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# Some Historical Aspects of *N*-Aryl Carcinogens and Their Metabolic Activation

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**This limited review of the early studies on the activation of the *N*-aryl carcinogens was written to provide a background for the current research to be presented at this conference on carcinogenesis by *N*-aryl compounds. Since the work of the past 10 years is too recent to be judged in a historical sense and because those data are much better known by current investigators, this review is generally limited to data up to 1972.**

## Discovery of *N*-Aryl Carcinogens

As for all history, the events of each period are rooted in the events of earlier times. Thus, the current interest in the carcinogenic activities of the N-aryl compounds can be traced to developments in organic chemistry in the mid-nineteenth century (*1*) (Table 1). The discoveries of Runge, Perkins, Verguin and Griess laid the foundations of the synthetic dyestuffs industry of Germany, which within a few decades had developed to such proportions that it was serving all of the industrial world. These dyes were generally based on aromatic amines or products made from aromatic amines, and accordingly their production necessitated the manufacture of these amines in large amounts. At first, the knowledge of their toxic effects was limited, and the need for protection of the work force and conditions suitable for this protection were not appreciated or generally available.

Table 1. The origins of synthetic dyes derived from aromatic amines.

| Year | Author | |
|---|---|---|
| 1840 | Runge | Synthesis of aniline blacks (quinonediimine complexes) |
| 1856 | Perkins | Synthesis of mauve (a phenazine) |
| 1859 | Verguin | Synthesis of fuchsin (a triphenylmethane dye) |
| 1858 | Griess | Diazotization of aromatic amines |
| 1864 | Griess | Azo dyes from coupling of diazonium salts with aromatic amines and phenols |
| 1860s | | Start of German synthetic dyestuffs industry and its role as a world supplier of dyes |

Subsequent events, starting with the classical clinical report of Rehn (*2*) in 1895 on the occurrence of urinary bladder cancer in 3 of 45 workers engaged in the manufacture of fuchsin from crude aromatic amines for 15-29 years, demonstrated the deleterious effects of the prolonged exposures to these aromatic amines. Rehn's understanding of the problem is reflected in the following translation of a portion of his paper:

> "For the majority of bladder cancers one can only imagine that substances were present in solution in the urine ... and that these caused a tumor to form by means of a chemical stimulus. ... For the time being these substances completely escape our knowledge. ... I am going to tell you about a number of illnesses which, with a high degree of probability, can be attributed to a chemical stimulus."

The various illnesses, in addition to bladder cancer, included hematuria, cyanosis and involuntary urination. By 1908, Rehn had reported 56 cases of urinary bladder cancer in workmen from seven dye factories in the area around Frankfurt, Germany, and as early as 1898 Leichtenstern (*4*) pointed to 2-naphthylamine as a possible cause. Occupational urinary bladder cancer from gross exposures to several aromatic amines occurred in over a dozen countries after 1900, especially after World War I (*3*). The carcinogenic activity of 2-naphthylamine in an

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experimental mammal was established in 1938 by Hueper et al. (*5*), who obtained benign and malignant tumors of the urinary bladder in 13 of 16 dogs administered the compound for 20-26 months. This observation was followed within a few years by data on the carcinogenic activities in rodent tissues of 2-acetylaminofluorene (AAF) (*6*), 4-aminostilbene (*7*), benzidine (*8*), *N,N*-dimethyl-4-aminobiphenyl (*9*) and 3-acetylaminodibenzothiophene (*9*). Subsequently the carcinogenicities of a variety of other *N*-aryl compounds, including heterocyclic derivatives, were observed (*10*).

