism of N-oxide drugs have been discussed recently (39, 41, 66, 101, 310, 489, 518). In some cases, N-oxides were postulated as intermediates in the dealkylation of N,N-dialkylarylamines, but as discussed elsewhere this concept appears untenable. The oxidation of dialkylarylamines to the corresponding N-oxides involves a microsomal liver preparation, requiring NADPH and oxygen. In rats, most of the enzyme activity was located in the liver, but in pigs activity was seen in kidney and lung. The livers of other species such as calf, rabbit, cat, bat, armadillo, brown squirrel, opossum, racoons, and a number of reptiles and birds also had N-oxidase activity. Several types of fish, frogs, and the English sparrow were less active. Cytochrome P-450 content failed to parallel the extent of N-oxide formation and the reaction *in vitro* and *in vivo* was not sensitive to SKF-525A, but the subsequent N-dealkylation step was, thus allowing N-oxides to accumulate. However, SKF-525A failed to alter the amount of N-oxides in urine when the N,N-dialkylaminoaryl N-oxides themselves were injected.

The enzyme system from pork liver microsomes was purified by a series of steps, including Triton extraction, protamine precipitation of contaminating proteins, ammonium sulfate precipitation steps, and finally a Sephadex column fractionation (518). The oxidase is a flavoprotein, with FAD as a prosthetic group, and requires NADPH and oxygen. Cytochrome P-450 and monoamine oxidase activity were absent, and the cytochrome b_5 content was small, variable, and of uncertain specificity. It is probable but not fully established that this system is dissimilar from the enzymes performing N-hydroxylation of primary or secondary arylamines or amides (8, 40).

VIII. Biochemical Reactions of N-Hydroxy Compounds

A. Formation of Glucuronic Acid Conjugates

Just as is true for phenolic compounds or aryl ring-hydroxylated derivatives, N-hydroxy derivatives conjugate readily with glucuronic acid, presumably through a reaction catalyzed by glucuronyltransferase to form glucosiduronic acids (187, 188). This type of conversion predominates in all species investigated except cats, where it occurs only to a small extent (479).

In addition to N-hydroxylation the formation of polar derivatives was noted when a 9000 × *g* supernatant of rabbit liver instead of washed microsome preparations was incubated with N-2-fluorenylacetamide (184). These polar derivatives yielded the N-hydroxy derivative after hydrolysis with β-glucuronidase. It must be assumed that in this instance the formation of the glucosiduronic acid was competitive with other side reactions such as deacetylation or reduction of the N-hydroxy compound. Species and other host factors controlling glucuronide formation with N-hydroxy derivatives may depend on the relative rates of these competing reactions. With other substrates and enzyme sources the desired N-hydroxy product is often reduced to the amine by a rapid reaction catalyzed by a soluble enzyme system in a 9000 × *g* fraction of liver (223, 232, 438). Also, Booth and Boyland (48) have described a soluble isomerase which converts N- to *o*-hydroxy amines.

The glucosiduronic acids of N-hydroxy derivatives of arylamines can be hydrolyzed readily by mammalian or bacterial β-glucuronidase under conditions similar to those under which ring-hydroxylated glucosiduronic acids are split (83, 179, 184, 495). No attempts are recorded to hydrolyze such glucosiduronic acids by the older acid procedures, probably because acid hy-

drolysis would give undesirable, unstable by-products, consequent to formation of reactive arylhydroxylamines.

Irving and Wiseman (198) reported that when labeled biosynthetic glucosiduronic acid of N-hydroxy-2-fluorenylacetamide was given to rats subcutaneously or intraperitoneally approximately 50% of the dose was in urine and 20% in feces. However, after oral intake only 33% was in urine and almost 40% in the feces. Thus the specific metabolites were related to the route of intake; the least alteration was seen after subcutaneous injection. This dependence on the path of entry is explained satisfactorily by the demonstration by Williams *et al.* (495) that the glucosiduronic acid is hydrolyzed readily by the bacterial flora in the gut and that the compound liberated undergoes substantial further changes. Among others reduction of the N-hydroxy derivative of N-2-fluorenylacetamide and resorption in the lower portion of the gastrointestinal tract lead to further metabolism. The data of Irving and Wiseman (198) and of Weisburger *et al.* (473) suggest that the glucosiduronic acid is absorbed from the gastrointestinal tract much less readily than the free unconjugated compounds. The mode of transport across tissue and cell membranes of glucosiduronic acids is not well known, but considering the biological and pharmacological importance of these metabolites, it deserves detailed examination.

B. Formation of O-Acetates

Apart from the high carcinogenicity of N-acetoxy-N-2-fluorenylacetamide, this synthetic compound has also proved useful as a model for investigations on the reactivity of such agents towards potential molecular and cellular targets (331–333).

A comparative study by Lotlikar and Luha (273) on the nonenzymic acylation of N-hydroxy-2-fluorenylacetamide by acetyl coenzyme A, carbamoyl phosphate, or acetyl phosphate showed acetyl coenzyme A was by far the best acylating agent. Substantial evidence was adduced that the product formed was N-acetoxy-2-fluorenylacetamide (274). In contrast, with N-hydroxyamides derived from stilbene, phenanthrene, or naphthalene, the reaction went several times better with carbamoyl phosphate than with acetyl coenzyme A (273).

The enzymic esterification of N-hydroxy-N-2-fluorenylacetamide and other hydroxamic acids by rat liver cytosol apparently varied markedly with the hormonal status of the animals. Liver from male rats had twice the activity of that from females; hypophysectomy decreased the level to that usually observed in female rat liver. Other endocrine ablations or treatments were less effective (269). These enzymic studies reinforce the report by Goodall (136) who found that hypophysectomy inhibited liver cancer induction by N-hydroxy-2-fluorenylacetamide.

Different from the system of Lotlikar (273, 274) seems to be one described by Booth (47) who reported on a specific enzyme system which could transfer acetyl groups from N-hydroxy-N-acetylarylamines to arylamines. The enzyme, found in the soluble fraction of rat liver, exhibited a broad pH optimum between 6 and 7.5 and required cystine or a thiol for maximal activity. N-Acetylphenylhydroxylamine had only slight activity but the N-acetyl-N-hydroxy derivatives of naphthylamine, 4-aminobiphenyl, and 4- and 2-aminofluorene were active donors. On the other hand, acetyl coenzyme A was inactive with the rat liver system. Polynuclear aromatic amines were better receptors than aniline or substituted anilines. Relevant to the system of Booth may be the observation that N-hydroxylation of arylamines facilitated the release of N-acyl groups (292).

Recently, a novel N- to O-transacetylation reaction by a soluble enzyme system from rat liver was described (29, 241). This enzyme converts N-hydroxy-N-acetylaminoaryl derivatives to reactive O-acetylhydroxylamino compounds which can arylamidate appropriate nucleophilic receptors

in cells such as membranes, proteins, and nucleic acids. The relationship of this enzyme system to the one described by Booth (47) needs clarification. The product of the reaction with a nucleic acid would be the aminoaryl derivative, which would mimic, in part, the situation found after *in vivo* studies on DNA. The transacetylase occurs in liver and, to a lesser extent, in kidneys and other tissues. Interestingly, the enzyme is inhibited by aniline (241), which may be related to the inhibition of carcinogenicity of N-hydroxy-N-2-fluorenylacetamide by acetanilide (491).

O-Esters of aromatic N-oxy compounds were shown to be generators of relatively stable free radicals (7). On a related topic (244) phenylhydroxylamine was a very active initiator of squalene autoxidation, usually inhibited by an antioxidant such as 2,6-di-tert-butylcresol. It was postulated that oxidation of free phenylhydroxylamine was the rate-determining initial step leading to radical formation.

C. Formation of O-Ethers

O-Methylation of N-hydroxy-N-2-fluorenylacetamide and other related N-hydroxy derivatives could be performed by a soluble fraction of rat liver with S-adenosyl-L-methionine as a methyl donor in the presence of cysteine; Mg^{2+} was not required (267). This reaction appeared specific for N-hydroxy but not phenolic compounds. The enzyme system was different from catechol O-methyltransferase methylating, for example, epinephrine, which requires Mg^{2+}, but not cysteine. Rat liver had most activity, but some was also seen in kidney. The order of activity as a function of species was: rat > hamster > rabbit > mouse > guinea pig. The significance of this reaction in the biological effect of N-hydroxy derivatives remains to be established.

D. Formation of Sulfuric Acid Conjugates

Evidence for the formation of sulfuric-acid esters of N-hydroxyarylamine derivatives is indirect. The synthetic sulfuric acid ester derived from the carcinogen N-hydroxy-N-2-fluorenylacetamide was not carcinogenic after injection into rats, probably because the half-life of this ester in water was of the order of seconds (332).

This limited lifespan in aqueous media and the fact that this hydrophilic compound very likely would exhibit low transfer potential across cellular membranes would account for the lack of carcinogenicity. Yet this compound was highly mutagenic in bacterial systems (287). Sulfate esters of other arylhydroxylamines have been reported as urinary metabolites of arylamines. In view of the now known high reactivity of such compounds, claims of their occurrence in an aqueous system must generally be discounted.

That the formation of esters may be related to the biological properties of N-hydroxy derivatives was first suspected on the basis of the following arguments.

Whereas the relationship between carcinogenicity of aromatic amines and biochemical activation has been explained to some extent by the first activation step, that is N-hydroxylation, there are instances where this has not provided a satisfactory answer. For example, it is well known that the hormonal environment affects the carcinogenicity of N-2-fluorenylacetamide. Male rats are much more susceptible, at least with respect to liver cancer formation, than female rats. Yet, there was relatively little difference in the amount of circulating N-hydroxy derivative in male and female rats or in the amount of tissue-bound carcinogen (467, 470). In fact, female rats excreted more glucosiduronic acid of the N-hydroxy derivative than male rats. Furthermore, while the level of this glucosiduronic acid was lower in hypophysectomized rats than in conventional controls, the difference did not seem to account for the sizable dissimilarity in biological effect. Thus, emphasis turned to a subsequent activation step, esterification of N-hydroxy derivatives. In this connection, De Baun *et al.* (88, 89) recently pro-

vided an additional rational explanation for the varying carcinogenicity as a function of sex or species by detailed study of the sulfotransferase reaction under these conditions. Male rats exhibited considerably higher levels of the enzyme system mediating this activation step than female rats. Hamsters, mice, rabbits, and guinea pigs had lower levels than the rat, in general agreement with the biological properties.

