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# SEMI-QUANTITATIVE DETERMINATION OF ASBESTIFORM AMPHIBOLE MINERAL CONCENTRATIONS IN WESTERN LAKE SUPERIOR WATER SAMPLES

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## ABSTRACT

The amphibole mineral, cummingtonite-grunerite, has been used as a tracer for taconite tailings discharged into Western Lake Superior. The discovery of many asbestiform amphibole fibers in the tailings and Western Lake Superior water lead to concern over fiber concentrations in municipal water supplies using this water. This concern was based on the association between human asbestos exposure and increased rates of cancer of the gastrointestinal tract and peritoneum. An x-ray diffraction external standard technique has been developed for rapid, inexpensive, semi-quantitative determinations of amphibole mass concentration in water. The average amphibole mass concentrations for different Western Lake Superior water intakes compare very well with the average electron microscope fiber counts for the same samples. Daily amphibole analysis of the Duluth water supply indicates an average amphibole concentration of 0.19 milligrams per liter.



## INTRODUCTION

For several years x-ray diffractometry has been the key analytical technique for National Water Quality Laboratory studies of the distribution and fate of taconite tailings which have been discharged into Western Lake Superior at Silver Bay, Minnesota since 1956. A major component of this 67,000 ton per day discharge, the amphibole mineral cummingtonite-grunerite, provides an ideal tracer for the tailings. The cummingtonite-grunerite (310) peak at 29.1° 2θ for copper $K_\alpha$ radiation (d = 3.07 Å) is not found in x-ray diffraction patterns for natural lake sediments or suspended

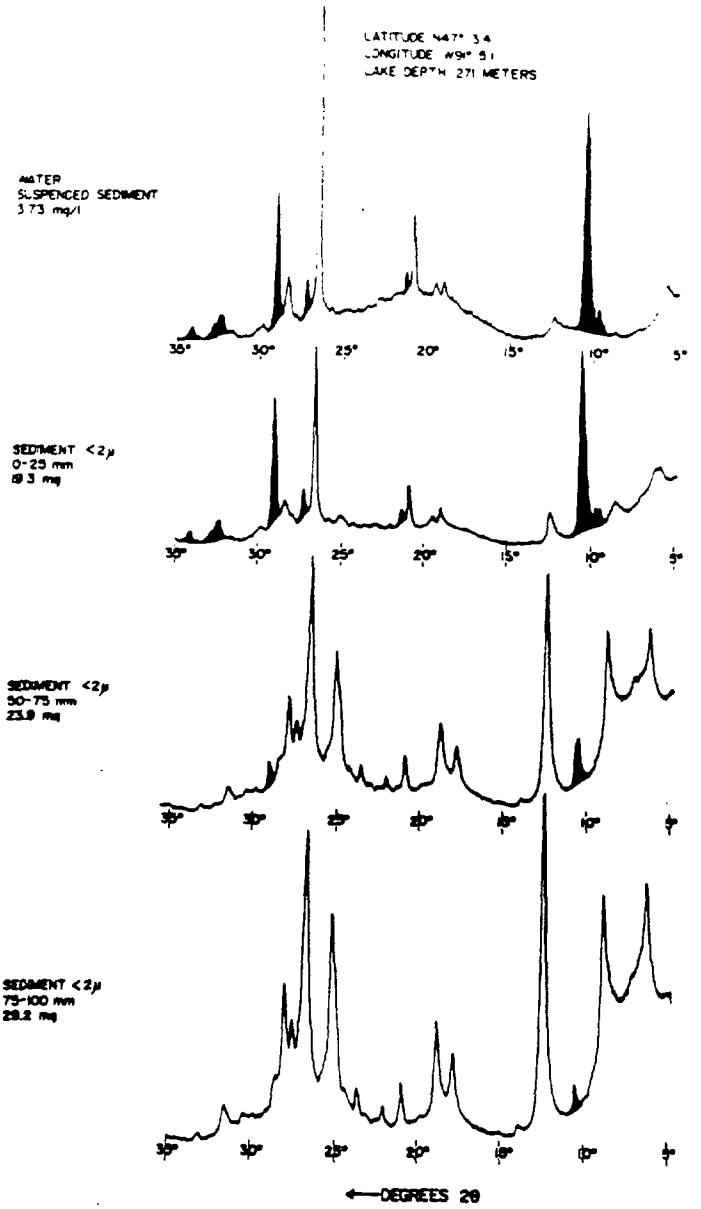


FIGURE 1---X-RAY DIFFRACTION PATTERNS (COPPER RADIATION) FROM SEDIMENT SAMPLES TAKEN AT SUCCESSIVE 25 MM INTERVALS IN AN AREA OF TACONITE TAILINGS DEPOSITION. CUMMINGTONITE-GRUNERITE, $(Mg,Fe)_7Si_8O_{22}(OH)_2$, PEAKS ARE SHADED. THE (110) PEAK AT APPROXIMATELY 10.6° 2θ IS COMMON TO MOST AMPHIBOLES

solids. X-ray diffraction patterns (Figure 1) of lake water suspended solids which contain taconite tailings and sediment from successive 25 mm sections of the lake bottom in an area of tailings deposition show a clear gradation from large amounts of cummingtonite-grunerite (shaded peaks) in very recent surficial sediments to no cummingtonite-grunerite and little amphibole in the older, underlying sediments (75-100 mm). X-ray diffraction study of hundreds of river suspended sediment samples also indicates no detectable cummingtonite-grunerite (<1%) and only 1-2% amphibole in the natural sediments entering Western Lake Superior. Much or all of the trace amphibole is the common, non-asbestiform mineral hornblende.

Further indication of the recent addition of cummingtonite-grunerite to Western Lake Superior water is provided by x-ray diffraction patterns of many suspended sediment samples saved from the years 1940, 1950, and 1964 (Figure 2). All samples from 1940 and 1950 did not contain detectable amounts of cummingtonite-grunerite and little if any other amphibole minerals as indicated by a (110) peak at 10.6° 2θ (d = 8.34 Å). All of the 1964 samples, however, contained large concentrations of cummingtonite-grunerite as shown by the appearance of large (110) and (310) peaks. The (110)/(310) peak ratios for these samples are typical of those found for taconite tailings samples.

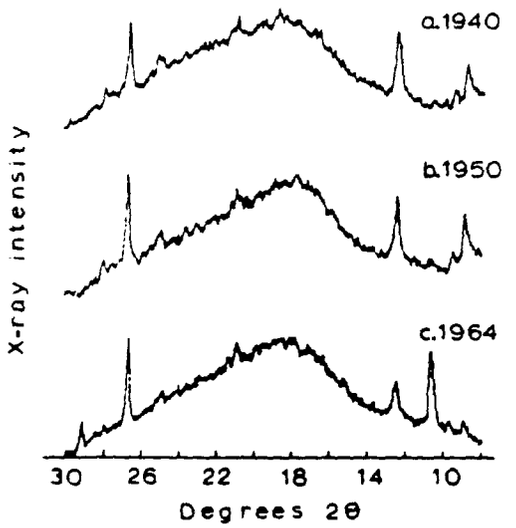


FIGURE 2——X-RAY DIFFRACTION PATTERNS FOR SUSPENDED SOLID SAMPLES OBTAINED FROM THE DULUTH MUNICIPAL WATER SUPPLY INTAKE: A HISTORICAL RECORD OF AMPHIBOLE CONCENTRATIONS IN DULUTH'S DRINKING WATER

In 1973, study of the morphology of amphibole particles in fine taconite tailings by transmission electron microscopy revealed the presence of many asbestiform fibers (Figure 3). The realization that many of these cummingtonite-grunerite fibers are indistinguishable from amosite asbestos fibers lead to concern over the use of Western Lake Superior water for municipal drinking water supplies. This concern was based on the association between human asbestos exposure and increased rates of cancer of the gastrointestinal tract and peritoneum (1) and daily x-ray diffraction analyses of Duluth, Minnesota drinking water samples which indicated the constant presence of high concentrations of taconite tailings. Transmission electron microscope analysis of Duluth water samples confirmed the presence of many amphibole fibers.

Since the discovery of asbestiform amphibole fibers in the water supplies of Silver Bay, Beaver Bay, Two Harbors, Duluth, and Cloquet, Minnesota, extensive sampling programs by the Environmental Protection Agency and other groups have been undertaken for electron microscope fiber counts. These analyses while in agreement with the x-ray diffraction results, are very expensive, time-consuming, and imprecise. At this time fiber counts done by different laboratories are not comparable and intralaboratory replicate results usually vary by ± 50% of the mean. The amphibole fiber concentrations generally correlate with the amphibole mass concentrations determined by x-ray diffraction. Thus x-ray diffraction monitoring of water samples combined with occasional electron microscope fiber counts offers a faster, less expensive, and probably more accurate measure of amphibole fiber contamination. This technique has been particularly useful for evaluating various filtration media's abilities to remove amphibole fibers from drinking water.

## AMPHIBOLE ANALYSIS OF WATER SAMPLES

Water samples from Western Lake Superior public water supplies, normally ten liters in volume, are pressure filtered through 0.45μ membrane fibers. When the turbidity of the sample is known, the volume filtered is adjusted to give a 4-8 mg sediment sample. The total suspended solids are determined by difference and a weighing correction applied to compensate for a small filter weight loss due to leaching (2). Distilled water blanks are run periodically to check for contamination. The dry membrane filter with sample is fastened to a glass slide with a thin film of lacquer, the filter edges trimmed, and the slide directly examined with a Norelco vertical diffractometer (copper $K_\alpha$ radiation) with a graphite crystal focusing monochromator.