A parallel series of findings led to demonstrations of the carcinogenicities of certain aminoazo dyes related to 4-aminoazobenzene (AB) (Fig. 1). Thus, in 1906, Fischer (*11*) observed that Scarlet Red caused a strong, but reversible, hyperplastic reaction in rabbit skin epithelium. These results were applied to clinical problems and between 1908 and 1911 several papers were published that demonstrated the clinical efficacy of Scarlet Red and *o*-aminoazotoluene (2′,3-dimethyl-4-aminoazobenzene) in promoting the healing of refractory skin lesions in the human (*12*). Adenomatous growths were observed by Schmidt (*13*) in the livers of mice fed Scarlet Red, and in the mid-1930s Yoshida and Sasaki (*14, 15*) and Kinosita (*16, 17*) reported the strong hepatocarcinogenic activities in rats of *o*-aminoazotoluene and *N,N*-dimethyl-4-aminoazobenzene (DAB), respectively.

## Protein Binding in Relation to the Carcinogenicity of Some Aminoazo Dyes

Against this background Rusch and Baumann at the University of Wisconsin initiated a series of studies on the effects of diet on carcinogenesis by DAB and other compounds in the 1940s. As collaborators in these nutritional studies (*18*) we developed an interest in the mechanisms of action of DAB as a hepatic carcinogen. The data of Stevenson et al. (*19*) in 1942 on the urinary excretion of high yields of *N*-acetyl-*p*-aminophenol and *N,N′*-diacetyl-*p*-phenylenediamine by rats fed this dye demonstrated that the carcinogen and its metabolites underwent reduction of the azo linkage, *N*-demethylation, and ring hydroxylation in unidentified sequences. Preliminary data by Kinosita (*17*) suggested that these monophenyl metabolites were not carcinogenic.

In addition to its carcinogenic activity, DAB had other properties that made it an especially good candidate for study during that period of biochemical science. One advantage was the relative ease of synthesis of DAB and its congeners. Of great importance in that period before $^{14}C$ and $^{3}H$ were available for laboratory studies were the acid-base indicator properties of DAB and its high molar extinction coefficients ($\varepsilon_{max}$ = 27,400 at 410 nm in ethanol and $\varepsilon_{max}$ = 42,800 at 518 nm in 4*N* HCl). The yellow dye was readily extractable from organic solutions into

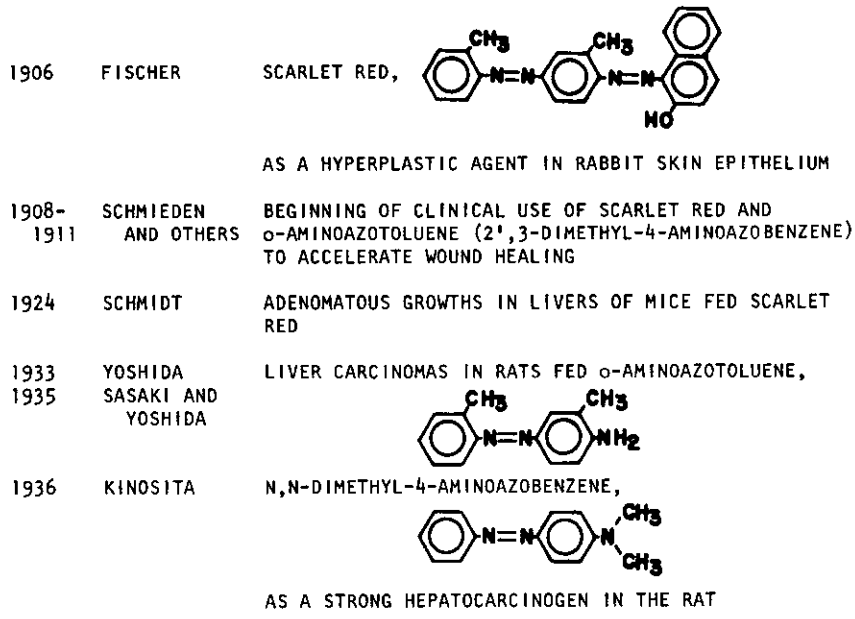


FIGURE 1. Early observations on the hyperplastic and carcinogenic activities of aminoazo dyes (*11-17*).

strong HCl, and the color intensity of the reddish-pink acidic solution was sufficient for detection of as little as 1 μg of the dye, even with the simple photoelectric filter colorimeters then available. It might be noted parenthetically that, in the early 1940s, Beckman or similar spectrophotometers were not available, visible absorption spectra were obtainable only with visual comparators, and data on ultraviolet spectra were obtained only by cumbersome photographic technics.