Although discussed as an appealing possibility (89, 327, 331), the actual demonstration that the sulfate ester of N-hydroxy-N-2-fluorenylacetamide is most likely the reactive "ultimate carcinogen" derived from N-2-fluorenylacetamide was based on the inhibition of the carcinogenicity of this material by acetanilide (491). Acetanilide is metabolized mainly to the *p*-hydroxy derivative, although N-hydroxylation also occurs to a small extent. Büch *et al.* (70) reported that in rats *p*-hydroxyacetanilide is excreted as a glucuronide and a sulfuric acid ester. With increasing dosage the glucuronide production remained relatively constant, but the sulfate ester formation increased and was limited only by the availability of inorganic sulfate or dietary sulfur. It was postulated therefore that one reason for the inhibition of carcinogenicity of N-2-fluorenylacetamide by large amounts of acetanilide was the unavailability of sulfate (327). That sulfate is a factor involved in the toxicity of N-hydroxy-N-2-fluorenylacetamide was demonstrated by De Baun *et al.* (88) who also noted that the amount of fluorene residue bound to protein, RNA, or DNA depended heavily on the presence of sulfate ion but not of other inorganic ions. Weisburger *et al.* (491) extended these findings to carcinogenicity by their observation that excess sulfate ion failed to restore this effect in animals fed N-2-fluorenylacetamide plus acetanilide (fig. 10, tables 3 and 4). In this situation acetanilide inhibited the N-hydroxylation of fluorenylacetamide (145). However, most significantly sulfate ion did restore carcinogenicity in rats given the N-hydroxy derivative and acetanilide. Thus, it would appear that the complex biological effects of N-2-fluorenylacetamide rest first on N-hydroxylation, a very well established reaction, and second on esterification of the N-hydroxy derivative with sulfate, a reaction reasonably well documented but for which additional support would be desirable.

For example, we found (490) that the metabolite of acetanilide responsible for

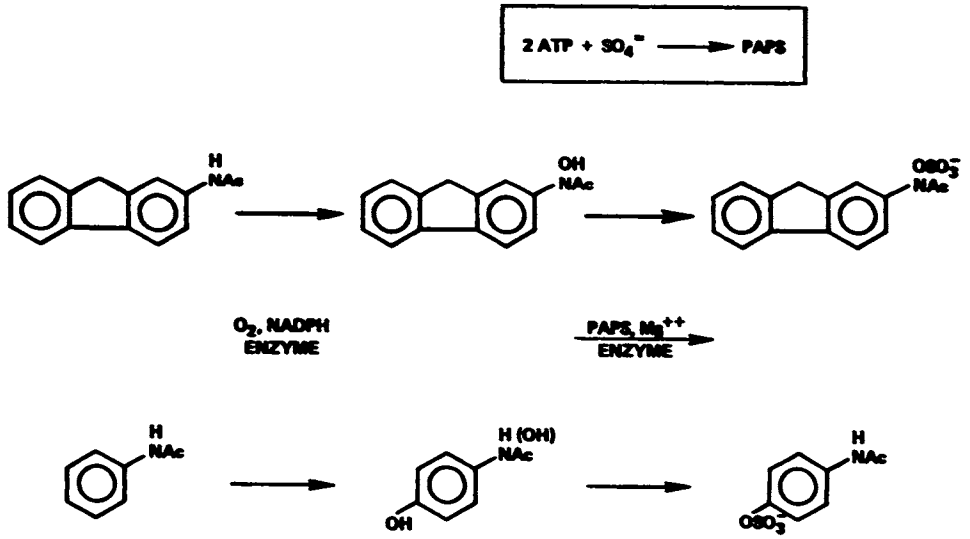


FIG. 10. Possible mechanism of interference by acetanilide metabolites with N-hydroxy amides (491).

TABLE 3

*Effect of dietary sodium sulfate on inhibition of hepatocarcinogenesis in rats by N-2-fluorenylacetamide or by N-hydroxy-N-2-fluorenylacetamide**

Experimental diets†	Final No. of Rats	Final Body Weight	Liver weight		Liver Histology						Rats with Liver Neoplasms
					Hyperplasia			Hepatoma			
					No	Area	Nodular	Focal in nodule	Small	Large	
		g	g	g%							%
FAA	2‡	260 ± 8§	19.2 ± 0.6	7.3						2	100
FAA + AA	10	315 ± 13	12.2 ± 0.8	3.8		9		1			10
FAA + AA + SO_4^-	10	298 ± 7	10.8 ± 0.4	3.6		10					0
N-OH-FAA	8¶	237 ± 4	18.6 ± 0.8	7.8				2		6	100
N-OH-FAA + AA	10	287 ± 7	11.6 ± 0.4	4.1		9		1			10
N-OH-FAA + AA + SO_4^-	10	291 ± 5	12.1 ± 0.3	4.3		3	1	1	1	4	60
AA	6	311 ± 17	9.2 ± 0.6	2.9	6						0
SO_4^-	5	312 ± 9	9.4 ± 0.4	3.0	5						0

* Groups of 6-week-old male Fischer F344 strain rats were fed the experimental diets containing carcinogens with or without acetanilide for 16 weeks, then continued on the control regimen of Wayne Laboratory Blox meal for 10 weeks longer (from ref. 491).

† Ingredients added in following concentrations: FAA, N-2-fluorenylacetamide, 0.03%; N-OH-FAA, N-hydroxy-N-2-fluorenylacetamide, 0.032%; AA, acetanilide, 0.8%; SO_4^-, sodium sulfate, 0.84%.

‡ Eight rats on FAA died from toxicity within 3 to 5 weeks. Toxicity of 0.03% FAA in Fischer rats was noted previously.

§ Weight ± standard error of the mean.

¶ Two rats on N-OH-FAA died from toxicity at 5 and 8 weeks.

binding sulfate (70), *p*-hydroxyacetanilide, is a less efficient inhibitor of the carcinogenicity of N-hydroxy-N-2-fluorenylacetamide (513) which moreover is influenced less by exogenous sulfate. Hence, the recently proposed acetyltransferase reaction, leading to active O-acetyl esters, may also be involved (29, 241). Of interest in this connection is the fact that this transferase is inhibited by aniline (241), which may explain the more effective lowering of the carcinogenicity by acetanilide than by the *p*-hydroxy metabolite. Very likely, all of the biochemical reactions are involved in yielding active metabolites, or ultimate carcinogens, for liver cancer induction (471).

Whether these same processes hold for other organs or other carcinogenic aromatic amines will have to be investigated. Thus, Irving *et al.* (190) found that the sulfotransferase levels in the mammary gland and Zymbal's gland (sebaceous gland of the auditory canal) of the rat were negligible Yet tumors are readily induced at these sites by 2-fluorenylacetamide and the N-hydroxy derivative. Other reactive intermediates may be acting in extrahepatic tissues. For example, Lotlikar and Wasserman (280) adduced that N-acetoxy-2-fluorenylacetamide and inorganic phosphate yielded an unreactive ring phosphate ester derived most likely from 5-hydroxy-2-fluorenylacetamide and a reactive N-phosphate. However, the actual synthesis of such an N-phosphate has not been achieved. Phosphate esters of aminonaphthols have been identified as metabolites of 2-naphthylamine in both human beings and dogs (431, 434).

E. Deacylation

Enzymic removal of the acetyl group, a metabolic pathway of aromatic acetamides, was also noted with N-hydroxy acetamides

TABLE 4

*Restoration by dietary sulfate of liver carcinogenesis in rats in inhibited system acetanilide + N-hydroxy-N-2-fluorenylacetamide**

Experimental Diets†	Final No. of Rats	Final Body Weight	Liver Weight		Liver Histology						Rats with Liver Neoplasms
					Hyperplasia			Hepatoma			
					No	Area	Nodular	Focal in nodule	Small	Large	
		g	*g*	*g%*							%
N-OH-FAA	7‡	262 ± 14§	26.2 ± 4.8	9.7				2		5	100
N-OH-FAA + AA	12	336 ± 9	11.4 ± 0.5	3.4	3	4	3	2			17
N-OH-FAA + AA + SO_4^-	13	337 ± 6	12.1 ± 0.4	3.6		1	9	1	1	1	23
N-OH-FAA + AA + SO_4^- (× 3)	14	334 ± 6	12.5 ± 0.4	3.7		2	4	5		3	57
N-OH-FAA + AA + PO_4^-	12	314 ± 11	10.8 ± 0.4	3.4	4	3	4			1	8.3
N-OH-FAA + SO_4^-	5	247 ± 6	22.4 ± 2.0	9.1						5	100
N-OH-FAA + $PO_4^=$	5	287 ± 9	23.8 ± 0.8	8.3						5	100
SO_4^-	6	361 ± 13	10.4 ± 0.4	2.9	6						0
SO_4^- (× 3)	5	379 ± 8	10.7 ± 0.2	2.8	5						0
$PO_4^=$	6	363 ± 12	10.2 ± 0.4	2.8	6						0

* The protocols were like those described in table 3, except for higher levels of carcinogen, additions of sodium sulfate or sodium phosphate in some groups, and a lengthened holding period of 16 weeks instead of 10 weeks on the control diet (from ref. 491).

† Ingredients were added in the following concentrations: N-OH-FAA, N-hydroxy-N-2-fluorenylacetamide, 0.032%; AA, acetanilide, 0.8%; SO_4^-, sodium sulfate, 0.84%; SO_4^- (× 3), sodium sulfate, 2.52%; $PO_4^=$, disodium hydrogen phosphate, 2.25%.

‡ Four rats died after 4 weeks on the diet; five others died after 24 weeks on experiment with enlarged livers and large hepatocellular carcinomas.

§ Weights ± standard error of the mean.

in a number of species. There appear to be two types of enzymes; one is a soluble deacylase (147, 152, 276) and another a microsomal enzyme (186). The microsomal enzyme was very high in the guinea pig and hamster, lower in the rabbit, and least in the rat with N-hydroxy-N-2-fluorenylacetamide as substrate. With acetanilide there was higher activity in the rat than in the guinea pig. The guinea pig microsomal enzyme was inhibited by SKF-525A, by fluoride, and diethyl-*p*-nitrophenylphosphate. On the other hand, pentacyanoamino ferrate, a reagent utilized to assess the product 2-fluorenylhydroxylamine, had little effect on the guinea pig enzyme and appeared to stimulate the rat and the rabbit enzyme. There was some activity in many tissues of the guinea pig, but kidney and, above all, liver excelled. Irving visualized the deacetylation system as an esterase. It could also be akin to an α-oxidative system leading to more readily hydrolyzed oxalyl derivatives (126, 226). Järvinen *et al.* (208) have isolated from guinea pig liver microsomes two enzymes capable of hydrolyzing N-hydroxy-2-fluorenylacetamide and the parent amide. Partial purification was accomplished by chromatography on Sephadex and hydroxylapatite or DEAE-cellulose. Enzyme I, of higher molecular weight, hydrolyzed the hydroxyamide 265 times faster than did the lower molecular weight enzyme II. Confirming Irving's premise, both enzymes hydrolyzed ester substrates such as 1-naphthylacetate very readily.