The amphibole fibers and cleavage fragments assume a preferred orientation such that the c-axis, which corresponds to the long dimension of the fiber, is parallel to the filter surface. This

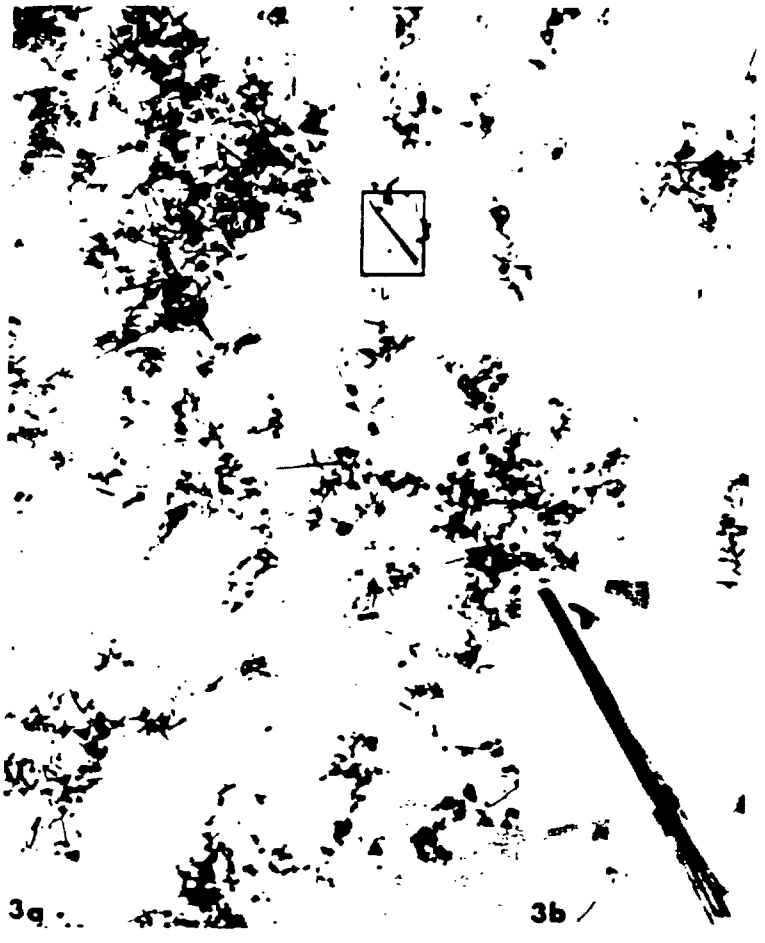


FIGURE 3——ELECTRON MICROGRAPH OF <2μ TACONITE TAILINGS. a) LOW MAGNIFICATION (2,500X). b) HIGHER MAGNIFICATION (12,500X) VIEW OF AN AMPHIBOLE FIBER BUNDLE

causes the (110) reflection and, to a lesser extent, the (310) reflection intensities to be enhanced, permitting the detection of trace amounts of amphibole. As little as 0.05 mg of <2μ cummingtonite-grunerite produces measurable (110) and (310) peaks.

A semi-quantitative measurement of the amphibole concentration is made by an external standard technique. This technique has been

used to estimate trace amounts of chrysotile asbestos and amphibole asbestos in dust samples on membrane filters (3,4) and fulfills the need for rapid, standardized estimates of amphibole concentration in samples which are not amenable to the use of an internal standard. Three potentially large sources of systematic error had to be considered before accepting the external standard model: variability of particle size, sample mass absorption coefficient, and amphibole preferred orientation.

The external standard chosen for the preparation of standard curves of x-ray peak intensity versus mass of amphibole was the amphibole mixture found in the <2μ taconite tailings. This choice was made since the predominant amphibole in Western Lake Superior water is cummingtonite-grunerite from taconite tailings and natural amphibole concentrations in Lake Superior water are normally not detectable by x-ray diffraction. The <2μ taconite tailings were determined by the x-ray diffraction of cummingtonite-grunerite/quartz mixtures to contain approximately 80% amphibole and 20% quartz. Most of the amphibole is cummingtonite-grunerite with some actinolite-tremolite. Larger size fractions of the tailings contain less amphibole and more quartz with a small percentage of magnetite.

Reference samples were prepared by adding known amounts of the <2μ amphibole standard to ten liter samples of Lake Superior water having no detectable amphibole minerals. This water, obtained from Grand Marais, Minnesota, contained 0.4 mg/l suspended solids which consisted primarily of organic debris, diatoms, quartz, and clay minerals. These standard samples were then filtered and analyzed by x-ray diffraction in the same manner as unknown samples. The resulting x-ray diffraction patterns are identical in appearance to those for Duluth water samples.

The <2μ amphibole particle size (by gravity settling) for the external standard was shown to be appropriate by a centrifugation size-separation of Duluth water suspended solids from samples taken on fifteen different days. Ninety-five percent of the suspended solids were in the <2μ fraction with only a small amount of amphibole in the 5% which was >2μ. Thus variability in diffracted x-ray intensity due to mineral particle size >2μ is insignificant.

The filtration of ten liters of Duluth water normally results in 4-8 mg of suspended solids retained on the 0.45μ membrane filter. When the suspended solids exceed 0.8 mg/l, smaller volumes are filtered. A sample weight of 8 mg and an average density of 2 $g/cm^3$ results in a hypothetical sample thickness of 3μ on the filter. This thin sample thickness should preclude variability due to differences in sample absorption coefficients. Direct evidence for this is provided by the linearity of a plot of percent amphibole versus x-ray intensity for samples in this weight range; the uniform

intensity of filter background in the x-ray diffraction pattern with increasing sample weight to 10 mg; and the linearity of a plot of quartz peak (d = 3.33 Å) intensity versus weight of quartz, regardless of total sample weight in the range 0–12 mg.

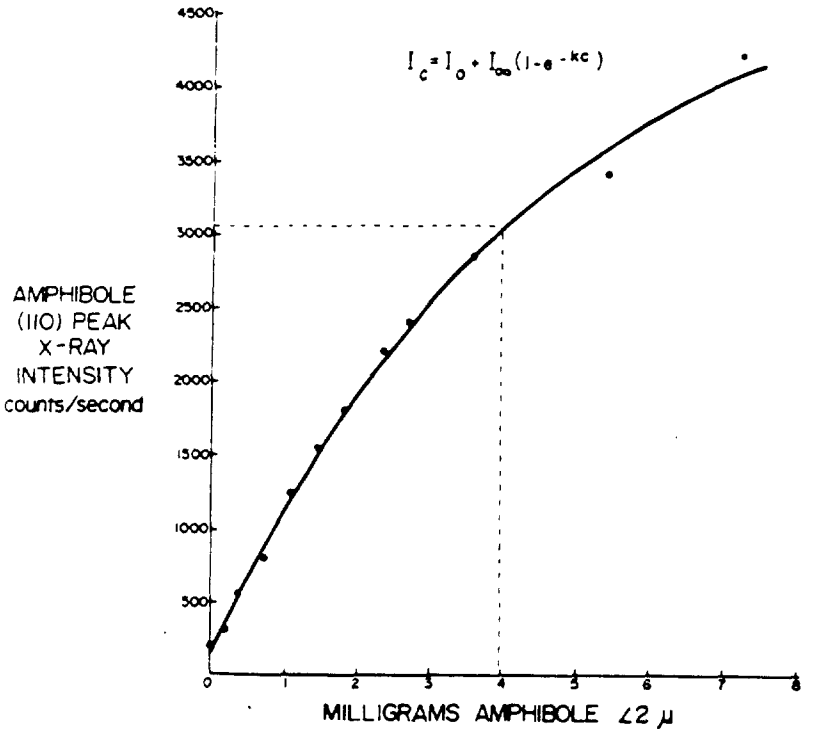


FIGURE 4—EXTERNAL STANDARD CURVE FOR AMPHIBOLE SEMI-QUANTITATIVE ANALYSIS

The non-linearity of the external standard curve (Figure 4) is due to a decreasing degree of preferred orientation as the amount of amphibole increases. This is indicated by decreasing amphibole (11Q)/(31Q) and amphibole (110)/quartz peak ratios with increasing weight of the standard amphibole–quartz mixture on the filter. The utility of the external standard curve depends on how well the curve models amphibole preferred orientation in environmental samples. Similar curves based on samples prepared with increased amounts of natural sediment agreed well with the standard curve used. With large amounts of natural sediment, the amphibole peak intensity is weakened which would cause an underestimation of amphibole concentrations. Other standard curves were employed to estimate the amphibole concentration in the few samples with a very high concentration of non-amphibole minerals.

External standard curves, such as Figure 4, were plotted from the non-linear least squares refinement of amphibole mass versus amphibole (110) peak intensity data points. The data fit an equation of the form: $I_C = I_0 + I_\infty(1 - \exp{-kC})$, where $I_C$ = intensity at concentration C (mg amphibole); $I_0$ = intensity at C = 0; $I_\infty$ = intensity at C = ∞; and k is a constant. This equation is consistent with a model in which the degree of preferred orientation decreases as more amphibole particles are placed on the membrane filter. Standard curves utilizing amphibole (110) peak height above background are identical to curves plotted from the (110) peak areas. Both measurements are used and give the same amphibole concentrations for environmental samples. Use of an amphibole (310) peak curve gives the same results but with less precision due to lower peak intensity.

Replicate (five) analyses of Duluth water samples indicate a standard deviation of ± 3% for determining amphibole concentrations in typical samples with 0.1-0.3 mg/l amphibole. For samples having lower amphibole concentrations (<0.1 mg/l) and high suspended solids (>1.0 mg/l), this precision is reduced to ± 25%. Overall suspended solids determinations have a standard deviation of ± 6% of the mean. Detection limits for determining amphibole concentration depend on the volume of water filtered and can be as low as 0.5 ug/l.

## WATER SUPPLY AMPHIBOLE ANALYSIS

Daily analyses of Duluth water samples for amphibole and suspended solids concentrations began in March 1973 and continues to date. Results through January of 1974 are shown in Figure 5 with climatological data and intake water temperatures. X-ray diffraction analysis provides a picture of daily and seasonal fluctuations in amphibole and suspended solids concentrations. For example, periods of heavy rainfall are followed by abrupt increases in suspended solids due to river run-off and shore erosion. These increases in suspended solids do not coincide with increases in amphibole, indicating a different source for amphibole sediment.

Maximum amphibole concentrations (up to 0.8 mg/l) occur in the late fall and spring. Minimum amphibole concentrations (0.04 mg/l) occur during the late summer and early fall when a thermocline is present in Western Lake Superior. The average amphibole concentration measured was 0.19 milligrams per liter with 0.83 milligrams per liter total suspended solids.