Our first studies, directed toward the determination of the "free" dyes in the liver, showed the occurrence of low levels of DAB, *N*-methyl-4-aminoazobenzene (MAB), and AB in the livers of rats fed DAB or MAB. Only AB was detected in the livers of rats fed this noncarcinogenic dye (*20, 21*). Assays soon showed that MAB and DAB had approximately equal carcinogenic activities in rat liver (*22*), while all of the other known metabolites of DAB were essentially inactive whether tested individually or as mixtures (*22, 23*). In the course of these studies, other dyes were found associated with the liver protein (*24*). Thus, the protein from perfused livers of DAB- or MAB-fed rats showed the acid-base indicator properties noted above for DAB. Since the dye could not be removed from the protein by procedures that did not involve hydrolysis of the protein and since the dye liberated by hydrolysis was much more polar than DAB or MAB, it was concluded that the protein-associated dye was covalently bound. These data thus pointed to the metabolism of DAB or a derivative to a chemically reactive form. Early correlations between the amounts of the protein-bound dye in the livers of rats fed a series of dyes, in various tissues of the rat and other species, and as a function of diet suggested its possible importance in the carcinogenicity of DAB (*24*). 2-Methyl-DAB was an early apparent exception. This dye did not produce tumors but it became bound to rat liver protein *in vivo* to the same level as did DAB (*25*). Later, 2-methyl-DAB was found by Warwick (*26*) and Kitagawa et al. (*27*) to be essentially only an initiator of liver tumors.

The above data and the finding that the primary tumors induced by the dyes had very little or no bound dye, even with continued administration of DAB or MAB, provided the basis for a "protein-deletion" theory of carcinogenesis (*24*). This theory received support from the finding with Sorof and Cohen (*28*) that the protein-bound dye was rather specifically associated with electrophoretically identifiable "h" proteins and from the data of Sorof and Cohen (*29*) that these proteins were present at only very low levels in the primary hepatic carcinomas induced by the dye. This protein-deletion hypothesis now appears to have only historical importance. Younger investigators may have little appreciation of the much better understanding in the 1940s of the biological activities of the proteins as compared to the understanding of the functions of the nucleic acids. Thus, the classical studies of Avery and his colleagues (*30*) on the ability of bacterial DNA to transform bacteria of one type to another type were first reported in 1944, and a number of years elapsed, even after the seminal paper of Watson and Crick in 1953 (*31*), before DNA was generally recognized as the basic information-containing molecule of the cell. Furthermore, while our limited analytical procedures were adequate to detect the protein-bound dyes, this was not the case for the nucleic acid-bound derivatives discovered later. Mixed nucleic acids isolated from the livers of dye-fed rats showed no pink color in acid (*32*). As became evident from later studies (*33, 34*), the structure of the major nucleic acid-bound derivative of DAB and MAB did not permit the resonance structure responsible for the distinctive red color of the dyes under acidic conditions.

From 1950 to 1962 we made repeated attempts to determine the structures of the protein-bound derivatives of DAB and related prime-ring-methylated dyes. By reduction of the azo linkage and steam-distillation of the resultant aniline or toluidine, we ascertained that the "prime" ring was apparently not involved in the linkage to protein (*25*). Accordingly, the presumed linkages to the amino acid residues were ascribed to the "diamine" ring or the nitrogen atom, and these sites of binding were documented later by Scribner et al. (*35*) and by Lin et al. (*36-38*) on characterization of the methionine, homocysteine, and tyrosine adducts. However, the structural studies were greatly hampered by the relatively small amounts (about 50 μg/liver), by the lack of any model reactive carcinogen derivative, and by the relatively limited state of the separation and organic characterization sciences as compared with those that soon became available.