In connection with bioassays of deriva-

tives of N-hydroxy-N-2-fluorenylacetamide, it was observed that the N-acetoxy derivative was even more carcinogenic than the N-hydroxy derivative, especially at the site of injection. This result provided one of the cornerstones for the concept that esterification of the N-hydroxy derivative was a second key activation reaction of a carcinogenic aromatic amine. Previously in view of the easy deacylation of N-hydroxy-N-acyl derivatives by soluble as well as microsomal preparations, it had been speculated that the hydroxylamine compounds or the corresponding nitroso derivatives were the relevant intermediates. The latter materials cannot yet be completely ruled out for at least two reasons. In rat liver DNA the main receptor was guanylic acid, and the residue attached to it appeared to be the amine rather than the acetylamino derivative. It had been suggested, therefore, that a glucuronide of the hydroxylamino derivative may react with DNA. Perhaps the newly discovered N,O-transacetylase discussed above plays a role in leading the relative ratios of amine/acetylamine bound (317, 326). Alternatively, binding to macromolecular receptors, particularly DNA, may occur during release of 2-fluorenylhydroxylamine from the N-acetyl compound by action of a deacylase system in the soluble portion of rat liver (238). 2-Nitrosofluorene did not participate. Binding to DNA under phosphorylating and especially sulfating conditions resulted in larger retention of the acetyl group, so that *in vivo* binding to macromolecular reactants may involve all of these mechanisms. Furthermore, in most species, while coenzyme A-mediated acetylation converts administered arylamines to the acetyl derivatives (481), the dog is deficient in this reaction. Yet, many aromatic amines which are carcinogenic in other species are also carcinogenic in the dog. Thus, in this species which can convert arylamines to the corresponding hydroxylamines, the ultimate reactant must involve a derivative of the arylhydroxylamine other than the acetohydroxamic acid.

Weisburger *et al.* (481) demonstrated that in the rat, as noted above for the rabbit, the reverse reaction, N-acetylation of aromatic N-hydroxylamines, occurred to a substantial extent. Thus, after intraperitoneal injection of synthetic N-2-fluorenylhydroxylamine, male and female rats excreted in the urine substantially similar amounts and types of urinary metabolites as when the N-acetyl derivative, N-hydroxy-N-2-fluorenylacetamide, was injected. As with the latter compound (467), males excreted less of a dose as glucosiduronic acids and more as sulfuric acid esters compared to females. Further extensive conversion to ring-hydroxylated metabolites was noted, probably because of the operation of the enterohepatic cycle.

Enzymic studies on N-2-fluorenylhydroxylamine in the presence of acetylcoenzyme A and liver cytosol indicated the rabbit, of all species tested, as most active in N-acetylation of the hydroxyl amine (275). However, enzymic reduction to aminofluorene with subsequent acetylation to form N-2-fluorenylacetamide appeared to be the predominant reaction.

F. Reduction

A typical arylhydroxylamine, N-hydroxy-N-2-fluorenylacetamide, can be reduced to the amide by a soluble fraction of rat liver which also contains a deacylase (147, 276). Thus, an incubation mixture of the substrate usually yielded N-2-fluorenylacetamide and 2-fluorenamine. The deacylase was inhibited substantially by fluoride, for under these conditions, the main product was N-2-fluorenylacetamide. Methylcholanthrene pretreatment failed to alter deacylase or dehydroxylase activity. On the other hand, Shirasu *et al.* (391–394) found that rats with an altered balance of pituitary hormones exhibited decreased capability of dehydroxylating N-hydroxy compounds in proportion to the excess pituitary hormone level. The exact mechanism is not known,

but Wilson (498, 499) reported that pituitary hormones decreased a number of drug-metabolizing enzyme systems, and that adrenals and gonads were not necessarily involved. On the other hand, Arrhenius (9) observed that prednisolone stimulated microsomal oxidation, which may also be relevant in the final balance of metabolites seen.

Enzyme systems which can reduce N-hydroxy compounds to the amides or amines are also elaborated by the bacterial flora in the lower intestinal tract. After an injection of N-hydroxy-N-2-fluorenylacetamide into germ-free rats, larger amounts of the glucuronide conjugate of this compound were eliminated in urine and some also in feces (142, 473). In contrast, conventional animals excreted lower amounts of the N-hydroxylated derivative in urine and only the reduced product, N-2-fluorenylacetamide, in feces. Coliform bacteria, and possibly other microbiological systems can convert N-hydroxy derivatives to the reduced components (495). The bacterial flora in the gut plays a substantial role in the over-all metabolism of numerous compounds. Thus, many conjugates such as glucuronides or sulfates delivered to the intestinal tract *via* the bile are hydrolyzed in the lower intestinal tract by cecal bacteria; then the aglycones are reduced to amines or amides. Absorption of the reduced compounds from the lower portion of the gut leads to further mammalian metabolism. Thus, enterohepatic circulation probably does not involve the excretion in the bile of metabolites which are then resorbed as such.

Additional experiments are required on the identity and possible similarity of the family of enzymes which reduce nitrobenzoic derivatives to the arylhydroxylamines and hydroxylamines to amines and also bacterial nitrate reductases or bacterial or mammalian reductases which convert azo dyes to aromatic amines.

Red blood cells can reduce N-oxy compounds rather quickly. Thus, red cells from guinea pigs or cattle produced 2-fluorenamine from 2-nitrosofluorene *in vitro* at the same time as hemoglobin was oxidized (236). Likewise, the red cells of several species reduced nitrosobenzene or nitrosonaphthalene (166). The reaction also occurred in cats. Shortly after an intravenous injection of N-hydroxy-N-2-fluorenylacetamide in rats, sizable amounts of the reduced N-2-fluorenylacetamide were present (282). Several coupled cyclic enzyme systems which participate in the reduction of methemoglobin are also instrumental in the oxidation-reduction steps between nitroso compounds and arylhydroxylamines and may be connected with the reduction of N-hydroxy derivatives (71, 72, 399, 451, 459).

Reductive dehydroxylation of N-hydroxy derivatives occurs also with arylhydroxamic acids such as anthranil- or other substituted benzohydroxamates (37, 306) and also arylacetohydroxamic acids including the important anti-inflammatory drug 4-n butoxyphenylacetohydroxamic acid (49, 122, 380). The connection between systems reducing this type of hydroxamic acid and those involved in the reduction of N-hydroxyarylamine derivatives remains to be investigated.

G. Isomerization

Administration to rats of unlabeled N-hydroxy-N-2-fluorenylacetamide together with 9-^{14}C-labeled N-2-fluorenylacetamide resulted in the urinary excretion of derivatives hydroxylated at the 5- and 7-positions of the fluorene ring with very little decrease in the specific activity (321, 328). However, the 3-hydroxy derivative and especially the 1-hydroxy derivative had specific activities much lower than that of the compound administered. It was concluded that the N-hydroxy derivative served as a precursor for the ring-hydroxylated compounds, more particularly the 1-hydroxy derivative, and that N-hydroxy compounds could undergo isomerization to *o*-substituted derivatives.

The underlying biochemical reaction was first studied by Booth and Boyland (48). A soluble fraction of rabbit liver converted N-hydroxyacetanilide (N-phenylacetohydroxamic acid) to *o*-acetamidophenol, N-hydroxy-2-acetaminonaphthalene to 2-acetamido-1-naphthol, N-hydroxy-4-acetamidobiphenyl to 4-acetamido-3-hydroxybiphenyl, and N-hydroxy-2-fluorenylacetamide to 1-hydroxy-2-fluorenylacetamide. NAD^+, NADH, or NADPH served equally well as cofactors. While no data were given, they indicated that the soluble fraction from rat liver could also isomerize the N-hydroxy compounds.

Gutmann and Erickson (152) examined the isomerization reaction in detail. With N-hydroxy-N-2-fluorenylacetamide as substrate, they observed no reaction with liver fractions from male adult Holtzman rats. However, injection of 3-methylcholanthrene or other enzyme inducers such as benzo(a)pyrene, dibenz(a,h)anthracene, and phenothiazine, in decreasing order of efficiency, 24 hr prior to isolation of liver fractions gave measurable isomerization. Several other enzyme inducers of the barbiturate type or male hormones were ineffective.

Under conditions where no ring-hydroxylation occurred (as when blocked by SKF-525A, or in the absence of components necessary for aromatic hydroxylation) the conversion yielded approximately twice as much N-(1-hydroxy-2-fluorenyl)acetamide than the 3 isomer when a $600 \times g$ supernatant fraction was used. N-Acetoxy-N-2-fluorenylacetamide could serve as substrate as well.

Apparently two steps were involved in the isomerization. The inducible enzyme was located in the microsomal fraction, because a soluble fraction from an untreated rat gave the same level of conversion as did a $600 \times g$ supernatant fraction from a methylcholanthrene-treated rat. Ethionine injection blocked induction demonstrating that the microsomal portion of the two-step enzyme system was the inducible component.

Resolution of the soluble fraction by DEAE-cellulose chromatography gave five fractions with activity but failed to resolve the component facilitating isomerization from a soluble N-hydroxy reductase. By use of ^{18}O-labeled N-hydroxy-N-2-fluorenylacetamide, Gutmann and Erickson (153) demonstrated that after incubation with the induced enzyme the hydroxy group was transferred intact to the carbons located ortho to the nitrogen. The isotopic content of either product, the 1-hydroxy- or 3-hydroxy-N-2-fluorenylacetamide isolated, was the same as that of the labeled N-[^{18}O]hydroxyfluorenylacetamide. Thus, an intramolecular rearrangement appeared responsible for the enzymic isomerization of this arylhydroxamic acid. Previously, a reactive amidonium ion was postulated as an intermediate. The fact that under conditions of N-dehydroxylation by a soluble fraction alone no evidence of attack by nucleophilic targets such as serum proteins was noted (147, 482) also favors the intramolecular rearrangement rather than an amidonium ion.

IX. Reaction of N-Hydroxy Compounds and Derivatives with Cellular Macromolecules

A. Reactions with Tissue and Cellular Constituents

There are few fields of pharmacology, in the broad sense of the word, in which so much information has accumulated on the interaction between exogenous "drugs" and tissue receptors as in the field of chemical carcinogenesis. The obvious aim of such studies is to gather leads on the mode of action of these agents. Inasmuch as many of the chemicals discussed in this review on N-hydroxylation are carcinogens derived from arylamines or certain azo dyes, there now exists a sizable body of knowledge on molecular interactions between N-hydroxy derivatives and cellular

receptors. Since such data may be useful to those interested in the general mechanism of drug action, some aspects of the present status of this area will be discussed (175).

B. Proteins

That chemical carcinogens of the azo dye and aromatic amine type combine with proteins has been known for a long time (319, 469). By means of the N-acetoxy derivative of N-2-fluorenylacetamide and the N-benzoyloxy derivative of N-methyl-4-aminoazobenzene, it has been possible to study this interaction in some detail.