During the period August 22-November 28, 1973, personnel from Region V of the Environmental Protection Agency obtained weekly water samples from municipal water supplies using Lake Superior water from Grand Marais, Minnesota to Marquette, Michigan. These

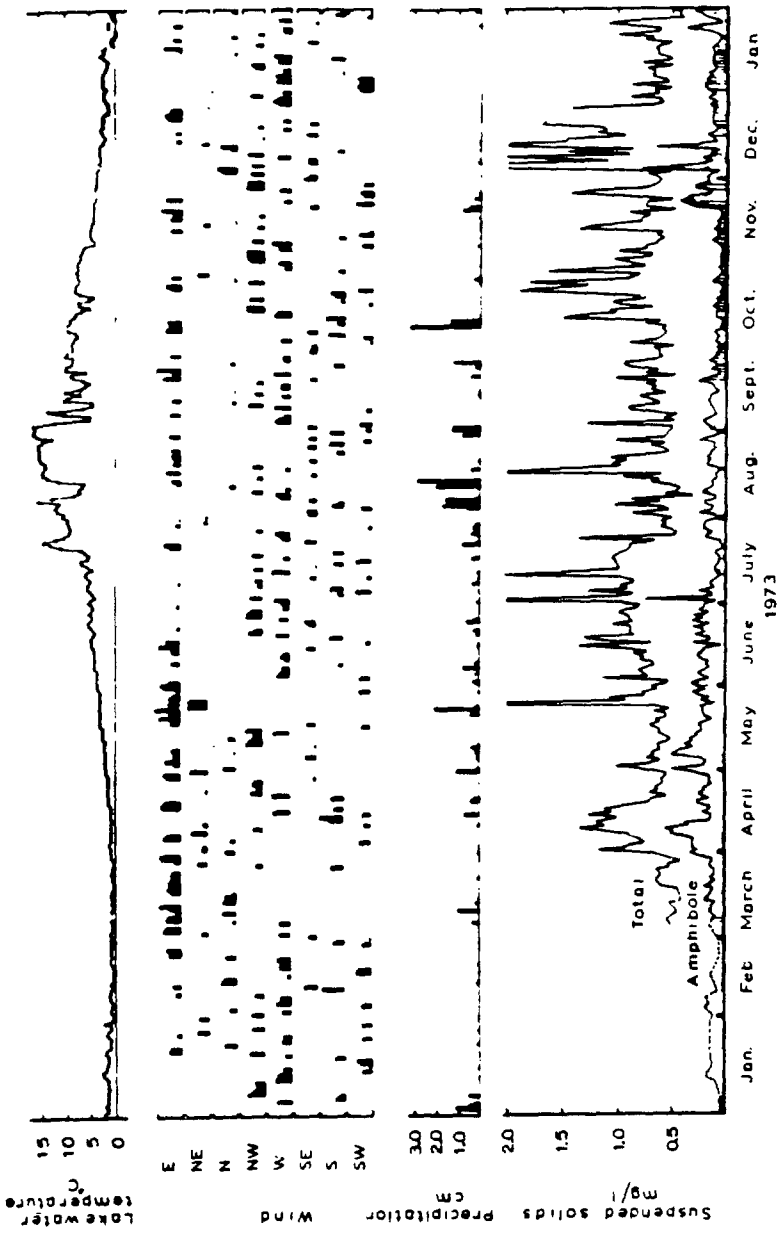


FIGURE 5---DAILY AMPHIBOLE AND SUSPENDED SOLIDS CONCENTRATIONS FOR DULUTH WATER WITH CLIMATOLOGICAL DATA AND WATER INTAKE TEMPERATURES

samples were analyzed for amphibole mass concentration at the National Water Quality Laboratory and amphibole fiber concentration by transmission electron microscopy at the Ontario Research Foundation in Sheridan Park, Ontario and McCrone Associates in Chicago, Illinois. Figure 6 depicts the average x-ray diffraction and electron microscope measurements for each station. The agreement between these two measurements is obviously very good. The pattern of maximum concentrations at Beaver Bay and decreasing concentrations in a counterclockwise direction around Western Lake Superior is consistent with large quantities of amphibole fiber discharged at a point between the Silver Bay and Beaver Bay, Minnesota water supply intakes and then transported towards Duluth (southwest) by the predominantly counterclockwise currents of Western Lake Superior (5).

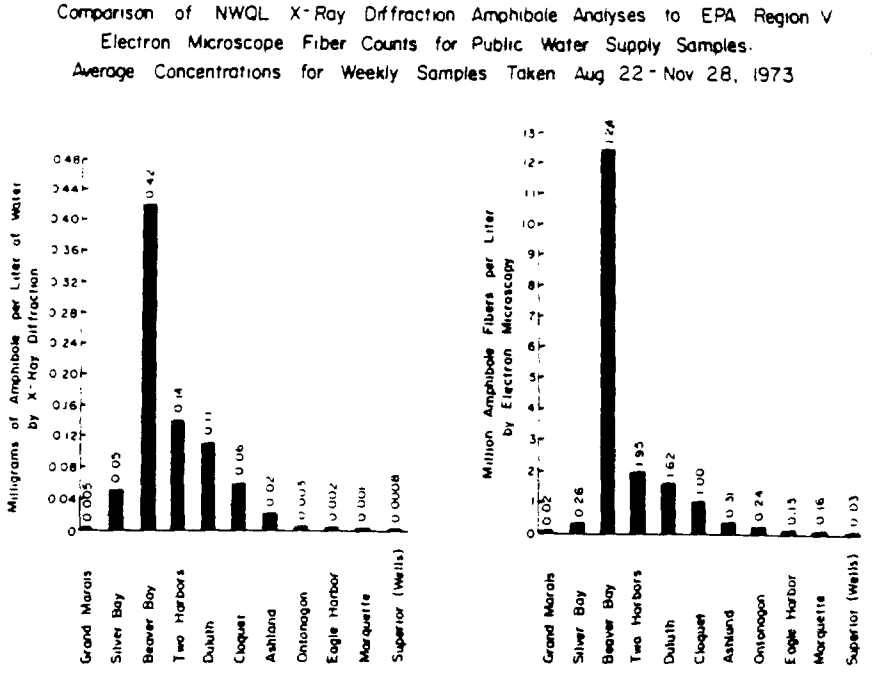


FIGURE 6---COMPARISON OF AMPHIBOLE MASS CONCENTRATION DETERMINED BY X-RAY DIFFRACTION TO TRANSMISSION ELECTRON MICROSCOPE AMPHIBOLE FIBER COUNTS FOR LAKE SUPERIOR WATER INTAKES

ACKNOWLEDGEMENTS

The author wishes to gratefully acknowledge the assistance of Mr. James Tucker of the National Water Quality Laboratory for electron microscope examinations of water and tailings samples; Mr. Robert Fulton and Mr. David Marklund for their excellent work in preparing many of the samples examined by x-ray diffraction; and Dr. Billy Fairless of the Environmental Protection Agency, Region V, Central Regional Laboratory, for providing water intake fiber counts.

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# Carcinogenesis by Chemicals: An Overview—G. H. A. Clowes Memorial Lecture[1]

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It is a tremendous honor to be chosen as the Ninth G. H. A. Clowes Memorial Lecturer and it is one that I share fully with my coworker Dr. Elizabeth Cavert Miller. I never cease to marvel at my good fortune in having her as a marital and research partner since our graduate student days. Our work together, particularly that of the past decade, forms the greater part of this talk. Both of us owe a great deal to our many collaborators over the years, and nothing can compare with the steadfast support and encouragement that we have received from Dr. Harold P. Rusch, Director and indeed the builder of the McArdle Laboratory since its inception in 1940. We know that our colleagues at McArdle join us in voicing our great esteem for Harold Rusch.

Dr. G. H. A. Clowes helped to found this Association in 1907 and was for many years Director of Research of Eli Lilly and Company. Clowes' essay in 1956 on "Cancer Research Fifty Years Ago and Now" (20) provides interesting perspective for these days of relative freedom and affluence in research. Clowes wrote: "In comparing conditions 50 years ago with those of the present, the greatest contrast is to be found in the freedom now granted to research workers in choosing a field of investigation and deciding what course to follow." " . . . whereas 50 years ago there was little or no incentive to engage in cancer research, today the results being obtained in this field are so important and the opportunities for research are so excellent that able young investigators need have no hesitation about engaging even in long-term projects in this most difficult and at the same time most fascinating field."

Dr. Clowes was intensely interested in understanding basic aspects of many facets of metabolism and growth of normal and malignant cells. This talk in his remembrance is directed in a modest way toward similar goals.

## CARCINOGENESIS IN MAN AND EXPERIMENTAL ANIMALS

As oncologists all of us seek to understand and control neoplasia in our species, and it is evident that the degree of understanding and control that any scientific generation achieves depends largely on the concepts and tools it can devise. Looking back, one sees that among the

[1] Presented at the 60th Annual Meeting of the American Association for Cancer Research, March 1969, San Francisco, Calif. The work of the author and his associates has been supported by Grants CA-07175 and CRTY-5002 from the National Cancer Institute, USPHS, and by grants from the Jane Coffin Childs Memorial Fund for Medical Research and the Alexander and Margaret Stewart Trust Fund.

major contributions of research in oncology in the latter half of the 19th century were the recognition and description of the great variety of malignant and benign neoplasms in man and other species as somatic cellular diseases. Likewise, among the successes of cancer research in this century stand the discoveries of the wide variety and large number of chemicals, viruses, and radiations that can induce cancer in mammalian species. Certainly, everyone would like to see cancer research in the remaining decades of this century reveal the molecular natures of carcinogenic processes and the molecular phenotypes of the neoplasms these processes produce. One cannot help but feel that even partial successes in these difficult tasks will spark major advances in the therapy and prophylaxis of cancer in the human. So let us hope that this Association will be out of business by the year 2000!

Any overview of chemical carcinogenesis should start with comments on the observations of the London surgeon Percivall Pott (125) on the occurrence of scrotal skin cancer in English chimney sweeps. Now almost 200 years old, Pott's implication of gross contact with soot as a cause of this neoplasm and his emphasis on its long latent period of development stand as milestones in oncology. Pott did not suggest avoidance of contact with soot as a means of prevention. Perhaps he thought it impractical for a chimney sweep—an essential worker in Pott's time. Still, as Clemmesen (19) has noted, Pott's book in 1775 with its brief paragraphs on the "soot wart" apparently inspired a ruling 3 years later by the Danish Chimney Sweepers' Guild that its members should bathe daily. The value of that ruling was revealed a century later when Butlin (16) investigated the relative rarity of scrotal skin cancer in chimney sweeps outside of England and found that on the continent it was apparently prevented by frequent bathing and protective clothing. How simple this seems today when we make only painfully slow progress toward the elimination of a personal form of air pollution that has led to an epidemic of lung cancer in the human and is tenaciously fostered by habit and the cigarette industry.

From Pott's time to our present chemical age, prevention of contact with harmful chemicals is a lesson that has had to be taught again and again. Violations of this simple but difficultly appreciated and applied rule have led repeatedly to the discovery of a variety of chemical carcinogens with man as the unwitting species at risk. These carcinogens are listed in Table 1. One can only admire the efforts of the epidemiologists who established these cause and effect relationships from thousands of human tragedies.