The possible relevance of protein-bound forms of the aminoazo dyes to their carcinogenic activities seemed great enough to warrant similar studies with other carcinogens. Within a few years a number of investigators had found protein-bound derivatives of two carcinogenic polycyclic aromatic hydrocarbons, of ethionine, and of AAF in the target tissues (*32, 39-42*) (Table 2). The advent of $^{14}$C-labeled carcinogens, first used by Heidelberger and Jones (*43*) in 1948, greatly facilitated the studies on protein-bound carcinogen derivatives and, probably more importantly, made possible similar searches for nucleic acid-bound carcinogen derivatives. Thus, nucleic acid-bound derivatives of the *N*- and *S*-mustards, dimethylnitrosamine, ethionine, AAF,

Table 2. Early findings on the covalent binding of carcinogens to proteins and nucleic acids *in vivo*.

| Carcinogen | Investigators |
|---|---|
| Protein-bound carcinogens | |
| DAB | Miller and Miller (1947) |
| Benzo(a)pyrene | Miller (1951) |
| Ethionine | Levine and Tarver (1951) |
| AAF | Miller and Miller (1952); Weisburger et al. (1953) |
| Dibenz(a,h)anthracene | Wiest and Heidelberger (1952) |
| Nucleic acid-bound carcinogens | |
| Nitrogen mustard | Wheeler and Skipper (1957) |
| Sulfur mustard | Brookes and Lawley (1960) |
| Dimethylnitrosamine | Farber and Magee (1960) |
| Ethionine | Farber and Magee (1960) |
| AAF | Marroquin and Farber (1962) |
| DAB | Marroquin and Farber (1963) |
| Polycyclic aromatic hydrocarbons | Brookes and Lawley (1964); Heidelberger (1964) |

DAB, and several polycyclic aromatic hydrocarbons were reported between 1957 and 1964 (*44-50*) (Table 2). However, none of the protein- or nucleic acid-bound derivatives of the carcinogens were characterized at that time.

## Metabolic Activation of *In Vivo* of 2-Acetylaminofluorene by *N*-Hydroxylation

The studies on the metabolism and carcinogenicity of the aromatic amines in Britain during the late 1940s and the early 1950s were directed primarily to the naphthylamines and the 4-aminobiphenyl derivatives and emphasized particularly the induction of cancer of the urinary bladder (*10, 51, 52*). This emphasis resulted in the development by Bonser and his colleagues (*51*) of an assay for urinary bladder carcinogens which depended on the luminal implantation in mouse bladders of pellets of cholesterol or wax containing the test compound. Their finding that a number of *o*-hydroxyamine metabolites induced urinary bladder tumors in this assay suggested to Clayson and his colleagues (*51, 53*) that *o*-hydroxyamines might be critical metabolites in aromatic amine carcinogenesis.

During this same period the Weisburgers and their colleagues (*54*) examined in considerable detail the urinary metabolites of AAF in the rat. They demonstrated that metabolic hydroxylation of the rings occurred at the 1, 3, 5, 6, 7 and 8 positions, including the two positions (1 and 3) *ortho* to the nitrogen atom. Furthermore, on the basis of the initial studies of Richardson et al. (*55*), the simultaneous administration of 3-methylcholanthrene or of certain other planar polycyclic aromatic hydrocarbons with 3′-methyl-DAB or AAF was found to inhibit the carcinogenicities of these amine and amide carcinogens (*56-58*). As an approach to probing the possible significance of the *ortho*-hydroxy derivatives of AAF to carcinogenesis by this compound, Cramer and we (*59, 60*) examined the urinary excretion of AAF metabolites when the AAF was fed to rats alone or simultaneously with 3-methylcholanthrene. A fortunate and unexpected result of this experiment was the detection of a new urinary metabolite of AAF, the amount of which gradually increased to about 15% of the dose after 18 weeks of administration of AAF in rats expected to develop liver tumors by 6 months. Much smaller amounts were excreted by rats in which the hepatocarcinogenicity of the AAF was greatly inhibited by administration of 3-methylcholanthrene.