Methionine, cysteine, tryptophan, and tyrosine in proteins appeared to be the main reactive centers. With the carcinogenic amino azo dyes the so-called "polar dyes" were the result of the reaction of an as yet unknown active N-hydroxy intermediate with such groups in proteins *in vivo*. Model studies have revealed the specific structures of such polar dyes synthesized from N-benzoyloxy-N-methyl-4-aminoazobenzene and the amino acids named above (262–264, 300, 367, 382, 416, 424). Upon hydrolysis of proteins containing such azo dye adducts or after reaction with methionine a new azo dye metabolite, 3-methylmercapto - N - methyl - 4 - aminoazobenzene, was obtained (fig. 11). Ketterer and Cristodoulides (219) studied two solu-

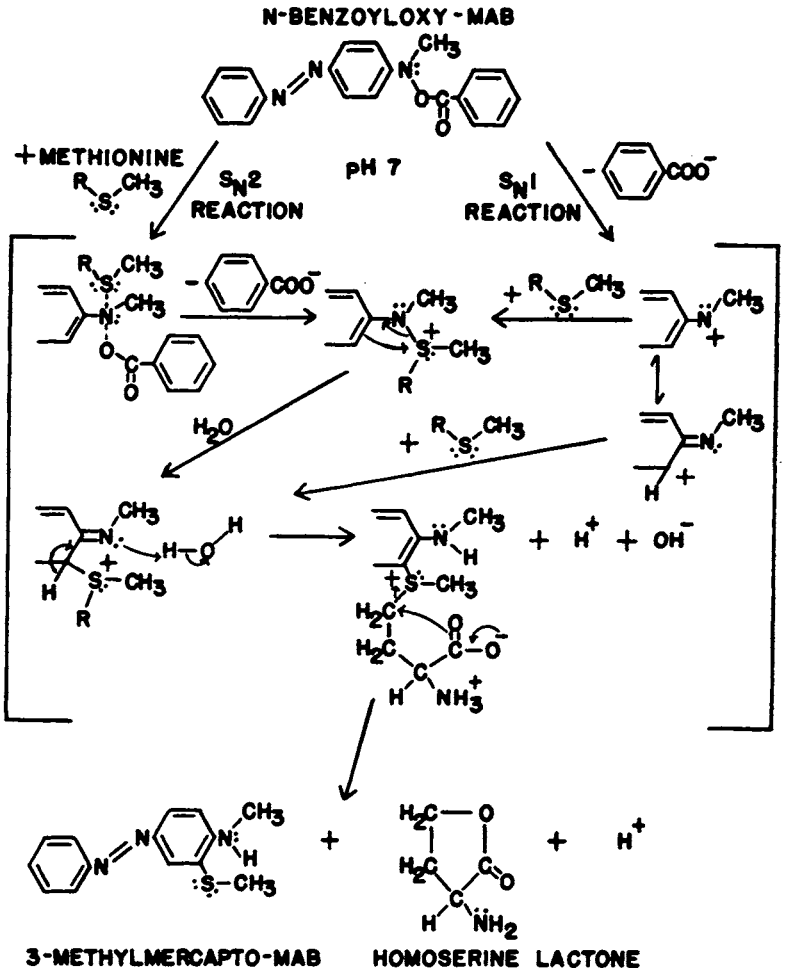


FIG. 11. Possible mechanisms for the reaction of N-benzoyloxy-N-methyl-4-aminoazobenzene (N-benzoyloxy-MAB) with methionine (367).

ble liver proteins which bound azo dye metabolites, a larger basic protein where binding appeared to occur chiefly through cysteinyl groups and a smaller protein where methionyl residues seemed to be the receptor sites.

With N-2-fluorenylacetamide and in particular the model ester derived therefrom, N-acetoxy-N-2-fluorenylacetamide, similar combinations with proteins were obtained (279, 331, 332). Also under conditions favoring *in vivo* or enzymic formation of the sulfuric acid ester like interactions were noted (88, 89). The reaction of methionine and the acetoxy compound, after elimination of homoserine and rearrangement, yielded two new sulfur-containing metabolites, the isomeric 1- and 3-methylmercapto derivatives of N-2-fluorenylacetamide. They were also obtained by the mild hydrolysis of liver proteins from rats fed the carcinogen, thus providing evidence for the reality of these reactions *in vivo*. Sulfotransferase levels in liver were correlated with the levels of protein-bound material and the carcinogenicity of N-hydroxy-2-fluorenylacetamide to the liver of various species (88, 90). Sorof *et al.* (402–404) had observed that certain classes of soluble liver proteins, h_2, were labeled more specifically when the carcinogen was fed chronically than when only a single dose was administered. After administration of N-hydroxy-N-2-fluorenylacetamide the soluble proteins of rat liver were resolved into five fractions (26). One of the fractions carried relatively more of the metabolites, but all of them yielded N - (*o* - methylmercapto - 2 - fluorenyl) - acetamide upon alkaline hydrolysis. Evidence was also obtained that some binding to proteins arose *via* 2-fluorenylhydroxylamine or the 2-nitroso compound (25). In this connection Miller *et al.* (318) demonstrated that nitrosobenzene, 2-nitrosotoluene, and 2-nitrosofluorene inhibited incorporation of amino acids in an *in vitro* system, but the N-hydroxy compounds were ineffective (see also 176 and 177). Only 2-nitrosofluorene was carcinogenic (163), the other two compounds were not.

It has been demonstrated that a basic protein which bound azo dye was similar to or even identical with the cortisol-binding protein (218, 266, 395). This may constitute evidence for overlapping sites of action of carcinogens and corticosteroids. However, the select proteins of Barry *et al.* (26) binding metabolites from N-2-fluorenylacetamide were different. It may be worth asking whether these interesting proteins serve as specific carriers of carcinogens and metabolites within the cell. For example, they might stabilize the reactive esters of N-hydroxy-N-2-fluorenylacetamide produced in the cytoplasm and transport them to the nucleus where they can react with receptors such as DNA. In the serum, only albumin, and to a lesser extent α_1-globulins, carried metabolites (93). Because the active metabolites also bound to these proteins, there is usually found in animals chronically treated with these carcinogens, such as aromatic amines and azo dyes, a substantial enlargement of the liver due to the functional demand to produce more serum proteins (92, 281). Liver carcinogens which do not appear to thus bind to serum proteins, such as diethylnitrosamine, fail to enlarge the liver. Evidence for the reactivity of the aromatic amine metabolites comes also from the fact that the methylmercapto derivative of N-2-fluorenylacetamide was detected in the blood of rats injected with this agent or its N-hydroxy metabolite (482). Its mechanism of formation stems from a reaction with methionine as such or in peptide linkage, followed by hydrolysis of the adduct.

The binding of N-hydroxy-2-fluorenylacetamide to the basic proteins or histones and acidic proteins of rat liver nuclei has been investigated by several groups. Lotlikar and Paik (278) reported that the compound was bound preferentially to the acidic nuclear proteins. Jungmann and Schweppe (209), who studied the time course of binding to histones and nuclear

acidic proteins, reported that one of the histone fractions had much higher label even after various treatments to remove possible contaminants. Previously, Barry *et al.* (27) had also implicated the histones, specifically those rich in arginine, in the binding of N-2-fluorenylacetamide to the nuclear proteins of rat liver.

Boyland *et al.* (56, 57) noted that phenyl- and naphthylhydroxylamine reacted with thiols such as cysteine or N-acetylcysteine to yield a number of adducts which converted readily to the corresponding mercapturic acids. Glutathionine also was sensitive to arylhydroxylamines, with formation of the corresponding glutathione derivative. N-Hydroxy derivatives of carcinogenic arylamines acted together with thiol reagents to effectuate an ATP-mediated mitochondrial swelling (157). N-Hydroxylation also appeared to be a key reaction in the acetaminophen-caused hepatic necrosis in rats (337). Enzyme inducers favoring N-hydroxylation also enhanced the necrosis. Covalent binding of isotope from labeled acetaminophen to tissue proteins required microsome-mediated oxidative reactions *in vivo* and *in vitro*, and the binding reaction was inhibited by glutathione, acting as a nucleophilic trap for a reactive electrophilic reagent, evolved from acetaminophen by metabolism. In this sense, the behavior of this drug is very much like that of typical aromatic amine carcinogens.

Boyland and Nery (60) also observed that after reaction with cysteine N-hydroxyurethan yielded an S-carbethoxycysteine by degradation. The reaction of proteins with the urethan metabolite thus might involve transfer of a carbethoxy link (347). In this case, N-hydroxylation can be regarded as an activation step in that it facilitates the leaving of the carbethoxy group, just as acyl cleavage is facilitated by N-hydroxylation of arylamides (292). As an alternative, the reaction with proteins could also occur *via* an O-ester of the N-hydroxy derivative. The most extensive binding *in vitro* to bovine plasma albumin (or salmon sperm DNA or yeast RNA) occurred with N-acetoxyphenacetin of a number of labeled phenacetin derivatives (349). It appears that "protein-binding," subject of numerous studies in the field of chemical carcinogenesis, is the result of conversion of aromatic amines to their N-hydroxy derivatives which are activated *in vivo* and then interact with select amino acid residues on protein. However caution is necessary in interpreting the results of such studies. The level of "protein-binding" in an organ may not correlate with the susceptibility of the tissue to the carcinogenic action or other pathological effect of an aromatic amine or its N-hydroxy derivative (207, 474). Likewise, Roberts and Warwick (378) observed that there was no clearcut correlation between binding of radioactivity from the labeled noncarcinogen aniline or the carcinogens 2-naphthylamine and 4-dimethylaminoazobenzene to rat liver or spleen proteins. Binding of the azo dye to guinea pig liver proteins occurred at the same level as in rat liver, even though the guinea pig is resistant to development of hepatomas on feeding the dye.

Recently, in a comparative study on the interaction between oxidation products of a model tertiary amine, N,N-dimethylaniline, or of a carcinogenic primary amine, 2-fluorenamine, this was described in relation to both N-oxidation and the subsequent reaction with proteins. With both agents N-oxidation was required for protein binding, but it was visualized that the binding resulted from transition state intermediates rather than activated metabolites (33).

C. Ribonucleic Acids

Combination of derivatives of arylamines, usually measured by isotope techniques, with total RNA has been demonstrated for N-2-fluorenylacetamide and for 4-dimethylaminoazobenzene under *in vivo* conditions (114). Investigation of these interactions under *in vitro* conditions yielded a greater insight into the mechanism. Kriek (246) observed that 2-fluorenyl-

hydroxylamine reacted with RNA at low pH, although much less at pH 7, chiefly with a guanylic acid residue. In contrast to what is seen with carbonium ions derived from the carcinogenic dialkylnitrosamines and indeed with other alkylating agents which attack the 7-position (285, 457), the fluorene derivative was unambiguously traced to the 8-carbon atom (251). This interaction was also observed at neutral pH with the model acetoxy derivative *in vitro* and has been demonstrated *in vivo* under conditions where specific activation of N-oxy esters with sulfuric acid or perhaps phosphoric acid could be implicated. Substitution at the 8-position stabilizes the guanine-ribose link, whereas substitution at the 7-position weakens it (430, 432, 433). The meaning of this crucial difference in reactivity of the adduct of guanylic acid and two types of carcinogens both affecting the liver remains to be explained.