Table 1
*Chemicals recognized as carcinogens in the human*

| Agent | Target |
|---|---|
| Certain soots, tars, oils (18) | Skin, lungs |
| Cigarette smoke (146) | Lungs |
| 2-Naphthylamine (18) | Urinary bladder |
| 4-Aminobiphenyl (18, 77) | Urinary bladder |
| Benzidine (18) | Urinary bladder |
| *N,N*-Bis(2-chloroethyl)-2-naphthylamine (155, 160) | Urinary bladder |
| Bis(2-chloroethyl)sulfide (162) | Lungs |
| Nickel compounds (18, 152) | Lungs, nasal sinuses |
| Chromium compounds (18) | Lungs |
| Asbestos (18, 163) | Lungs, pleura |

Table 2
*Some early landmarks in experimental chemical carcinogenesis*

| | | |
|---|---|---|
| 1915<br>1918 | Induction of skin cancer in rabbits and mice by coal tar | Yamagiwa and Ichikawa (173, 174), Tsutsui (158) |
| 1930 | Tumor induction by the first pure chemical carcinogen-1,2,5,6-dibenzanthracene | Kennaway and Hieger (71) |
| 1933 | Isolation of the carcinogen 3,4-benzpyrene from coal tar | Cook, Hewett, and Hieger (23) |
| 1933<br>1936 | Induction of liver cancer in rats by *o*-aminoazotoluene and by *p*-dimethylaminoazobenzene | Yoshida (175), Kinosita (75) |
| 1937 | Induction of urinary bladder cancer in dogs by 2-naphthylamine | Hueper, Wiley, and Wolfe (60, 61) |
| 1941 | Initiation and promotion stages in skin carcinogenesis with tar and 3,4-benzpyrene | Berenblum (4, 5); Rous, MacKenzie, and Kidd (93, 135) |

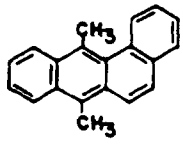
Experimental confirmation of the first recognized chemical carcinogens for the human was sought quite early, but the use of limited dosages and times of exposure coupled with unfortunate choices of species led to many failures (59, 149). The first breakthrough was reported from Japan in 1915, and a number of important landmarks in experimental chemical carcinogenesis occurred in the subsequent 3 decades (Table 2). The preoccupation with aromatic carcinogens then gave way to the discovery of a wide variety of aliphatic carcinogens and some carcinogenic metals. From the beginning, the chemical carcinogens have provided tools for studies on mechanisms of action of carcinogenesis. The discovery of new chemical carcinogens continues unabated and still occasionally derives from exposures of humans to various compounds. Concomitant with these discoveries of chemical carcinogens, a wide variety and large number of carcinogenic or oncogenic viruses (14, 50, 161) have been revealed. Likewise, X-rays and ultraviolet light, early shown to be carcinogenic in man and then in experimental animals, have been joined by a variety of carcinogenic ionizing particulate radiations (6, 55, 130).

So today we know that we are confronted with a host of carcinogenic chemicals, viruses, radiations, and complex interplays between these agents as models and examples of causes of cancer in man. It seems certain that many new chemical and viral carcinogens will be found in future work. Recent work of the geographical pathologists (57, 121) has raised strong suspicion that a high proportion of human cancer is of environmental origin. The chemical carcinogens in our total environment, through lifetimes of exposure to small amounts of these compounds, may rank as major causes of human cancer. This in no way minimizes the possible role of viruses in carcinogenesis in the human. Present knowledge on the

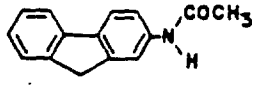
oncogenic viruses makes it almost inconceivable that our species could be exempt from such causes of cancer. It seems far more likely that the important question will be the degree to which such agents act alone, in concert with, or following the action of chemical and physical carcinogens in the human. If, for example, some or many chemical carcinogens activate or act with oncogenic viruses, the removal of the chemical carcinogen might frequently be the most feasible course of action. The recognition and control of chemical, viral, and physical carcinogens in man's external and internal environment has become a principal task of cancer research.

## CHEMICALS AS CARCINOGENS

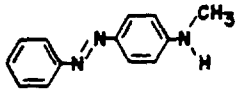
Chemical carcinogens now comprise a very diverse group of nonviral and nonradioactive organic and inorganic structures with various species and tissue selectivities. Some of these agents have always been present in inanimate nature, many of them are products of man's day to day activities, still more of them derive from man's great synthetic capabilities in the laboratory, and some of the newest chemical carcinogens are metabolites of living cells.



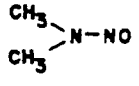
7,12-DIMETHYL-BENZ(a)ANTHRACENE (9,10-DIMETHYL-1,2-BA)



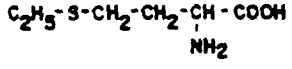
2-ACETYLAMINOFLUORENE



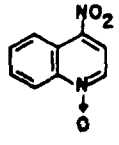
N-METHYL-4-AMINOAZOBENZENE



DIMETHYLNITROSAMINE

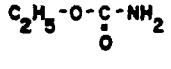


ETHIONINE



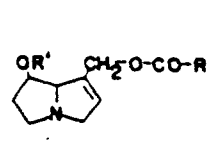
4-NITROQUINOLINE-1-OXIDE

CARBON TETRACHLORIDE

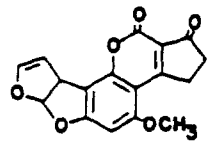


ETHYL CARBAMATE (URETHAN)

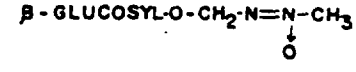
Chart 1. Some synthetic chemical carcinogens for experimental animals (18).



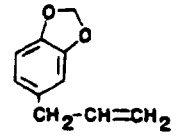
PYRROLIZIDINE ALKALOIDS (SENECIO, CROTOLARIA AND HELIOTROPIUM GENERA)



AFLATOXIN $B_1$ (ASPERGILLUS FLAVUS STRAIN)



CYCASIN (CYCAD NUTS)



SAFROLE (OIL OF SASSAFRAS)

Chart 2. Some naturally occurring compounds which are carcinogenic for experimental animals (110, 112).

Some of the variety to be found among the synthetic chemical carcinogens is shown in Chart 1. The very potent hydrocarbon 9,10-dimethyl-1,2-benzanthracene, the versatile aromatic amide AAF,[2] the aminoazo dye *N*-methyl-4-aminoazobenzene, and the nitrosamine dimethylnitrosamine are each representative of large groups of similar compounds with various species and tissue specificities. The heterocyclic compound 4-nitroquinoline-1-oxide and its close relatives were discovered to be carcinogenic relatively recently and have carcinogenicities similar to those of the polycyclic aromatic hydrocarbons. Ethionine, an amino acid which is hepatocarcinogenic in the rat, was originally designed as an antagonist of methionine. The mouse and hamster hepatocarcinogen carbon tetrachloride and the versatile water-soluble carcinogen ethyl carbamate further exemplify the variety of structures found among chemical carcinogens.

The variety of carcinogenic structures seems almost endless, and some of the newest and most exotic structures have been found as metabolites of fungi and green plants (110, 112). Some of them, such as the aflatoxins, the pyrrolizidine alkaloids, and cycasin (Chart 2), have very high hepatocarcinogenicities in the rat. The aflatoxins are suspected as being among the causes of the high incidences of primary hepatic cancer in native population groups in central and southern Africa and elsewhere (2, 7, 76, 170).

An important aspect of carcinogenesis by the synthetic and naturally occurring carcinogens shown in Charts 1 and 2 is that studies on their mechanisms of action indicate strongly that most if not all of these compounds are not carcinogenic as such. They appear instead to be *precarcinogens* which are converted in the host into carcinogenic and reactive structures. In this respect, they differ from the carcinogenic alkylating agents shown in Chart 3. These agents are essentially in their final reactive forms as administered and they generally take part in $S_N2$ (substitution, nucleophilic, bimolecular) reactions in

[2] The abbreviations used are: AAF, 2-acetylaminofluorene; MAB, *N*-methyl-4-aminoazobenzene; AF, 2-aminofluorene.

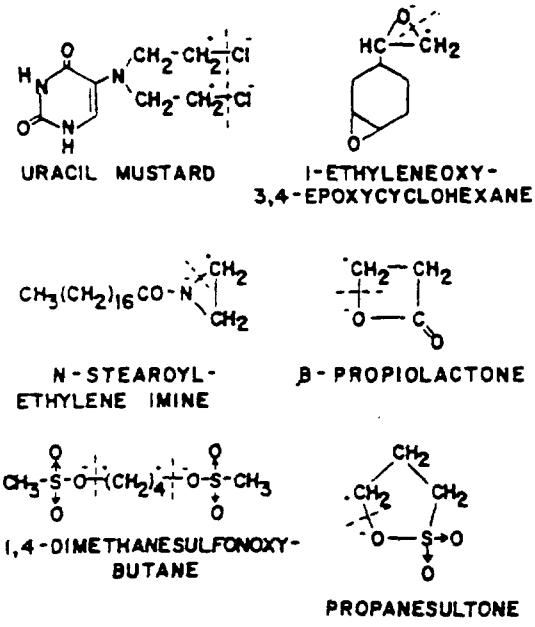



Chart 3. Some alkylating agents which are carcinogenic for experimental animals. *Dashed lines*, bonds which are cleaved in the alkylation reactions.

which a relatively positive or electrophilic atom in the alkylating agent combines with relatively negative or nucleophilic atoms of the molecules attacked in cells (129, 134, 141). During administration to tissues, these carcinogenic electrophilic reactants encounter extracellular nucleophiles such as water and protein before their entry into cells. This may account in part for the observations that the alkylating agents are frequently not strong carcinogens and may require multiple large dosages at local tissue sites to reveal their activity as carcinogens. This is not always true, however, for the uracil mustard shown in Chart 3 is a potent lung carcinogen in the mouse (1). Similarly, propanesultone, one of the newest carcinogenic alkylating agents (30), is a potent carcinogen in the subcutaneous tissue of the rat and even yields some tumors in distant neural tissue in this species.

The small class of carcinogenic metals (18, 43) such as beryllium, cadmium, cobalt, nickel, and lead is another group of agents which, in their ionic forms, are electrophilic reactants and can react with various nucleophiles. Organic electrophilic derivatives of metals may also be formed *in vivo*. Carcinogenesis by metals, especially with regard to mechanisms of action, has been a relatively neglected area. Studies on nickel carcinogenesis (43, 152) are an important exception, and similar investigations should aid in an understanding of carcinogenesis by these seemingly simple chemicals.

## MECHANISMS OF ACTION OF CHEMICAL CARCINOGENS: GENERAL ASPECTS

Since it seems axiomatic that carcinogenic agents must induce neoplasia through interaction with tissue components, efforts to understand the mechanisms involved in chemical carcinogenesis have been directed primarily toward studies of the chemical reactivities of the carcinogens and their metabolites. Thus, the determination of the active form(s) (ultimate carcinogens) of a chemical carcinogen comprises a logical first step in the elucidation of its mechanism of action. Most of the current work *in* chemical carcinogenesis is now focused in this area and at the next step, *i.e.*, the elucidation of the nature of the interactions of these active form(s) with tissue constituents. These studies are increasingly providing possible mechanisms by which chemical carcinogens may produce tumors, but the really difficult problems are still ahead. That is, we must determine which of these interactions are critical to particular carcinogenic processes *and* the mechanism(s) by which these critical interactions induce neoplasia. These are formidable problems which must still be met not only in the study of chemical carcinogenesis, but in the elucidation of the mechanisms involved in viral and radiation carcinogenesis as well. So far, it has not been possible to guide these determinations from a knowledge of the biochemical nature of the neoplasms produced by these agents, for the neoplastic properties of no tumor are understandable as yet in terms of its molecular phenotype. These problems in carcinogenesis at the molecular and cellular levels have been discussed in detail recently in an excellent review by Farber (38).