Although a new metabolite of a xenobiotic compound may be of disappointingly low interest if its identification demonstrates only one more detoxification route, characterization of this metabolite of AAF provided a new focus in the understanding of the metabolic activation of aromatic amine and amide carcinogens and of the toxicity of aromatic amines and amides in general. The new metabolite was isolated and characterized as a conjugate (presumably the glucuronide, since it was cleaved by crude β-glucuronidase) of *N*-hydroxy-AAF (*59*). The structure of this metabolite was deduced from its acidic character, its elementary analysis (only the addition of one oxygen atom to the elementary formula of AAF), and its separability from all of the ring-hydroxy derivatives of AAF, most of which had been prepared and studied by the Weisburgers. An unequivocal synthesis of *N*-hydroxy-AAF from 2-nitrofluorene and demonstration of the identity of the synthetic and metabolic products provided definitive evidence for the structure of the metabolite (*59*). Since, on administration, *N*-hydroxy-AAF proved to be more active as a carcinogen than AAF in a number of rat tissues and in a number of species (*61, 62*), *N*-hydroxy-AAF was thus identified as a proximate carcinogenic metabolite of AAF. This was the first recognition of a proximate carcinogenic metabolite. Subsequent studies with a variety of aromatic amines and amides have strongly implicated *N*-hydroxylation as a first step in their activation for carcinogenic activity (*63*). At the present time there appears to be no exception to the generality that aromatic amine and amide carcinogens are *N*-hydroxylated as the first stage in their metabolic activation to reactive electrophiles, although oxidation to free radicals, early suggested as an alternative pathway (*64-67*), requires further investigation.

## Discovery of Metabolically Derived Electrophilic *N,O*-Derivatives

The concept of the electrophilicity of reactive forms of chemical carcinogens was curiously slow in developing in studies on chemical carcinogenesis. Although by the early 1950s a number of alkylating agents were shown to have carcinogenic activity (*68*), their activities were relatively weak and these carcinogens received only limited study. To our knowledge the alkylating agents were not regarded as prototypes for the reactive forms of chemical carcinogens in general. The concept of electrophilic reactivity as a feature of the activities of other chemical carcinogens was forced on workers in the field by direct observations, and the first of these were made with the *N*-aryl carcinogens. Thus, the activation of hydroxylamines by protonation, already known to the organic chemists, was applied to *N*-hydroxy-AF by Kriek (*69*), who demonstrated its acid-catalyzed reaction with guanine residues in nucleic acids in 1965. That formation of *N*-hydroxy-AF was a metabolic possibility was demonstrated the next year by Irving (*70*). The degree of protonation at physiological pH of most aromatic hydroxylamines is relatively low, and the significance of these proton-catalyzed reactions of aromatic hydroxylamines in amine carcinogenesis requires further study.