At neutral pH, 2-fluorenylhydroxylamine reacted with rRNA, DNA, or poly(G) but on continued incubation loss of the 8-(N-2-fluorenylamino)guanine moiety occurred to a considerable extent in RNA, less in poly(G), and least of all in DNA (240). This reaction may represent a mechanism by which release of the bound carcinogen leads to an altered nucleic acid, with as yet unknown physiological or pathological consequences. Significantly, this changed nucleic acid no longer carried the carcinogen residue. Hence, analytical techniques utilizing tagged carcinogen as a marker would fail to detect this possibly crucial modified receptor.

Study of the reaction of N-acetoxy-N-2-fluorenylacetamide with tRNA *in vitro* has been intensively pursued by Weinstein and associates (463). Purification of the tRNA (from *Escherichia coli*) after the reaction indicated that 1 to 2 acetaminofluorene residues were bound per molecule of tRNA (465), a level approximating that for the binding to DNA. This altered tRNA showed changes in the ability to accept amino acids in the aminoacyl-tRNA synthetase reaction and selective decreases in the ability of the tRNA to recognize the normal codons (149, 463, 464).

Another *in vitro* study (519) compared the extent of binding of N-hydroxy-2-fluorenylacetamide, a strong liver carcinogen, and N-hydroxy-3-fluorenylacetamide, a mammary carcinogen but not a hepatocarcinogen, to yeast tRNA in the presence of soluble rat liver proteins. Whereas the 2 isomer bound to tRNA and its acetoxy derivative formed an adduct with methionine, the 3 isomer was unreactive and bound to tRNA to a negligible extent. Thus, the binding seemed to correlate with the hepatocarcinogenic activity. Furthermore, Irving and Veazey (195) determined that, in male rats which are more susceptible to liver cancer induction by 2-fluorenylacetamide than females, higher levels of the carcinogens were bound to tRNA or rRNA in male rat liver than in females. Although DNA-bound carcinogen levels were the same in males and females, there were differences between them in the retention of the acetyl group on the bound fluorenylamine residue.

In the case of guanylic acid and of tRNA it would seem quite certain that attachment of fluorenylacetamide residues to the 8-position has sizable stereochemical effects in specifically modifying the conformation of the polymers. These changes may be expected to affect rather appreciably the transcriptional or translational properties of the resulting DNA and RNAs, respectively (150). Additional investigations with such carcinogens will not only provide an accounting for their carcinogenicity on the basis of specific molecular interactions but will also lead to an understanding of how chemicals interact with vital macromolecules in cells and membranes to yield their pharmacological and pathological effects.

D. Polyribonucleotides

Marroquin and Coyote (296) first reported that N-hydroxy-2-fluorenylacetamide bound to different polyribonucleotides in small amounts, appreciably to poly(G) and

even more to poly(G:U), probably at the favored 8-position of guanosine. However, Kriek and Reitsema (252) found that N-acetoxy-2-fluorenylacetamide could also react with the pyrimidine ring of adenine if the poly(A) were in the form of a stacking coil. When the poly(A) was in the double helical form, binding was greatly decreased (148).

It has been emphasized that conformational changes are necessary for the 8-position of guanosine to react (148, 216, 313). Rotational distortion of the modified guanosine about the glycosidic bond followed by stacking of the fluorenylamine residue with the base adjacent to the guanine was suggested as one possible mode (346). Incorporation of the fluorenylamine residue into polynucleotides such as poly(U_3,G), poly(U_3,A), or AAG inactivated the ability of the triplets to stimulate ribosomal binding of their respective aminoacyl transfer RNAs. In a protein-synthesizing system, the modified poly(U_3,G) blocked incorporation of valine or phenylalanine into polypeptides (149). The conclusion was that in the modified fluorenylamine-containing synthetic mRNAs translation was impaired because codon-anticodon interaction was inhibited.

The evidence from *in vivo* studies indicates that the active metabolites of this type of carcinogen react mainly with guanylic acid residues in nucleic acids and to a much lesser extent with adenylic acid residues. Recently it was demonstrated in a model system that N-acetoxy-N-2-fluorenylacetamide reacted with polyadenylic acid in which the major portion of the adenosine residues was altered. Although this modifying polymer did complex with polyuridylic and polyinosinic acids, it did not form structures such as those seen with polyadenylic acid itself. Furthermore, the complex was changed by long wavelength ultraviolet irradiation to a fluorescent material. Likewise, DNA-containing carcinogen residues were susceptible to similar changes by ultraviolet irradiation which appear to be the result of cross-linking (313).

The data suggest that the carcinogen is phased in DNA in a geometry such that it is perpendicular to the axis of the helix and thus parallel to the base pairs, a feature also postulated independently as a result of observation of circular dichroism spectra (148).

E. Deoxyribonucleic acids

Under *in vivo* conditions the reaction of active metabolites of arylhydroxylamines with DNA has been somewhat more difficult to demonstrate than with RNA because it usually occurs to a lesser extent (94, 180, 197, 297, 302, 332, 422). However, although the combinations with proteins or with RNA have a limited half-life, the combination of carcinogen metabolites with DNA is more stable. N-2-Fluorenylacetamide was the model agent used in most such studies (fig. 12). Electron microscopy and ultraviolet spectroscopy provided evidence of a considerable alteration of DNA-bound carcinogen (107). Also, in contrast to RNA, where acetamidofluorene was the residue bound, on DNA more than 70% of the entity attached to the 8-carbon of guanylic acid was aminofluorene, *i.e.*, a loss of acetyl had occurred (196, 238, 248). The component responsible for the persistent binding to DNA has been partially characterized (250). The major component (80%) of bound carcinogen, N-(deoxyguanosine-8-yl)-2-fluorenylacetamide, had a half-life of 7 days but the other 20% remained on the DNA for periods of up to 8 weeks. Although this material was also apparently bound to guanine, the amino nitrogen of fluorenylacetamide may not be involved, inferring possible attachment at the 1 or 3 positions on the fluorene molecule.

With N-hydroxy-N-acetyl-4-aminobiphenyl more of the RNA-bound material was deacetylated than was the case with the N-hydroxy-2-fluorenylacetamide (249). For DNA-bound material, the level of retention of the N-acetyl group was almost the same for either carcinogen. However, the overall binding of the biphenyl deriva-

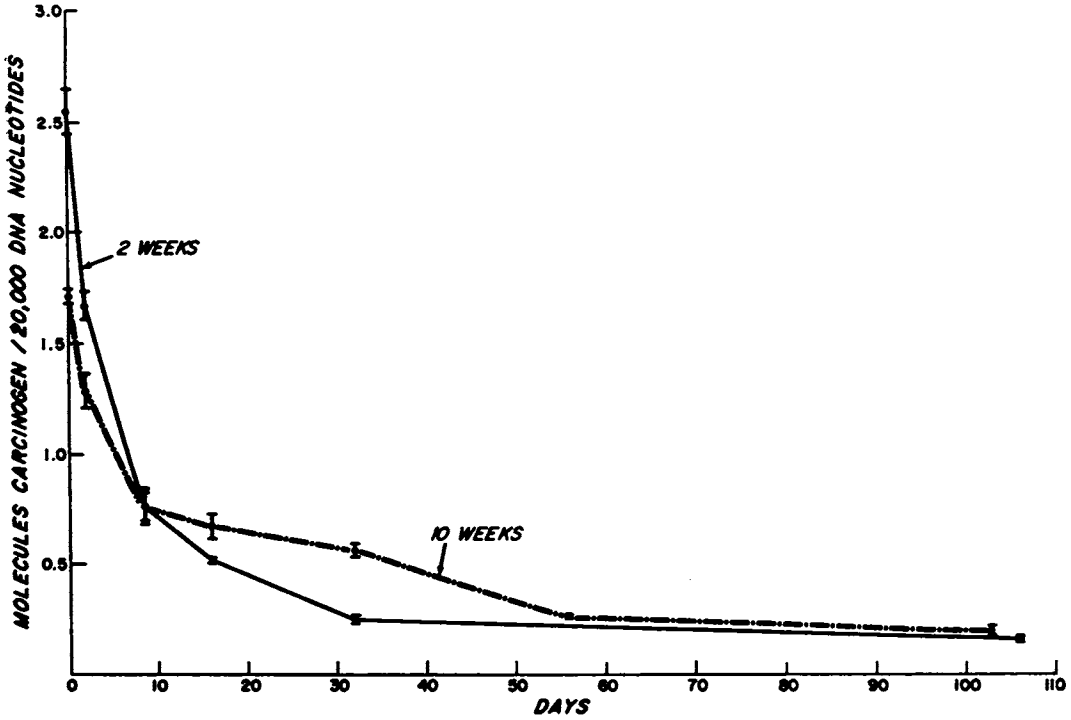


FIG. 12. Carcinogen bound to DNA after 2 and 10 weeks of feeding 160 ppm of [9-^{14}C] N-hydroxy-N-2-fluorenylacetamide in the diet and maintenance on control diet for the time shown on abscissa (418).

tive was much lower than that of the fluorene compound, implying a lower reactivity of the intermediate or a lower rate of formation of an activated intermediate. DNA, particularly at higher pH values, mimicked the relative relationship of aminofluorene *versus* acetylaminofluorene residues bound to the 8-position of the guanylic acid as found in DNA after *in vivo* tests (194, 196, 248). However, arguments have been presented against the direct involvement of this particular main metabolite of N-hydroxy-N-2-fluorenylacetamide in the reaction leading to nucleic acid-bound metabolite. Nonetheless, the glucosiduronic acid (192) or the N-acetoxy derivative (29, 241) of the corresponding hydroxylamine, highly reactive, needs further scrutiny in this regard. The glucosiduronic acid from N-hydroxy-4-acetylaminobiphenyl was less reactive than that of the fluorene derivative (193), just as the corresponding O-acetoxy compound was also of lower activity (387). Of interest is the higher stability of the glucuronide of the biphenyl *versus* that of the fluorene compound with increasing pH, which may be related to the greater tendency of the fluorene compound to lose N-acetyl with production of the highly reactive fluorenylhydroxylamine glucuronide. In favor of this concept is the finding that incubation of tRNA with the glucuronide of N-hydroxy-N-4-acetylaminobiphenyl followed by enzymic degradation of the adduct led to a guanylic acid with attached aminobiphenyl rather than acetylaminobiphenyl, also implicating the deacetyl compound as the reactive species.

4-Nitroquinoline-1-oxide combined *in vitro* with DNA, probably by intercalation, and did so also with poly(A) and poly(dAT) but not with poly(CG) and only minimally with denaturated DNA. However, under *in vivo* conditions, or with cells in culture, a metabolite of 4-nitroquinoline-1-oxide, identified as the hydroxylamino derivative, was

firmly bound to DNA. Under these conditions the agent caused strand scission in the DNA. When treated DNA was used as template for the biosynthesis of RNA more but shorter molecules of RNA were produced (101). Chemical degradation of the DNA or RNA 4-nitroquinoline-1-oxide adducts indicated that guanine and adenine were the purine bases involved (420).