**Macromolecule-bound Derivatives of Chemical Carcinogens *in Vivo*.** The first covalent interactions of chemical carcinogens with proteins of target tissues were noted over 20 years ago (105, 106), and the earliest studies on the reaction of nucleic acids with alkylating agents *in vivo* were made in 1957 (167). Covalent bindings of residues of chemical carcinogens with macromolecules (DNA, RNA, and/or proteins) *in vivo* have now been noted in all cases which have been adequately examined (38, 107, 115). These studies have most frequently involved examinations of the liver or skin of treated animals, but macromolecules from a wide variety of other tissues have been studied after treatment with specific chemicals. Likewise, these studies have utilized a wide variety of chemical carcinogens: the aromatic amines, aromatic polycyclic hydrocarbons, alkylating agents, and potential alkylating agents. Interactions of derivatives of these and other chemical carcinogens with other macromolecules [e.g., glycogen (35, 38)] and with lower-molecular-weight compounds of tissues surely also occur. However, the protein and nucleic acid interactions have been the focus of interest since, in our present state of knowledge, we look chiefly to the proteins and nucleic acids for those changes which could explain the heritable and at least quasipermanent partial or complete loss of growth controls that characterizes neoplasms. Indeed, a number of correlations have been obtained in which the amount of 1 or more of these covalent bindings appears to correlate with the ultimate incidence of tumors (12, 56, 106, 107). However, these correlations with binding to total DNA, RNA, or protein are not exact and need considerable refinement before far-reaching assessments of their roles in the carcinogenic process can be made.

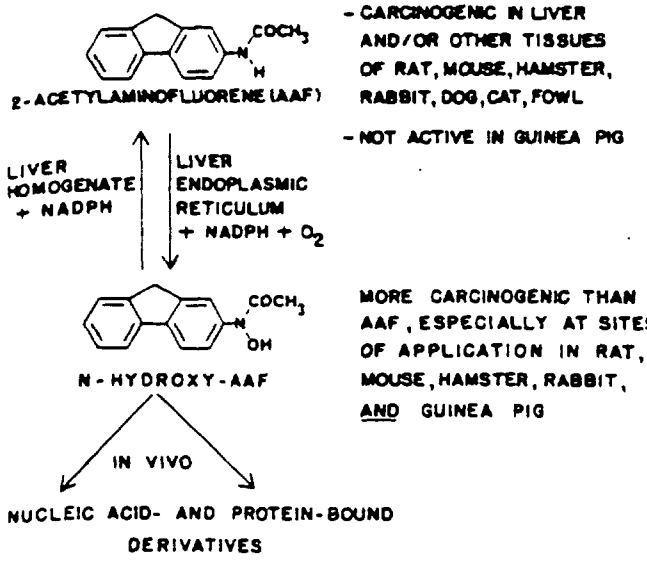



Chart 4. The primary activation of the carcinogen AAF by *N*-hydroxylation (25, 109).

**Reactive Forms of Chemical Carcinogens.** Although the studies on the covalently bound forms of chemical carcinogens in cells have not revealed the molecular aspects of carcinogenesis that we seek, these studies have provided considerable insight into the reactive forms of chemical carcinogens *in vivo*. As noted later in this talk, an important product of this work in the past few years has been the realization that, despite their chemical dissimilarities, many chemical carcinogens are metabolized *in vivo* to potent electrophilic reactants. The properties of these reactive forms of the carcinogenic aromatic amines and amides are considered next in some detail, since they form a well-documented example.

## CARCINOGENESIS BY AROMATIC AMINES AND AMIDES

***N*-Hydroxylation of Aromatic Amines and Amides.** The propensity of the aromatic amine and amide carcinogens to induce tumors in tissues distant from the sites of entry, but not at these sites, in man and experimental animals has been the basis for many years of the idea that these compounds require metabolic activation. Evidence that *N*-hydroxylation is the initial activation step for the carcinogen AAF was first obtained in our laboratory in 1960 with Dr. John Cramer (25) (Chart 4), and this conclusion is now supported by studies from several laboratories with a variety of carcinogenic aromatic amines and amides (113, 114). In some cases, the inability of the animal to *N*-hydroxylate a sufficient amount of an amide appears to be the crucial factor which prevents an amide from being a carcinogen. For instance, the guinea pig has little or no ability to *N*-hydroxylate AAF; AAF is not carcinogenic for this species while *N*-hydroxy-AAF does produce tumors (108). Similarly, Gutmann *et al.* (53) have shown that the synthetic *N*-hydroxylation of 7-hydroxy-AAF, a noncarcinogenic metabolite of AAF (58, 118), converts it into a potent carcinogen for the rat (53). On the other hand, the introduction of a *N*-hydroxy group into an aromatic amine or amide is not necessarily sufficient to convert it into a carcinogen; as shown in both our studies (104, 136) and those of Gutmann *et al.* (53, 54), the aryl group is a very important determinant.

The greater carcinogenic activity of the metabolite *N*-hydroxy-AAF, as compared to that of the parent amide (109), and the higher yields of macromolecule-bound fluorene derivatives (Chart 4) in the livers of rats given *N*-hydroxy-AAF rather than AAF (27, 63, 97), implicate *N*-hydroxy-AAF as an intermediate in the carcinogenicity and *in vivo* reactivity of AAF. However, the fact that *N*-hydroxy-AAF, like AAF, has very little ability to react under physiological conditions with proteins or nucleic acids or their derivatives (3, 48, 64, 73, 102, 103) indicates that some further metabolic step(s) are necessary to convert *N*-hydroxy-AAF to ultimate carcinogenic and reactive forms. Since our first evidence that this metabolic activation depended on esterification of the *N*-hydroxy group to yield strong electrophilic reactants came from studies on MAB, a hepatocarcinogenic aminoazo dye, these data will be considered first.

**Metabolic Activation of MAB *in Vivo*.** The first hints as to the nature of the reactive forms of MAB *in vivo* came from studies with Drs. Scribner, Poirier, and Lotlikar. The first step was the finding that treatment with cold alkali of the liver proteins from rats fed MAB yielded

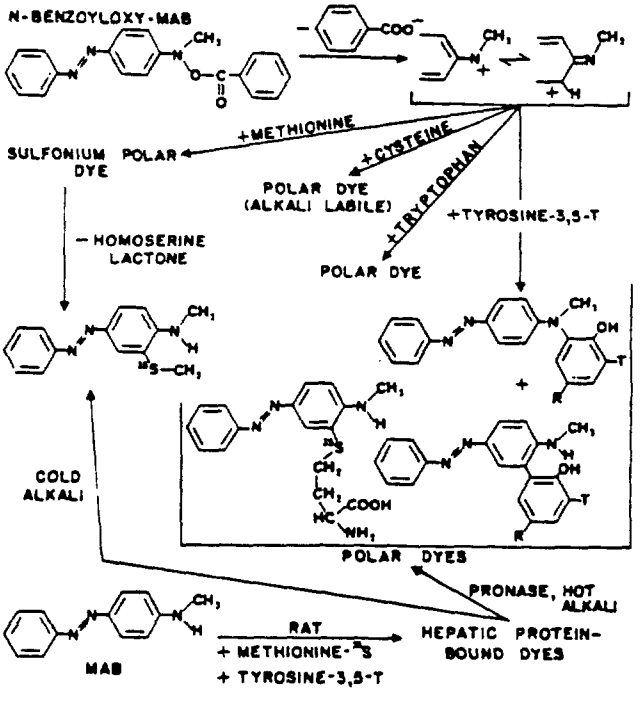



Chart 5. The nonenzymatic reactions of the synthetic ester *N*-benzoyloxy-MAB with certain amino acids at neutral pH and the identity of some of these *in vitro* products with derivatives released on hydrolysis of the liver protein from rats fed MAB (88, 89, 92, 124, 137).

3-methylmercapto-MAB, in which the thiomethyl group was derived from methionine (114, 137) (Chart 5). Shortly afterward, *N*-benzoyloxy-MAB, synthesized as a possible precursor of *N*-hydroxy-MAB (124), was shown to be a potent electrophilic reactant. It reacts with methionine nonenzymatically at pH 7 to yield a water-soluble derivative which decomposes to 3-methylmercapto-MAB (92, 124). These observations led to the suggestion that the metabolically active form of MAB in the rat liver is an ester of *N*-hydroxy-MAB.

Further evidence that a reactive form of MAB *in vivo* might be an ester of *N*-hydroxy-MAB has come from the studies with Dr. Lin (88, 89) on the characterization of the protein-bound dyes in the livers of rats fed MAB. The polar dyes released by hydrolysis of the liver proteins can also be formed by reaction of *N*-benzoyloxy-MAB with the appropriate amino acid (Chart 5). The major polar dye released by this procedure is 3-(homocystein-*S*-yl)-MAB; this dye presumably arises *in vivo* through the *S*-demethylation of protein-bound 3-(methion-*S*-yl)-MAB. It can be formed nonenzymatically by reaction of *N*-benzoyloxy-MAB with homocysteine. Two other polar dyes derived from the hepatic protein-bound dyes are identical with 3-(3-tyrosyl)-MAB and *N*-(3-tyrosyl)-MAB synthesized by reaction of *N*-benzoyloxy-MAB with tyrosine. *N*-Benzoyloxy-MAB also reacts with tryptophan and cysteine, but no polar dyes containing these amino acids were detected. The lack of detection of a cysteinyl polar dye is in accord with the extreme lability of the cysteinyl derivative prepared by reaction with *N*-benzoyloxy-MAB to the alkaline hydrolysis used in the preparation of the polar dyes. By the use of other procedures, Ketterer and Christodoulides (72) have obtained evidence for a cysteinyl dye derivative in the liver protein of rats given 3'-methyl-4-dimethylaminoazobenzene.

Very recent studies with Dr. Lin[3] have shown that the nucleic acid-bound derivatives formed from MAB in the rat liver *in vivo* are also derived from esters of *N*-hydroxy-MAB or derivatives with similar reactivity. Thus, degradation of the RNA and DNA from the livers of rats given injections of MAB-prime ring-$^3$H yields derivatives which are chromatographically identical with the compounds prepared by reaction of *N*-benzoyloxy-MAB with guanosine or deoxyguanosine. The latter compounds have been characterized as *N*-(guanosin-8-yl)-MAB and *N*-(deoxyguanosin-8-yl)-MAB.