Also in the mid-1960s a reinvestigation of the "free" dyes from the livers of rats fed DAB provided unexpected insight into the structures and derivation of the bound dyes. Scribner et al. (*35*) found that what was termed DAB from the liver was actually a mixture of DAB and another dye, which arose from decomposition of a protein-bound dye. This new dye was characterized as 3-methylmercapto-MAB and was deduced to be derived from protein-bound 3-methion-S-yl-MAB residues. About the same time Poirier, unable to synthesize *N*-hydroxy-MAB, synthesized its benzoic acid ester (*71*); we hoped that administration of *N*-benzoyloxy-MAB would result in liberation of *N*-hydroxy-MAB *in vivo*. However, *N*-benzoyloxy-MAB proved to be an electrophilic reactant at neutrality, and on incubation with methionine it yielded a polar dye that decomposed to 3-methylmercapto-MAB (*71, 72*) (Fig. 2). Analogous studies with *N*-acetoxy-AAF showed that it had similar, but stronger, electrophilic reactivity. Thus, *N*-acetoxy-AAF reacted at neutrality with methionine to yield 1- and 3-methylmercapto-AAF (*72*) and with guanine residues in nucleosides or polynucleotides (*73, 74*); under appropriate conditions nearly all of the guanine residues in DNA or RNA could be modified. *N*-Benzoyloxy-MAB and *N*-acetoxy-AAF proved to be carcinogenic in the subcutaneous tissue of rats, a site where the respective parent compounds MAB and AAF did not produce tumors (*71, 75*).

Further studies emphasized the possible importance of the reactivity of the electrophilic esters of

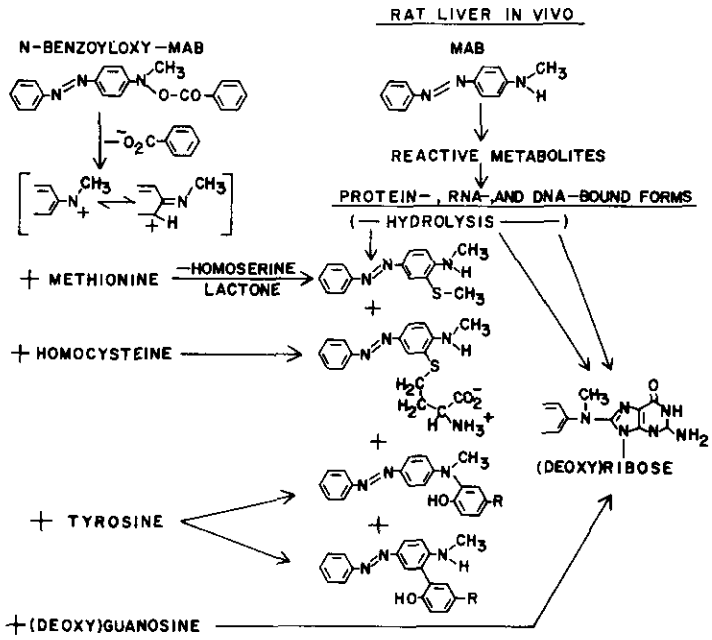


FIGURE 2. Nonenzymatic reactions at neutrality of *N*-benzoyloxy-MAB with methionine, cysteine, tyrosine, homocysteine, guanosine and deoxyguanosine. Cochromatography showed that the adducts formed in these reactions are identical to derivatives obtained from the liver protein or nucleic acids of rats administered MAB.

*N*-hydroxy-MAB and *N*-hydroxy-AAF in relation to the biological activities of MAB and AAF or *N*-hydroxy-AAF. Thus, by analogy to the finding of 3-methylmercapto-MAB as a degradation product of the protein-bound dyes from DAB and MAB (*35, 71*), 1- and 3-methylmercapto-AAF were obtained on degradation of the liver proteins from rats fed *N*-hydroxy-AAF (*76*). Homocysteinyl and tyrosinyl derivatives, which were identical to those synthesized from the amino acids and *N*-benzoyloxy-MAB, were isolated from the livers of rats treated with MAB (*37, 38*). Somewhat later, Ketterer and Christodoulides (*77, 78*) found dye residues linked to cysteine residues in ligandin and to methionyl residues in a fatty acid binding protein in the liver cytosol of rats fed DAB. Furthermore, major adducts in the RNA and DNA from rats given AAF or *N*-hydroxy-AAF were found to be identical to the *N*-(guan-8-yl)-AAF derivatives obtained on reaction of guanosine or deoxyguanosine with *N*-acetoxy-AAF (*74, 79, 80*). Accordingly, tissue preparations, especially rat liver, were examined for their abilities to form these reactive esters enzymatically.