After a single dose, metabolites from aniline or acetanilide bound only slightly to DNA (378). However, after dietary intake of labeled acetanilide, increasing amounts of isotope were firmly bound to DNA of rat liver (144). The significance of this interaction is not clear, for acetanilide appears to have no adverse effect on rat liver even in life-time tests. In any case, the nature of the aryl residue plays a key role in the reactivity of the "activated intermediates" and may relate in an important but as yet unknown way to the biological properties, including the organotropism of the carcinogen. Extrapolation of such findings to the field of structure-activity relationships in the general area of pharmacology suggests that apparently minor changes in molecular structure may exert pronounced effects on activated forms of drugs, influencing their pharmacodynamic action.

Active DNA replication, induced by partial hepatectomy, did not influence the rate of removal of the 2-fluorenylacetamide moiety from DNA after administration of labeled N-hydroxy-2-fluorenylacetamide to rats (501). Similarly Jackson and Irving (204) found that there was no predominant binding of N-hydroxy-2-fluorenylacetamide to newly synthesized DNA but that the bulk of the bound carcinogen was on the nonreplicating DNA.

Feeding 3-methylcholanthrene in the diet of rats did not influence the extent of persistent binding of metabolites of either 2-fluorenylacetamide or N-hydroxy-2-fluorenylacetamide to DNA 4 weeks after single injections of these compounds. However, when methylcholanthrene was fed prior to injection of either compound, there was a sizable decrease in the binding of 2-fluorenylacetamide to rRNA and DNA but no decrease in binding of N-hydroxy-2-fluorenylacetamide (191). These results substantiate the fact that feeding of methylcholanthrene inhibits the carcinogenicity of 2-fluorenylacetamide but not that of N-hydroxy-2-fluorenylacetamide (328).

Model experiments with N-acetoxy-N-2-fluorenylacetamide and DNA *in vitro* showed no loss of acetyl residue (251). With the glucosiduronic acid ester of N-hydroxy-N-2-fluorenylacetamide, however, *in vitro* reaction with tRNA and with DNA at pH 7.4 led to a 65 to 80% loss of acetyl groups. Between pH 7.0 and 9.5 the proportion of nucleic acid-bound fluorene residues with N-acetyl groups decreased from 44% to 1% (196). Furthermore, King and Phillips (238) noted that binding of metabolite to DNA under deacetylating conditions led to almost complete loss of acetyl group on the fluorene residue bound to DNA and also to protein; under phosphorylating conditions some acetyl retained, and under sulfating conditions most was incorporated. However, there are complications in relating the *in vitro* tests to the *in vivo* situation. Kriek (248) and King and Phillips (239, 240) reported that nucleic acid residues containing the fluorenylamine group lost the fluorene moiety to yield an unknown guanylic acid residue, although the relative proportion of bases in the nucleic acid was unchanged. The adduct obtained with guanosine and N-hydroxy-N-2-fluorenylacetamide under deacetylating conditions (or with the hydroxylamine) on the one hand, or under phosphorylating, sulfating or acetylating conditions on the other, was different as measured by mobility on thin layer chromatograms, fluorescence, ultraviolet absorption, and content of acetyl group. Thus, differences in the loss of carcinogen bound to macromolecular constituents *in vivo* may be due not only to the intrinsic half-life of the macromolecule or the operation of repair enzymes but also to the chemical stability of the adducts.

In attempts to develop information of the mechanisms of DNA repair following damage of human lymphocytes by alkylating agents or proximate carcinogens like N-acetoxy-N-2-fluorenylacetamide or the N-hydroxy derivative, Lieberman *et al.* (260, 261) found that such treatment led to unscheduled DNA synthesis measured by thymidine incorporation in the presence of hydroxyurea. Most of the thymidine was in the DNA and was mainly internal and not much the result of terminal addition. Procarcinogens like N-2-fluorenylacetamide did not stimulate such unscheduled DNA synthesis. More information is needed on the precise mechanisms underlying these interesting findings, for the DNA synthesis involved mainly pyrimidine, not purine, incorporation, whereas current data indicate carcinogens like N-acetoxy-N-2-fluorenylacetamide attack mainly the purines. Another approach deals with the systematic dissection of the complex repair process by isolating the various responsible enzymes, such as was done with an endonuclease from rat liver cytosol which can operate on DNA modified by N-acetoxy-N-2-fluorenylacetamide (458). Repair mechanisms for DNA are deficient in cells from xeroderma pigmentosum patients. Exposure of such cells to 4-nitroquinoline-1-oxide in culture showed the repair-deficient cells had a 10-fold excess of chromosome aberrations over the level of normal skin cells (411). After N-acetoxy-N-2-fluorenylacetamide such cells had lower unscheduled DNA synthesis, just as they would after ultraviolet irradiation damage (388).

Contemporary evidence suggests that the hepatocarcinogenic azo dyes combine with macromolecular receptors, in particular DNA, by similar mechanisms, namely N-hydroxylation followed by further activation forming a reactive N-O ester (332, 367). Additional reactions can occur however with other arylamino carcinogens. For example, whereas fluorene carcinogens react chiefly with guanylic acid and very slightly with adenylic acid in DNA or RNA, appreciable reaction with adenylic and cytidylic acid was detected with derivatives of 4-aminostilbene and 4-hydroxylaminoquinoline-1-oxide (322). Furthermore, in addition to reacting with the guanine residue at the C-8 position, N-acetoxy amines from aminobiphenyl and aminophenanthrene appeared to yield an acetylated guanosine derivative (331). The aminostilbene derivative afforded still another incompletely characterized adduct. On the other hand N-acetoxy-1- and 2-naphthylacetamide failed to alter DNA according to Troll and Berkowitz (432). These compounds all possess an individual tumor spectrum as regards organotropy, which may or may not be related to the individual characteristic reactivities with cellular macromolecules. Such specificities may assist in ultimately relating biological effect and biochemical interaction (258).

A similar diversity of ideas abounds as to the specific cellular targets in relation to biological action, including toxicity and carcinogenicity. Most of the developments refer to events in liver, the organ most studied since it is affected quite often but not exclusively by the agents discussed here (331). Thus emphasized but not necessarily as sole receptor system are proteins (129, 364, 402, 424), RNA (114), rRNA (191, 247, 248), tRNA (4, 121, 463), DNA (51, 303, 406, 432, 460), or others such as glycogen (108, 114).

The consequences of attachment of carcinogen metabolites to nucleic acids are manifold. There are changes in the absorption spectrum, the melting curve, buoyant density, the susceptibility to nuclease action, and the priming ability for RNA synthesis which in turn is reflected in the biochemical specificity of cellular and enzymic reactions (34, 127, 129, 287, 406, 432).

The carcinogenicity of N-hydroxyarylamines like that derived from N-2-fluorenylacetamide is usually expressed only after chronic treatment of animals. However, even a single dose exerts important effects on macromolecular synthesis in target organs

such as the liver, especially when this normally static organ is forced into division as after partial hepatectomy. Thus, N-hydroxy-N-2-fluorenylacetamide inhibits incorporation of labeled precursors into liver RNA and DNA (298). A carcinogenic azo dye increases DNA repair synthesis (137). Previously a number of investigators had shown by nonisotopic techniques that mitosis in liver after partial hepatectomy was severely depressed after administration of such carcinogens (289). Even the rate of regeneration as measured by the liver weight increase was lower (131). The role played by this inhibition at the tissue and at the molecular level requires further study to explain the toxic and carcinogenic effects of these agents. Also relevant to this question are well documented findings that the liver of newborn animals (243) or liver after partial hepatectomy is sensitive and eventually develops cancer with agents which do not affect adult liver. Examples are urethan (74, 255), polycyclic aromatic hydrocarbons (242), or β-propiolactone (75).

F. Glycogen

It was observed that hyperplastic nodules, precursors of liver cancer in animals fed N-2-fluorenylacetamide or the N-hydroxy derivative, contained sizable amounts of glycogen (108, 114). After fasting the adjoining normal liver exhibited no glycogen. The glycogen in nodules was not as susceptible to enzymic hydrolysis as normal glycogen, and it appeared to contain a bound derivative of N-hydroxy-N-2-fluorenylacetamide. The specific nature of this interaction remains to be established. However, the stability of the modified glycogen suggests that bonds in the molecule normally susceptible to hydrolytic splitting become unavailable, perhaps because they have reacted with the active carcinogenic intermediate, or are in the vicinity of a carbohydrate-carcinogen adduct.

G. Conclusions

It appears fairly certain that formation of N-hydroxylated derivatives is a necessary but not sufficient reaction for converting arylamines, certain azo dyes, 4-nitroquinoline-1-oxide compounds, and perhaps ethyl carbamate (urethan) to a biologically reactive compound. The exact nature of the subsequent activation step most likely is esterification of the N-oxy compound by acetylation (274, 275), transacetylation (29, 241), glucuronic acid conjugation (187, 193, 196, 317), sulfuric acid conjugation (88, 90, 491), phosphoric acid conjugation (237, 238, 280, 421), conversion to arylhydroxylamine by deacetylation (238, 246, 481), or ion and radical formation (7, 152, 343, 493), or of course, a combination of these or other mechanisms.

X. Immunological and Allergic Reactions

N-Oxy derivatives of carcinogenic or noncarcinogenic aromatic amines are reactive materials. Thus, under some conditions, certain of their interactions *in vivo* with proteins or membranes may yield specific antigens which lead to immunological reactions. This scheme probably accounts for allergies occasionally encountered in sensitive individuals exposed to aromatic nitro compounds or arylamines, both of which can be converted to N-hydroxy compounds (305, 446). Also, administration of some of these compounds, mostly carcinogenic azo dyes or arylamine derivatives which are known to require biochemical activation to N-hydroxy derivatives, leads to a derepression of the synthesis of fetal proteins shortly after exposure or when the induced tumors appear. There are several discussions and reviews on this topic (1, 2, 14, 16, 91, 202, 253, 461).

XI. Carcinogenicity of N-Hydroxy Compounds

Since recent overviews on the literature in this field are available (77, 151, 327, 332,

471), only a few relevant points will be made. Because carcinogenic aromatic amines rarely give cancer at the point of application, it was assumed that the compounds themselves were not the active carcinogenic principles. After the importance of N-hydroxylation was realized in the case of N-2-fluorenylacetamide, it was demonstrated that N-hydroxylation was a key activation reaction with other carcinogenic aromatic amines and azo dyes. The N-hydroxy derivative, in contrast to the amide itself, did cause subcutaneous sarcomas at the point of injection or cutaneous carcinomas in the area of application to the skin (325). The N-hydroxy derivative is somewhat more acidic than the corresponding 7- or 5-hydroxylated compounds but less so than the *o*-hydroxy metabolites (146). Metal chelates were prepared and their complexing constants evaluated (477), but when tested they usually exhibited local oncogenic properties inversely related to their solubility (35, 329, 366, 407). Also, species, for example guinea pigs, not susceptible to the carcinogenicity of N-2-fluorenylacetamide or of fluorenamine probably are so because they do not form or accumulate N-hydroxy derivatives in sufficient amounts. Such species were responsive when treated with large amounts of the N-hydroxy derivative.