These studies on the structures of the end products formed by reaction of dye derivative(s) with proteins and nucleic acids *in vivo* cannot establish the identity of the reactive metabolite(s). However, the suggestion that ester(s) of *N*-hydroxy-MAB are metabolic intermediates is supported by the similar reactivities of *N*-benzoyloxy-MAB and the reactive metabolite(s) *in vivo* and by the data correlating the formation of the sulfuric acid ester of *N*-hydroxy-AAF with the hepatocarcinogenicity of this hydroxamic acid (see below).

**Metabolic Activation of *N*-Hydroxy-AAF *in Vivo*.** The initial studies on the reactivity of *N*-benzoyloxy-MAB led quickly to similar studies with synthetic esters of *N*-hydroxy-AAF with Drs. Lotlikar, Scribner, and DeBaun. Like *N*-benzoyloxy-MAB, *N*-acetoxy-AAF and *N*-benzoyloxy-AAF are potent electrophilic reactants. They react with methionine and methionyl peptides, and the resulting sulfonium derivatives decompose to yield a mixture of 1- and 3-methylmercapto-AAF (27, 92, 103) (Chart 6). The liver proteins from rats given AAF or *N*-hydroxy-AAF yield these same *o*-methylmercapto-AAF's on treatment with cold alkali (27). Degradation of the peptide-bound sulfonium derivatives can occur by an internal nucleophilic attack which causes cleavage of the peptide linkage (Chart 6) in a manner analogous to that reported by Gross and Witkop (49) and Gundlach *et al.* (52) for alkylated methionine residues in peptides. To the extent that this type of decomposition occurs *in vivo*, the fluorene residues would be detached, the protein would be cleaved at the former methionyl peptide linkage, and one of the resulting peptides would contain homoserine instead of methionine. The protein-bound derivatives in the rat liver which give rise to 1- and 3-methylmercapto-AAF account for only about 15% of the total protein-bound fluorenyl derivatives. By analogy with the protein-bound forms of MAB in rat liver and from the known reactivity of esters of *N*-hydroxy-AAF, fluorene derivatives of homocysteine, cysteine, tyrosine, and tryptophan may also occur in the livers of rats given *N*-hydroxy-AAF. These have not yet been studied in detail.

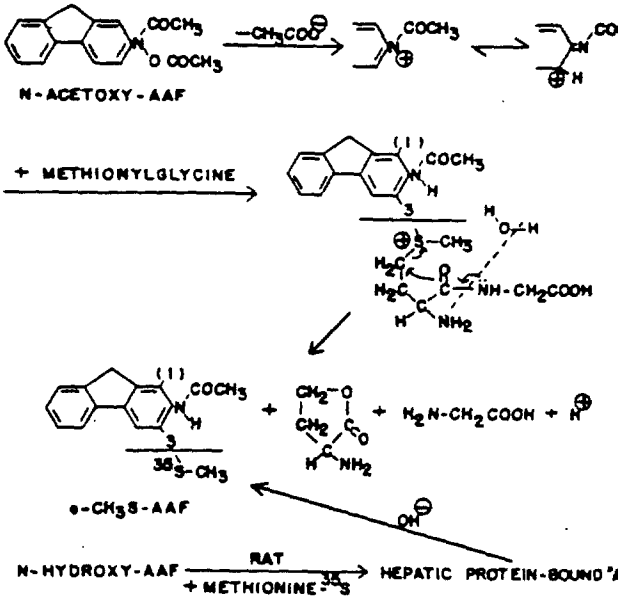



Chart 6. The nonenzymatic reaction at pH 7 of the synthetic ester *N*-acetoxy-AAF with methionylglycine to yield 1- and 3-methylmercapto-AAF, homoserine lactone, and glycine and the liberation of 1- and 3-methylmercapto-AAF from the liver proteins of rats given *N*-hydroxy-AAF (27, 92).

[3] Lin, J.-K., Miller, J. A., and Miller, E. C. *N*-(Guanosin-8-yl)- and *N*-(Deoxyguanosin-8-yl)-*N*-methyl-4-aminoazobenzene: Components of Hepatic rRNA and DNA in Rats Given *N*-Methyl-4-aminoazobenzene, submitted for publication.

The synthetic esters of *N*-hydroxy-AAF react readily with guanine residues in nucleosides, nucleotides, or nucleic acids and, to a very limited extent, with adenine residues (82, 102, 113, 114). In contrast to the attack of alkylating agents at the N-7 position of guanine, studies with Dr. Kriek showed that the esters of *N*-hydroxy-AAF react at the C-8 position (82) (Chart 7). Thus, the reaction products of *N*-acetoxy-AAF with guanosine and deoxyguanosine were characterized as *N*-(guanosin-8-yl)-AAF and *N*-(deoxyguanosin-8-yl)-AAF, respectively. Further, the enzymatic hydrolysis of the liver ribosomal RNA from rats given AAF or *N*-hydroxy-AAF to the nucleoside or nucleotide levels yields derivatives indistinguishable chromatographically from *N*-(guanosin-8-yl)-AAF or its 5'-phosphate derivative (79–81, 114) (Chart 7). These findings are in agreement with the earlier work of Irving *et al.* (65) that no loss of the *N*-acetyl group occurred in the binding of fluorene residues from *N*-hydroxy-AAF to ribosomal RNA in the liver of the rat. Thus, it was of interest that Kriek (79) noted subsequently that a large loss of the *N*-acetyl group occurred in the binding of fluorene residues from AAF to the guanine bases in hepatic DNA in the rat. Recently, Irving and his associates (63) and Kriek (80, 81) have further analyzed the situation in the rat liver and find that the majority (about 70%) of the fluorene residues bound to the rat hepatic ribosomal RNA retain the *N*-acetyl group and that the majority (about 70%) of the fluorene residues in the rat hepatic DNA do not retain the *N*-acetyl group (Chart 7).

The occurrence of the *N*-(guan-8-yl)-2-acetylaminofluorene derivatives appears to be most readily explicable in terms of the *in vivo* reaction of an ester of *N*-hydroxy-AAF with guanine residues in the nucleic acids, and evidence for this will be presented below. The reactive form which serves as the precursor of the nonacetylated derivatives is not clear, but several possibilities can be outlined. The possible importance of reaction *in vivo* of *N*-hydroxy-AF with guanine residues in nucleic acids has been suggested by Kriek (78) on the basis of the nonenzymatic, acid-catalyzed reaction of *N*-hydroxy-AF with guanine residues and by King and Phillips (74) from their studies on the binding of *N*-hydroxy-AF to tRNA with rat liver preparations *in vitro*. Esters of *N*-hydroxy-AF, if they are formed *in vivo*, would be expected to be strong electrophilic reactants, but the study of this reaction has not been possible because of our inability to synthesize the model compounds. Collaborative studies in our laboratory with Dr. Irving showed that the *O*-glucuronide of *N*-hydroxy-AAF reacts with nucleophiles in a manner similar to that of the esters of *N*-hydroxy-AAF, but at a very much slower rate (103). However, in addition to the differences in rate of reaction, another important difference was noted; thus, while the esters of *N*-hydroxy-AAF yield essentially only *N*-(guanosin-8-yl)-AAF on reaction with guanosine, about one-third of the product formed in the reaction with the glucuronide is *N*-(guanosin-8-yl)-AAF and about two-thirds is the deacetylated product *N*-(guanosin-8-yl)-AF (103). Furthermore, recent studies by Irving and his associates (62) have shown that the glucuronide of *N*-hydroxy-AF is very reactive with guanylic acid; this compound is not known to be formed *in vivo*, but, in view of the large amounts of the glucuronide of *N*-hydroxy-AAF formed metabolically, the presence of some of the deacetylated derivative would not be unexpected. Finally, the possibility that the initial product formed on reaction of the DNA *in vivo* is an *N*-acetyl derivative, which is subsequently deacetylated, cannot be ignored.

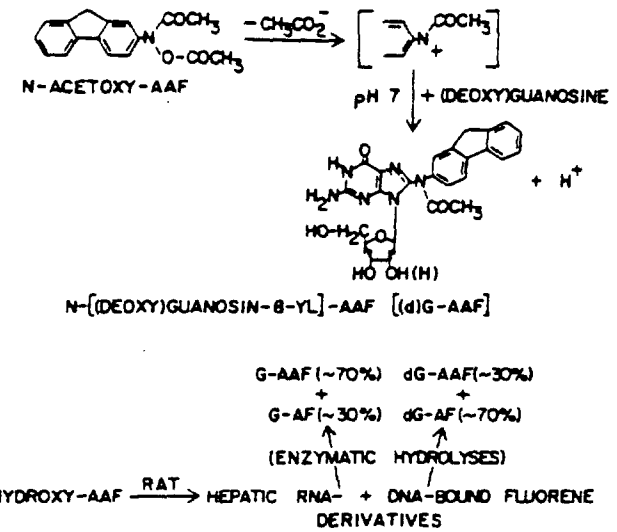



Chart 7. The nonenzymatic reaction at pH 7 of the synthetic ester *N*-acetoxy-AAF with guanosine or deoxyguanosine (82), and the (deoxy) guanosin-8-yl derivatives of AAF and AF found by Kriek (79–81) and Irving *et al.* (63) in the hepatic RNA and DNA of rats given labeled *N*-hydroxy-AAF.

**Evidence That the Sulfuric Acid Ester of *N*-Hydroxy-AAF Is an Ultimate Reactive and Carcinogenic Derivative of *N*-Hydroxy-AAF in the Liver.** From 20 to 50% of administered *N*-hydroxy-AAF is excreted by the rat in the urine and bile as the *O*-glucuronide (66, 111, 145). However, this metabolite is similarly excreted in large amounts by other species, such as the mouse, hamster, and rabbit (67, 108, 145), which are much less susceptible to the carcinogenic activity of this hydroxamic acid (68, 108). This fact suggested that the *O*-glucuronide is not the major metabolite responsible for the carcinogenic activity and *in vivo* reactivity of *N*-hydroxy-AAF in the rat and prompted a search for other metabolic forms.