Five enzymatic systems for the conversion of *N*-hydroxy-AAF to electrophilic reactants had been demonstrated in rat liver preparations by 1972. The first of these is the microsomal deacetylation of *N*-hydroxy-AAF to *N*-hydroxy-AF (*70*), which was demonstrated by Kriek (*69*) to undergo an acid-catalyzed reaction with nucleic acid guanine residues (Fig. 3). The glucuronide of *N*-hydroxy-AAF, which is a major metabolite (*60, 83, 84*), has weak electrophilic reactivity at neutral pH (*85, 86*). The extent of reaction of the glucuronide increases very rapidly with small increases in pH; this increase in activity apparently results from loss of the *N*-acetyl group and the markedly greater reactivity of the glucuronide of *N*-hydroxy-AF as compared to that of *N*-hydroxy-AAF (*87*). The next metabolic pathway to be identified was the sulfotransferase reaction for the formation of *N*-sulfonoxy-AAF (*88, 89*). This ester of *N*-hydroxy-AAF is a stronger electrophilic reactant than *N*-acetoxy-AAF, and the sulfotransferase activity for *N*-hydroxy-AAF in liver parallels quite well the susceptibility of the livers of males of various species or of rat liver under various conditions to carcinogenesis by *N*-hydroxy-AAF (*76*). Shortly thereafter Bartsch et al. (*65, 66*) observed that a 1-electron oxidation of *N*-hydroxy-AAF by purified peroxidases yielded a free nitroxide radical which on dismutation gave *N*-acetoxy-AAF and 2-nitrosofluorene. Finally, cytosols from liver and a wide variety of other tissues carry out an acyl transferase reaction which, in effect, transfers the *N*-acetyl groups of *N*-hydroxy-AAF or other aromatic acethydroxamic acids to the oxygen atoms to yield *N*-acetoxyarylamines (*90, 91*). Yet another mechanism was demonstrated by Tada and Tada (*92, 93*), who observed the activation of 4-hydroxyaminoquinoline 1-oxide by its esterification with serine activated by seryl-t-RNA synthetase.

The reactivity of these electrophilic compounds

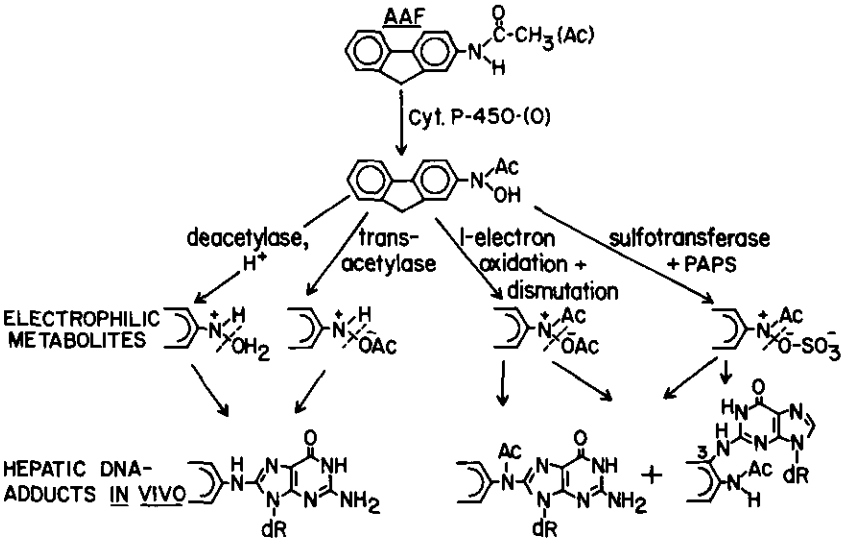


FIGURE 3. Pathways for the metabolism of AAF to electrophilic reactants in rat liver. In addition to the reactions shown, *N*-hydroxy-AAF is also metabolized to its *O*-glucuronide. The latter metabolite, although a much weaker electrophile, also reacts with nucleic acids to yield both *N*-acetylated and nonacetylated adducts. The adducts *N*-(guan-8-yl)-2-aminofluorene and 3-(guan-$N^2$-yl)-2-acetylaminofluorene were characterized only within the past few years (81, 82). Citations for the other reactions and structure shown are given in the text.