N-Hydroxylation is a necessary but not sufficient biochemical reaction to elicit carcinogenic properties in aromatic amines. Although aniline or acetanilide were subject to N-hydroxylation in a number of species, adequate tests revealed that aniline, acetanilide, even phenylhydroxylamine, N-phenylacetohydroxamic acid (35, 151, 316), or N-acetoxy-4-acetylaminobibenzyl (351) were not carcinogenic. However, as discussed elsewhere, aniline exhibits appreciable pathological effects, particularly in the hematopoietic system because of N-hydroxylation.

Further evidence for the importance of N-hydroxylation is derived from the fact that aromatic amines such as 2-biphenylamine (138), 1-naphthylamine (372, 373), and 1-fluorenamine which for as yet unknown reasons apparently do not undergo significant N-hydroxylation are also not carcinogenic. On the other hand, the corresponding synthetic N-hydroxy compounds are active (35, 154–156, 291, 516). In fact, Radomski *et al.* (374) obtained tumors in rats with 1-naphthylhydroxylamine while the 2 isomer had no effect, but Belman *et al.* (35) reported both isomers of naphthylhydroxylamine to be active carcinogens. However, in neonatal mice, as had been shown by Boyland *et al.* (52), 2-naphthylhydroxylamine was active. Moreover in dogs 2-naphthylhydroxylamine was decidedly more potent as a bladder carcinogen than the parent 2-naphthylamine.

Within the interesting series of the isomeric arylamines, it has been generally found that when the amino group was ortho to another ring substitution, such as in 2-biphenylamine, 1-naphthylamine, 1-fluorenamine, and 4-fluorenamine, they were generally not carcinogenic. On the other hand, para-substituted agents, or broadly speaking, molecules where the amino group is not ortho to the adjoining ring, are carcinogenic (487). As discussed elsewhere in this review, it is quite certain now that the underlying reason stems from the stereospecificity of the biochemical N-hydroxylation reaction, which requires further exploration. The main documentation for this concept rests on the fact that the corresponding synthetic N-hydroxyarylamines or properly substituted arylhydroxylamines like N-acyl derivatives are carcinogenic. The best known series of analogous and isomeric chemicals is that of the arylamines derived from fluorene. 2-Fluorenamine and related compounds are all carcinogenic because they are converted to the proximate carcinogen 2-fluorenylhydroxylamine and derivatives. While the 1-, 3-, and 4-fluorenamine compounds are not active, the N-hydroxy-3-fluorenylacetamide is an excellent carcinogen for the mammary gland in female and even in male rats (155, 156). Parenthetically, it is a poor carcinogen

for liver, which suggests that either this agent does not reach the liver in adequate concentration, an unlikely supposition, or that the sulfotransferase apparently involved in liver carcinogenesis also requires certain stereochemical arrangements of substrate. The N-hydroxy derivative of 3-fluorenylacetamide may not be a good substrate. Of further interest is the fact that the N-hydroxy derivative of 1-fluorenylacetamide was an even less active carcinogen than the corresponding 3 isomer, which was in turn less potent than the well known 2 isomer. This was true also when the acyl substituent was benzoyl rather than acetyl. On the other hand, within the series of compounds derived from *p*-substituted arylamines such as 2-fluorenamine or 4-biphenylamine, the N-hydroxy derivatives of the N-benzoyl or N-tosyl derivatives were quite powerful mammary carcinogens. Some of these agents were also active at the point of injection, suggesting that these molecules were close to the ultimate carcinogen.

Agents like the N-hydroxy-N-2-fluorenylbenzenesulfonamide readily underwent deacylation with conversion to similar metabolites as found after injection of the better known N-acyl derivatives. Thus, chemical N-hydroxylation weakens the N-acyl link and subjects it to biochemical attack, helping to account for the carcinogenicity of these compounds. There is good evidence that the potent synthetic carcinogen, N-hydroxy-2-fluorenylbenzenesulfonamide, was desulfonylated *in vivo* to N-2-fluorenylhydroxylamine which was presumed to be the actual carcinogenic intermediate (291). Further evidence for this premise came from the fact that N-phenyl-2-fluorenylhydroxylamine and N-phenyl-4-biphenylylhydroxylamine were only weakly active or inactive as carcinogens. On the other hand, the synthetic O-glucuronide of N-2-fluorenylhydroxylamine was only marginally active (199). The carcinogenicity of the O-glucuronide of N-hydroxy-2-fluorenylacetamide depended on the vehicle used; it yielded no tumors when injected in tricaprylin but produced tumors when injected in physiological saline solution (199).

As noted, Gutmann and associates (155, 156) reported that amides from 2-fluorenamine which exhibited low or indeed no carcinogenicity, like the N-benzoyl or N-benzenesulfonamido derivatives, were converted to highly potent carcinogens when the N-hydroxy group was inserted into these molecules by chemical synthesis. Previously it was presumed that the reason for the low carcinogenicity of these compounds stemmed from their resistance to the hydrolytic enzymes which could liberate 2-fluorenamine (472). The real reason now appears to be that the benzoyl or tosyl and related derivatives do not N-hydroxylate readily.

The N-hydroxy derivative of 4-aminostilbene showed pronounced carcinogenic activity at the target tissues such as mammary gland and ear duct but also induced tumors in the forestomach, intestine, or at subcutaneous injection sites (5, 15). After oral dosing both N-acetoxy- and N-hydroxy-4-acetylaminostilbene produced ear duct tumors (351). Furthermore, the tumor inhibitory properties of the stilbenamine derivatives were found to be largely due to the N-hydroxy metabolite.

The carcinogenic activity of 4-nitroquinoline-1-oxide is probably mediated through the reduced form 4-hydroxyaminoquinoline-1-oxide (for review see 101). Presumably the active form may be an O,O-diacetyl derivative (106), analogous to the situation with N-acetoxy-2-fluorenylacetamide. However, tests of the two quinoline compounds in mice and rats showed that the carcinogenicity of either 4-hydroxyaminoquinoline-1-oxide and its diacetyl derivative was equivalent in mice; the latter compound was much less active than the parent hydroxylamine as an inducer of subcutaneous sarcomas in rats (103). The diacetyl compound may be too reactive to reach the proper target organ.

4-Hydroxyaminoquinoline-1-oxide is a fairly potent carcinogen, producing various types of tumors in many organs, depending on the mode of administration and the type of animal used. For example, skin painting gives papillomas and carcinomas, subcutaneous injection sarcomas (101, 103), intragastric instillation afforded stomach and intestinal tumors (338, 339), and a single intravenous injection in young Sprague-Dawley rats yielded benign exocrine pancreatic tumors, a type rare in experimental animals, but of importance in man (161).

Whether the carcinogenic action of urethan can be ascribed to metabolic conversion to N-hydroxyurethan remains open (335, 336), particularly since the N-hydroxy compound was not appreciably more active than the parent urethan. Pretreatment of mice with various enzyme inducers such as phenobarbital or chlordane lowered lung tumor response after urethan (511). A related N-hydroxy compound, hydroxyurea, a useful drug in cancer chemotherapy because it specifically affects the mitotic cycle in S phase, is not carcinogenic (336, 341, 510), nor is N-hydroxysuccinimide (86).

With the carcinogenic azo dyes derived from 4-methylaminoazobenzene (N-methyl-*p*-phenylazoaniline) it has been long known that the N-methyl group was required for the expression of carcinogenicity (330). Nonetheless, this dye or the parent aminoazobenzene is metabolized to an N-hydroxy derivative in rats, mice, and hamsters and excreted in urine as a conjugate (382). However, bioassays of N-hydroxy-4-aminoazobenzene, N-hydroxy-4-acetylaminoazobenzene, or the N-acetoxy derivative, as such or as the cupric chelate, uniformly failed to yield tumors. It was not possible to prepare the postulated intermediate N-methyl-N-hydroxyaminoazobenzene from 4-methylaminoazobenzene, but the N-benzoyloxy ester could be synthesized chemically (367). This compound was highly carcinogenic at the site of injection and yielded a number of cancers at remote sites. Taken together with other evidence, it would appear that the mechanism of activation of carcinogenic azo dyes involves a monodealkylation of the N,N-dialkyl derivative, followed by N-oxidation and esterification, just as is true for the aromatic amines. The specific requirement for an N-methyl group for the carcinogenicity of these azo dyes is not yet clear. On the other hand, *o*-aminoazotoluene (3,2′-dimethyl-4-aminoazobenzene) is carcinogenic without N-methyl substitution, and there is evidence of direct N-hydroxylation with this dye (299).

There is suspicion that certain tryptophan metabolites or tryptophan itself, particularly when present in abnormal amounts, may have some carcinogenic or cocarcinogenic effect (375). Since the discovery of N-hydroxylation of arylamines, attention was directed to similar metabolites of tryptophan. However, Boyland and Fahmy (53) observed that 2-hydroxylaminobenzoic acid was not present in the urine of rats or rabbits given anthranilic acid or tryptophan. On the contrary, these species metabolized the administered N-hydroxylamino derivative to the same metabolites seen after anthranilic acid, indicating that the bulk of the material was reduced.

XII. Mutagenicity of N-Hydroxy Compounds

If the mechanism of the carcinogenic process should involve a direct permanent alteration of a cellular or molecular constituent which is heritable, it follows that carcinogens might also be mutagens. Early tests of this concept failed, for a number of chemical carcinogens were not mutagenic in microbiological systems as well as in the classic fruit fly (Drosophila) tests. It is now clear that these historic experiments failed simply because the materials tested were not the active carcinogenic entities, and the indicator systems were devoid of biochemical activation mechanisms. Recent approaches utilizing not the parent amines but proximate or ultimate carcinogens did reveal that such compounds proved to be

excellent mutagenic agents in a variety of systems.

Thus, the synthetic sulfate, acetate, or benzoate esters of N-hydroxy-N-2-fluorenylacetamide or the benzoate ester of N-oxy-4-methylaminoazobenzene exhibited mutagenic effect on transforming DNA for *Bacillus subtilis* (287) (fig. 13). The glucosiduronic acid of N-hydroxy-N-2-fluorenylacetamide, or the free N-hydroxy compound itself as well as nitrosofluorene or 2-fluorenylhydroxylamine were inactive. Other active compounds included the sulfuric acid ester of N-hydroxy-4-acetylaminobiphenyl and the acetyl derivatives of N-hydroxy-4-acetylaminobiphenyl, N-hydroxy-4-acetylaminostilbene, and N-hydroxy-2-acetylaminophenanthrene (288). The parent amides or amines, N-hydroxyamides, and N-hydroxyamines had little or no effect on inactivation or mutation of the transforming DNA under similar conditions.

Using a different indicator, bacteriophage T4, Corbett *et al.* (80) determined the mutagenic activity and types of mutational events produced for numerous carcinogens and some noncarcinogenic analogs. Various N-hydroxylamines (N-hydroxy-1- and 2-naphthylamine, N-hydroxy-2-aminofluorene) were toxic but not mutagenic. However, N-acetoxy-2-fluorenylacetamide and 7 - fluoro - N - acetoxy - 2 - fluorenylacetamide were active mutagens, producing adenine//thymine → guanine//cytosine base pair transitions and frameshift mutations. Of various nitroquinoline-1-oxide derivatives, only 4-hydroxylaminoquinoline-1-oxide markedly affected phage T4 (201).