In view of the short half-lives of the esters of *N*-hydroxy-AAF in water, *e.g.*, about 7 min for *N*-acetoxy-AAF and less than 1 min for AAF-*N*-sulfate at 37° (114, 138), it did not appear feasible to attempt the isolation of such esters from tissues or excreta. However, their high reactivity did make it feasible to capture the esters by reaction with nucleophiles as they were formed *in vitro*. In this manner evidence was obtained by King and Phillips (74) and in our laboratory with Dr. DeBaun (27, 28) for the formation of the sulfuric acid ester of *N*-hydroxy-AAF by soluble liver proteins. Thus, incubation of soluble rat liver proteins with *N*-hydroxy-AAF, 3'-phosphoadenosine 5'-phosphosulfate, $Mg^{++}$ or $Mn^{++}$, and methionine resulted

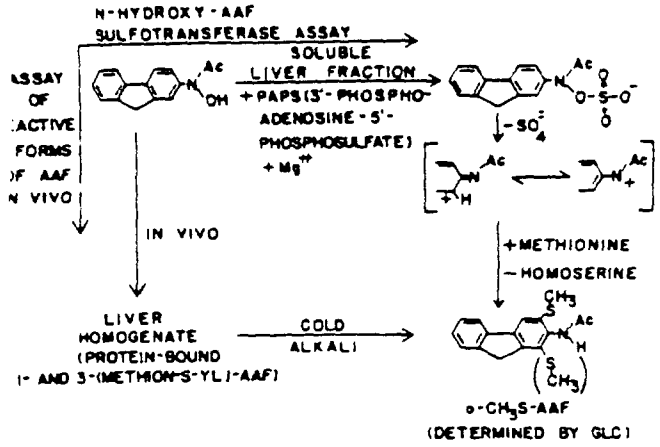



Chart 8. The *in vitro* assay system for *N*-hydroxy-AAF sulfotransferase in the soluble fraction of rat liver and the release of 1- and 3-methylmercapto-AAF from the liver protein of rats given *N*-hydroxy-AAF (27, 28).

Table 3

*Comparison of hepatic N-hydroxy-AAF sulfotransferase activity and the in vivo formation of hepatic protein-bound methionyl-AAF derivatives in animals susceptible and resistant to hepatocarcinogenesis by N-hydroxy-AAF*

| Species and sex | o-Methylmercapto-AAF from: Liver sulfotransferase *in vitro*[b] (μg/0.04 ml supernatant/30 min) | Liver protein *in vivo*[c] (μg/5 g liver) | Hepatocarcinogenicity of *N*-hydroxy-AAF[a] |
|---|---|---|---|
| Rat (M) | 23 ± 2 (13)[d] | 38 ± 9 (17) | +++++ |
| Rat (F) | 4 ± 0.5 (3) | 4 ± 1 (5) | + |
| Mouse (M) | <0.5 (3) | <1.5 (3) | + |
| Hamster (M) | <0.5 (3) | <0.3 (3) | + |
| Rabbit (M) | 2 ± 0.5 (3) | <0.5 (3) | − |
| Guinea pig (M) | <0.3 (3) | <0.3 (3) | − |

[a] Hepatocarcinogenicity data from References 68, 108, 109.
[b] Assays were carried out on livers of untreated animals.
[c] Animals were given i.p. injections of 5 mg *N*-hydroxy-AAF in 0.5 ml dimethyl sulfoxide-corn oil (1:6) emulsion/100 g body weight and were killed 16 hr later.
[d] Data are given as average values ± 1 S.D. with the number of analyses in parentheses.

in the accumulation of 1- and 3-methylmercapto-AAF, while little or none of these products was detected when 3'-phosphoadenosine 5'-phosphosulfate was omitted from the medium (27, 28) (Chart 8). 3'-Phosphoadenosine 5'-phosphosulfate is recognized as a specific sulfate donor in the enzymatic sulfation of various physiological hydroxy compounds (133).

In order to assess the possible importance of the sulfuric acid ester in the carcinogenic activity and *in vivo* reactivity of *N*-hydroxy-AAF, correlative studies were carried out with Dr. DeBaun on (*a*) the hepatic sulfotransferase activity (measured by the above assay with limiting amounts of sulfotransferase), (*b*) the amounts of 1- and 3-methylmercapto-AAF which could be released from the liver protein of animals given intraperitoneal injections of a standard dose of *N*-hydroxy-AAF, and (*c*) the hepatocarcinogenicity of *N*-hydroxy-AAF under a variety of conditions. Strong correlations were obtained between these 3 parameters (27). Thus, the liver of the male rat is far more susceptible to the carcinogenic activity of *N*-hydroxy-AAF than are the livers of female rats or the livers of male animals of a number of other species. Similarly, the male rat liver has higher levels of sulfotransferase activity for *N*-hydroxy-AAF and higher levels of protein-bound methionyl-AAF derivatives than the livers of the other species studied (Table 3). Hepatic carcinogenesis in the male rat by AAF and its derivatives is susceptible to modification by hormonal manipulation (46, 131, 166). With male rats, thyroidectomy, hypophysectomy, or castration plus administration of estrogen all markedly inhibit hepatic carcinogenesis by AAF and *N*-hydroxy-AAF. Each of these endocrine alterations also lowers the sulfotransferase activity for *N*-hydroxy-AAF and reduces the amount of protein-bound methionyl-AAF derivatives in the livers of rats given *N*-hydroxy-AAF (Chart 9) (27).

The correspondence between the *in vitro* sulfotransferase activity and the *in vivo* reactivity suggests, but does not prove, that AAF-*N*-sulfate is a major reactive form of *N*-hydroxy-AAF in the liver. This premise is also strengthened by our inability to detect appreciable amounts of a

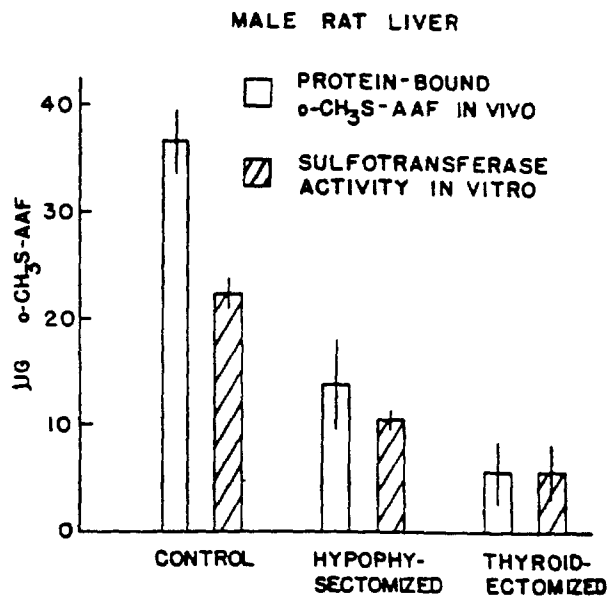



Chart 9. The effects of hypophysectomy and of thyroidectomy of adult male rats on the hepatic *N*-hydroxy-AAF sulfotransferase activity and on the amount of hepatic protein-bound methionyl derivatives formed *in vivo* from *N*-hydroxy-AAF (27). The endocrine organs were removed 5 weeks before the assays were carried out. The protein-bound methionyl derivatives are expressed as μg *o*-methylmercapto-AAF released/5 g liver 16 hr after an i.p. injection of 5 mg *N*-hydroxy-AAF/100 g body weight. The sulfotransferase activities were determined on the livers of rats not given *N*-hydroxy-AAF and are expressed as *o*-methylmercapto-AAF formed/0.04 ml liver supernatant/30 min.

Table 4

*Stimulation by sulfate ions of the binding in vivo of AAF residues to protein-bound methionine in the livers of male rats given injections of N-hydroxy-AAF*

The injection schedules were as follows. Experiment I: *p*-hydroxyacetanilide in 1 M NaCl or 0.5 M $Na_2SO_4$ solution was injected at 0 and 3 hr; *N*-hydroxy-AAF was injected at 4 hr. Experiment II: *p*-hydroxyacetanilide in 2 M NaCl or 1 M $Na_2SO_4$ solution was injected at 0 hr, and *N*-hydroxy-AAF was injected at 4 hr. Experiment III: water, 1.5 M NaCl + 0.25 M $MgCl_2$ solution or 0.75 M $Na_2SO_4$ + 0.25 M $MgSO_4$ solution were injected at 0 hr, and *N*-hydroxy-AAF was injected at 3 hr. Experiment IV: injection schedule as for Experiment III, except that *N*-hydroxy-AAF was injected at 2 hr. All injections were intraperitoneal (0.5 ml/100 g body weight). *N*-Hydroxy-AAF was suspended in 1.75% gum acacia, which in Experiments I, III, and IV contained the same concentrations of salts as the solutions injected at 0 time.

| Experiment no. | *p*-Hydroxyacetanilide injected (total) (mg/100 g body weight) | *N*-Hydroxy-AAF (mg/100 g body weight) | Time rats killed (hr after *N*-hydroxy-AAF injection) | μg *o*-methylmercapto-AAF liberated/5 g liver from rats given injections of: | | |
|---|---|---|---|---|---|---|
| | | | | Sulfate | Chloride | No anion |
| I | 38 | 5 | 16 | 29 ± 6 (6)* | 8 ± 4 (6) | |
| II | 19 | 5 | 16 | 38 ± 8 (5) | 14 ± 3 (6) | |
| III | 0 | 12 | 2 | 78 ± 19 (13) | 55 ± 13 (7) | 51 ± 23 (6) |
| IV | 0 | 20 | 2 | 98 ± 14 (4) | 63 ± 19 (4) | 68 ± 12 (4) |

* Mean ± 1 S.D.; number of rats is in parentheses.

phosphotransferase (27) or acetyltransferase for *N*-hydroxy-AAF in rat liver. Further evidence that the sulfuric acid ester of *N*-hydroxy-AAF is involved in its reactivity in rat liver *in vivo* was obtained in experiments in which rats were depleted of sulfate ion by administration of *p*-hydroxyacetanilide [on the basis of the experiments of Büch *et al.* (13)]. In these animals, supplementation with sulfate ion prior to the administration of *N*-hydroxy-AAF resulted in 2 to 3 times greater levels of protein-bound methionyl-AAF derivatives in the liver than if no sulfate ion was administered (29) (Table 4). When *N*-hydroxy-AAF-9-$^{14}$C was administered, the overall amounts of protein-bound and RNA-bound fluorene derivatives in the livers were approximately twice as great in rats administered both *p*-hydroxyacetanilide and sulfate ion as in control rats given *p*-hydroxyacetanilide and chloride ions. The level of DNA-bound fluorene derivatives was approximately one-third greater in the sulfate-treated rats than in the controls (Table 5). These treatments with *p*-hydroxyacetanilide and salt solutions did not alter the *N*-hydroxy-AAF sulfotransferase activity of the liver, and the levels of bound fluorenyl derivatives thus appear to reflect the availability of sulfate for the synthesis of AAF-*N*-sulfate. Furthermore, when very high levels of *N*-hydroxy-AAF were given, injection of sulfate ion, without prior administration of *p*-hydroxyacetanilide, caused approximately 50% increases in the amounts of protein-bound methionyl-AAF derivatives.