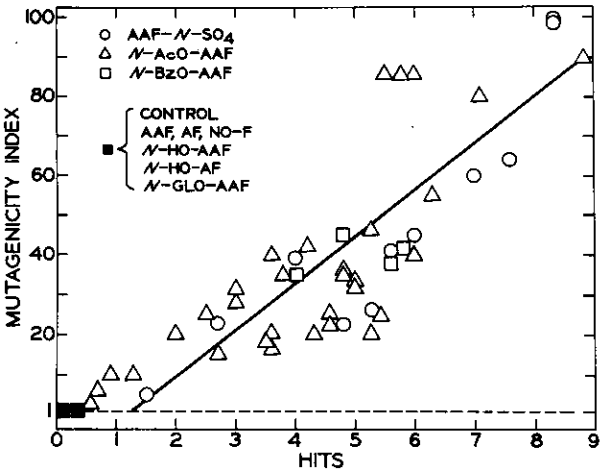


FIGURE 4. The mutagenicities of esters of N-hydroxy-AAF for *Bacillus subtilis* transforming DNA. The nonelectrophilic metabolites shown were not mutagenic (*94*).

with nucleic acids naturally led to the question of the biological consequences of these reactions. A suitable test for these very reactive compounds appeared to be one that utilized transforming DNA, and studies with Maher and Szybalski (*94*) showed that esters of *N*-hydroxy-AAF and *N*-hydroxy-MAB were strong mutagens for *Bacillus subtilis* transforming DNA (Fig. 4). This first demonstration of the mutagenicity of ultimate carcinogenic and electrophilic derivatives of carcinogens, together with the deduction that the ultimate forms of all or most carcinogens are electrophilic reactants (*75, 95*), provided a strong catalyst for the development of mutagenicity assays for the detection of potential carcinogens (*96-99*) and for the provision of metabolic activating systems in these assays.

## Concluding Remarks

Because of the time frame that we have used (i.e., data up to 1972), this historical review covers the period of the recognition of the carcinogenicities of a variety of aromatic amines and amides and the development of the concept that the carcinogenicity of these compounds depends on the metabolic formation *in vivo* of electrophilic reactants that covalently bind to informational macromolecules in target cells. The characterizations of the products formed in cells from the electrophilic metabolites and the biological consequences of these reactions are more recent areas of study. Current approaches in these studies are the principal subjects in most of the subsequent chapters of this book.

The current understanding of the separation of tumor induction in many, and quite possibly all, tissues into stages of initiation and promotion (*100*) should facilitate future studies of chemical carcinogenesis. It is now evident that some chemicals may be active in only one or the other step and that many carcinogens may have both activities, but in different proportions. This clarification of these qualitatively different biological activities will presumably make it easier to establish which reactions are essential to each stage of carcinogenesis.

The recent quantum leaps in the molecular characterization of the retroviruses and of the conditions which make possible the expression of the naturally occurring c-onc genes (*101*) have provided a new understanding of the expression of cellular genetic information and new models for the initiation of carcinogenesis. Whereas much of the earlier thought on the ability of chemicals to initiate carcinogenesis centered on the causation of frameshift or base-substitution mutations or of major deletions, these recent observations raise the question of whether or not chemicals may initiate carcinogenesis through facilitating genomic rearrangements that result in the expression of previously unexpressed c-onc or other genes with similar potential (*102, 103*). We may be on the threshold of major advances in our understanding of the molecular mechanisms of action of chemical carcinogens.

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