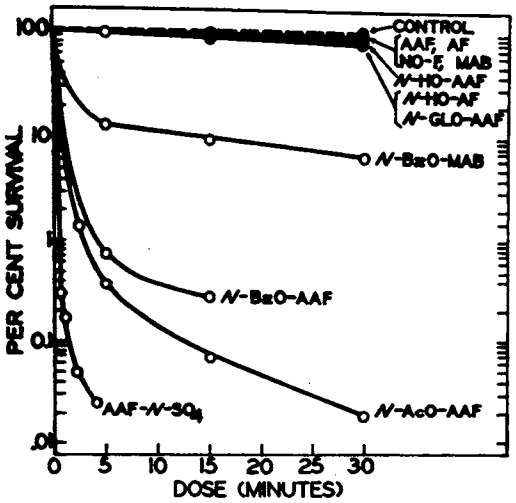


FIG. 13. Inactivation of *Bacillus subtilis* SB 19 DNA as assayed by its ability to transform the tryptophan-requiring strain T3, in the presence of various derivatives of N-2-fluorenylacetamide (AAF) or of methylaminoazobenzene (MAB) (287).

On the other hand, in a related test system, prophage induction in lysogenic *Escherichia coli*, N-acetoxy-N-2-fluorenylacetamide and N-hydroxy-2-fluorenylacetamide were not active, even at very high concentrations. Simpler hydroxylamines such as N-hydroxy-1-naphthylamine (but not N-hydroxy-2-naphthylamine) and oxamylhydroxamic acid were most effective in prophage induction (165). Since phage induction has been advocated as a method for detection of mutagenic, carcinogenic, and other toxic materials (100, 164), the discrepancy demonstrates the need for continued efforts on this problem. Undoubtedly, a test system in which mutagenicity of any substance could be correlated with its carcinogenic activity would be a great boon for rapid detection of carcinogens. However, an ideal test system has not yet been devised (323, 396). Many future efforts to assess mutagenicity-carcinogenicity relationships are in order. Very likely a number of mutagenic assays will, however, serve as reasonably good yet rapid prescreens of the vast number of environmental chemicals for their carcinogenic potential.

Tests of various aromatic amine derivatives on yeast cells, *Saccharomyces cerevisiae*, also led to the conclusion that only the N-acetoxy compounds among the aminofluorenes or stilbenes were strongly active in inducing mitotic gene conversion (295). On the other hand, *p*-tolylhydroxylamine was more effective than N-acetoxyaceto-*p*-toluidide, N-hydroxyaceto-*p*-toluidide, and *p*-nitrosotoluene in causing gene conversion.

However, the unstable *p*-tolylhydroxylamine was readily oxidized to *p*-nitrosotoluene in dilute aqueous solution. The question of whether the hydroxylamine or the nitroso compound was the active entity was not resolved.

On the basis of an extensive study of a variety of chemicals, Fahmy and Fahmy (111) concluded that certain classic alkylating agents give evidence of mutations due to intramolecular damage (point mutations) to DNA in Drosophila. On the other hand, aromatic amines, including active derivatives like N-acetoxy-N-2-fluorenylacetamide, or N,O-diacetyl-N-hydroxyurethan (112, 113) did not generally yield point mutations, but rather produced small chromosome deletions giving the *Minute* phenotype. The interpretation seems to hinge on differences in the mode of attack of these agents on cellular macromolecules. Alkylation at position 7 in guanine, for example, weakens the nucleic acid skeleton and leads to breaks in the backbone. Reactive derivatives of an arylamine and possibly of urethan, on the other hand, attack cytosine or the 8-position in guanine, which does not necessarily affect the structural backbone of nucleic acid but may eventuate in secondary disturbances in the chromosome structure. Although Belman *et al.* (35) favor a G:C to A:T transition, some have interpreted the attack as resulting from an A:T to C:G change, revealing perhaps an attack on adenine rather than guanine as the significant interaction (80) Perez and Radomski (363) and Mukai and Troll (340) compared carcinogenic, mutagenic, and initiating potency for skin tumor formation in mice of a series of chemicals. Their conclusions were that 1- and 2-naphthylhydroxylamine possessed all three properties. N-Acetoxy-N-2-fluorenylacetamide and N-2-fluorenylhydroxylamine were carcinogenic and mutagenic; 1-hydroxy-2-naphthylamine and N-hydroxy-N-2-fluorenylacetamide were carcinogenic, but they were not mutagenic or initiators of skin tumors. Although both N-acetoxy-1- and -2-naphthylacetamide were mutagenic and had initiating activity for skin, their overall carcinogenicity had not yet been measured. None of the corresponding arylamines were mutagenic. From the metabolic studies on urethan (335) there remain serious reservations as to whether N-hydroxylation plays a role in the biological effect of this drug. On the other hand, the studies of Boyland *et al.* (61) and of Freese (125) unequivocally demonstrated that only N-hydroxyurethan, but not urethan, produced chromosomal aberrations or inactivated transforming DNA. In this case action on heritable matter depended on N-hydroxylation. As alluded to previously, Fahmy and Fahmy (112) postulated that the small chromosome deletions caused by urethan derivatives most likely arise through further esterification of the N-hydroxy derivative. They noted the broadest spectrum of mutagenicity with the model N-acetoxy ester, even though it was anticipated that an indirect mechanism would operate.

Toxicological problems related to the artificial sweetener cyclamate have aroused considerable interest. It is fairly well established that cyclamate itself (cyclohexylamine sulfamate) is hydrolyzed, most likely by the bacterial flora in the gut to cyclohexylamine which can be metabolized further to cyclohexylhydroxylamine (135, 377). As in the case of urethan, there is controversy as to whether metabolites are responsible for the pathological effects. In a culture of human leukocytes little difference was noted in the potential to induce chromosomal aberrations between the amine and the cyclohexylhydroxylamine (414).

With Syrian hamster embryo cells in culture, N-acetoxy-2-fluorenylacetamide was also very active in transforming the cells into neoplastic ones as determined by morphological conversion (95). N-Hydroxy-2-fluorenylacetamide had only weak activity. Previously Süss *et al.* (417) reported that N-acetoxy-2-fluorenylacetamide was more effective than either the N-hydroxy- or parent amide in inhibiting DNA,

RNA, or protein synthesis in hamster embryo or HeLa cells in culture. Additional studies demonstrated that not only was N-acetoxyfluorenylacetamide capable of transforming cells but it was also highly mutagenic to Chinese hamster cells. Neither 2-fluorenylacetamide nor N-hydroxy-2-fluorenylacetamide had any mutagenic effect in this mammalian system (174).

Although neither urethan nor N-hydroxyurethan had any effect *in vitro*, urethan was significantly active transplacentally, demonstrating the need for metabolic activation. Upon treatment with a variety of carcinogens, among them N-hydroxy-N-2-fluorenylacetamide, epithelial rat liver cells in long term culture were transformed to cells which led to tumors upon injection into syngeneic hosts (494).

Another mutagen, 4-hydroxylaminoquinoline-1-oxide (109), was fairly active in transforming hamster embryo cells *in vitro* to malignant lines, as defined by morphological alteration and the tumorigenicity of these altered cells (254, 514). The earliest effects of the hydroxylaminoquinoline-1-oxide were to induce chromosomal gaps, breaks, and deletion, followed by heteroploid and polyploid karyotypic changes. This compound likewise inactivated *Salmonella typhimurium* phage P22 *in vitro* whereas the parent 4-nitroquinoline-1-oxide did not, pointing toward the hydroxylamine as the active intermediate responsible for the carcinogenic activity of 4-nitroquinoline-1-oxide (505).

XIII. Summary

In the past most metabolic reactions on exogenous materials were considered to be detoxification reactions. However, during investigations on adverse effects of aromatic amines and related materials, it was discovered in the last 15 years that certain of these chemicals have pronounced effects on the hematopoietic system, are carcinogenic, or have other pharmacological or pathological properties because of a novel toxication reaction. This reaction consists in substituting one hydrogen on the amine nitrogen with a hydroxyl group, N-hydroxylation. N-Hydroxylation has been studied extensively both *in vivo* and *in vitro*. N-Hydroxy compounds can be produced by a number of methods. Chemically, selective reduction of the corresponding nitro compounds under conditions where the hydroxylamine can be isolated is generally most useful. Similarly, nitroaryl compounds can serve as substrates for an *in vivo* or *in vitro* biochemical reduction in mammalian as well as in bacterial systems.

Biochemically N-hydroxy compounds are generally the product of specific N-hydroxylation reactions on the corresponding aromatic amines. In a few instances aliphatic amines were so studied, but relatively little success was achieved in demonstrating N-hydroxylation except by indirect criteria.

Biochemical N-hydroxylation is subject to a number of limitations. With aryl amines N-hydroxylation generally proceeds better when there are no substituents ortho to the amino group. Many species including man have varying capability to perform N-hydroxylation, but the guinea pig and the steppe lemming have the least capability. Furthermore, although many tissues can N-hydroxylate *in vitro*, the liver usually excels in this respect. In many but not all instances chronic administration of an arylamine yields increasing levels of an N-hydroxy derivative. In some cases this increase has been attributed to the higher production of N-hydroxy compounds by damaged tissue such as liver. Although there are relatively minor effects due to sex, endocrine-modified animals do sometimes exhibit different capacities for N-hydroxylation.

N-Hydroxylation appears to be performed by membrane-bound enzymes on the endoplasmic reticulum just as are other types of biochemical hydroxylations. However, more information is needed on certain differences between C- and N-hydroxylation. Carbon monoxide and other selective inhibitors have less effect on N-hydroxylation than

on some types of C-hydroxylation. Thus, it is not yet established that the now classic cytochrome P-450 system is involved in all types of N-hydroxylation reactions.

As with other hydroxylated derivatives, N-hydroxy compounds are good substrates for further conjugation reactions with glucuronic or sulfuric acid. Thus, N-hydroxy compounds are transported *in vivo* and are excreted into urine chiefly as glucuronic acid conjugates, which probably are detoxification forms. On the other hand, there is evidence that sulfate ester formation yields unstable activated metabolites which are reactive entities. These compounds can be mutagenic, carcinogenic, and highly toxic. Other esters like O-acetates are similarly endowed. However, the effect on the hematopoietic system appears to be mediated by arylhydroxylamines reacting directly with select constituents of this system or acting as catalysts in transferring oxygen to form methemoglobin.

Thus, N-hydroxylation is a key reaction accounting for many of the pharmacological, pathological, and other adverse effects of aromatic nitro compounds, aromatic amines, and their derivatives.

Acknowledgments. The secretarial assistance of Mrs. Nancy Branch, Miss Flodella Mitchell, Mrs. Anna Parker, Miss Diane Weisburger, and Mrs. Frances Williams is gratefully acknowledged.

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