**Carcinogenic Activity of Esters of *N*-Hydroxy-AAF and Related Hydroxamic Acids.** When ultimate carcinogenic metabolites can be isolated or synthesized, it is to be expected that they will exhibit reactivity in the proper tests and that, under favorable conditions, these metabolites will be more carcinogenic and will be carcinogenic in a wider range of species and tissues than the parent precarcinogen. However, it is also apparent that an ultimate reactive metabolite may not show the expected carcinogenic activity when administered exogenously if its reactivity inhibits its entry into the cells. This problem has

Table 5

*Stimulation by sulfate ions of the binding in vivo of fluorene residues to total protein, ribosomal RNA, and DNA in livers of male rats*

*p*-Hydroxyacetanilide (19 mg/100 g body weight) in 2 M NaCl or 1 M $Na_2SO_4$ solution was injected into adult male rats at 0 hr, and *N*-hydroxy-AAF-9-$^{14}$C (5 mg/100 g body weight) was injected at 4 hr. All injections were intraperitoneal (0.5 ml/100 g body weight); the *N*-hydroxy-AAF was suspended in 1.75% gum acacia solution.

| | Fluorene residues bound (pmoles/mg) of: | | |
|---|---|---|---|
| | Protein | Ribosomal RNA | DNA |
| Rats pretreated with *p*-hydroxyacetanilide and sulfate ions | 1470 ± 220* | 325 ± 90 | 280 ± 95 |
| Rats pretreated with *p*-hydroxyacetanilide and chloride ions | 780 ± 140 | 155 ± 70 | 200 ± 70 |

* Mean ± 1 S.D.; 5 rats/group. The differences between the amounts of bound fluorenyl derivatives for the livers of rats given injections of sulfate or chloride ions had $p$ values of $<0.05$ for DNA and ribosomal RNA and $<0.01$ for protein.

already been encountered in tests with the carcinogenic alkylating agents, and it appears to be exemplified again in the tests on the carcinogenicity of AAF-*N*-sulfate and of several synthetic neutral and lipid-soluble esters of *N*-hydroxy-AAF. Thus, while AAF-*N*-sulfate is strongly implicated in hepatocarcinogenesis with *N*-hydroxy-AAF, this ester has induced very few tumors on application to the skin and subcutaneous tissue of the rat.[4] It seems likely that the high reactivity of this ester and possibly also its ionic nature preclude the entry of sufficient amounts of the compound into the cell before it reacts with extracellular or cell membrane components. The situation is more favorable with the neutral and lipid-soluble synthetic esters such as *N*-acetoxy-AAF and *N*-benzoyloxy-AAF. These compounds, with somewhat longer half-lives, have

[4] E. C. Miller, and J. A. Miller, unpublished data.

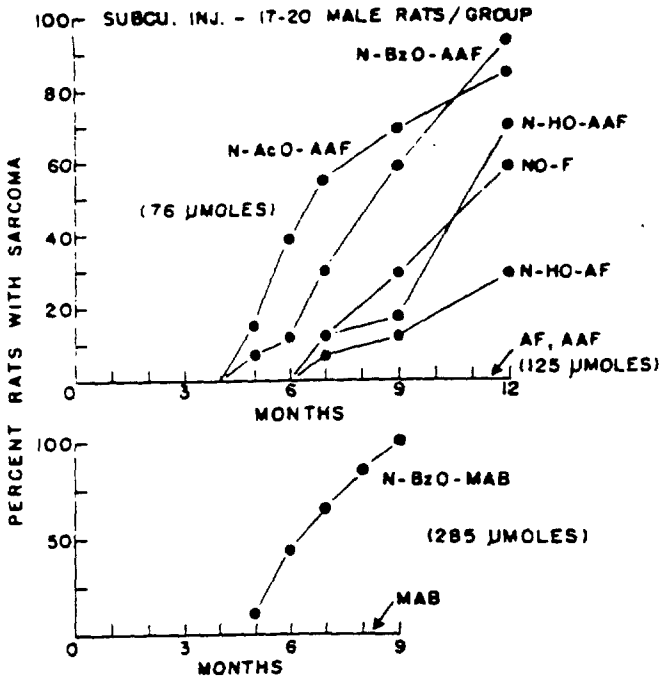



Chart 10. The induction of sarcomas at the site of repeated s.c. injections of various fluorene and aminoazo dye derivatives in male rats (the total doses are given in parentheses).

proven to be stronger carcinogens than *N*-hydroxy-AAF at sites of application, especially the subcutaneous tissue of the rat (114) (Chart 10). *N*-Hydroxy-AF and 2-nitrosofluorene were also less carcinogenic than *N*-acetoxy-AAF and *N*-benzoyloxy-AAF under these conditions, and similar doses of AAF and AF induced no tumors at the site of injection. The *O*-glucuronide of *N*-hydroxy-AAF also has little or no carcinogenic activity under these conditions (103).

Other esters of hydroxylamines and hydroxamic acids have also shown greater carcinogenic activity at the site of subcutaneous injection than the parent amines or hydroxamic acids. Thus, *N*-benzoyloxy-MAB is carcinogenic at the subcutaneous site while MAB is inactive (124) (Chart 10). Likewise, the synthetic *N*-acetoxy derivatives of 4-acetylaminobiphenyl, 2-acetylaminophenanthrene, and 4-acetylaminostilbene have each induced more tumors at the site of subcutaneous injection in rats than the corresponding *N*-hydroxy metabolites (Chart 11) (114). On the other hand, the more reactive (less stable) and ionic sulfuric acid ester of *N*-hydroxy-4-acetylaminobiphenyl has exhibited little or no carcinogenic activity under these conditions.

**Mutagenic Activity of *N*-Hydroxy Esters.** Since heritable alterations in the information coded in the DNA of a cell could be expressed phenotypically by a partial or complete loss of control of its multiplication, somatic mutations form a reasonable theoretical basis for the induction of tumors. Accordingly, an analysis of the mutagenic activities of various metabolic and synthetic derivatives of AAF and MAB was undertaken in collaboration with Drs. Maher and Szybalski (96). The mutagenic system chosen was that of Freese and Strack (41), in which naked transforming DNA from wild type *Bacillus subtilis* is exposed to the test compound *in vitro*, reisolated free of the compound, and used for the transformation of a strain of *B. subtilis* which lacks gene B for tryptophan synthetase (Chart 12). Gene B is closely linked to the genes which code for the synthesis of indole from anthranilic acid, and mutations in the latter genes are readily detected by the fluorescence of the anthranilic acid and 1-(*o*-carboxyphenylamino)-1-deoxyribulose which accumulate in the mutant cells. Hence, the frequency of transformants is a measure of the survival of gene B, and the frequency of fluorescent colonies is a measure of the mutagenicity of the compound to which the transforming DNA was exposed *in vitro*. The esters of *N*-hydroxy-AAF inactivated and caused mutations in the transforming DNA, and these 2 biological effects were proportional to each other (Chart 13) and to the extent of reaction of the esters with the DNA as determined from the density of the treated DNA or its content of $^{14}C$ from *N*-acetoxy-AAF-9-$^{14}C$. *N*-Benzoyloxy-MAB was likewise mutagenic in this system (96). AAF, AF, and their metabolites 2-nitrosofluorene, *N*-hydroxy-AAF, *N*-

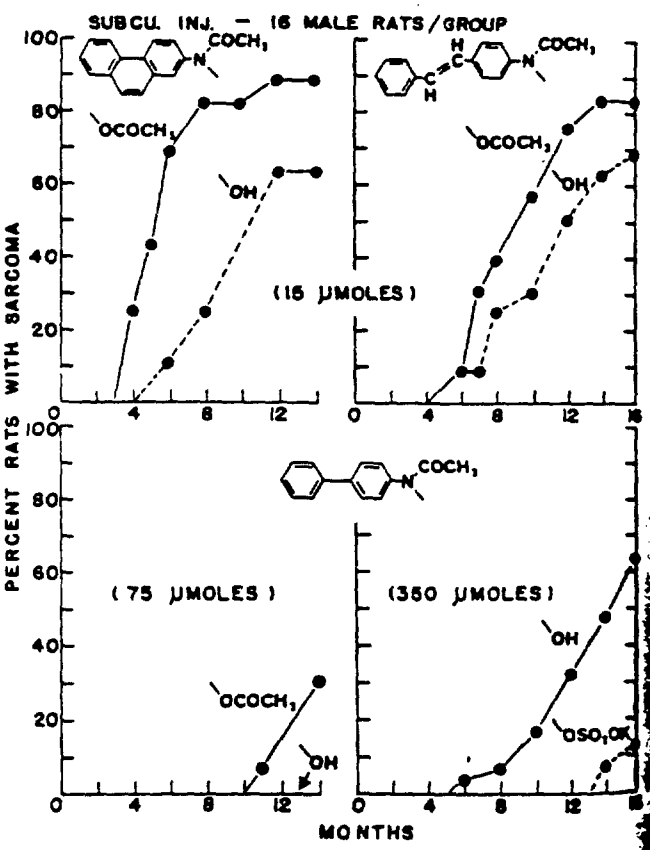



Chart 11. The induction of sarcomas in male rats at the site of repeated subcutaneous injections of various *N*-acetoxy and *N*-hydroxy aromatic amides; data are also included for the sulfuric acid ester of *N*-hydroxy-4-acetylaminobiphenyl (the total doses are given in parentheses).

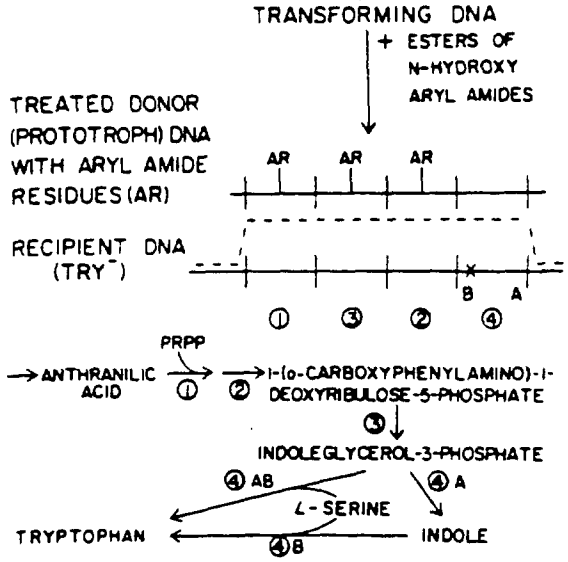



Chart 12. Procedure for the detection of linked mutations in the tryptophan operon of *B. subtilis* transforming DNA by the method of Freese and Strack (41). ①, ③, ②, and ④ indicate the linked genes and the enzymes for which they code (17). The recipient cells of *B. subtilis* carry an inactivating mutation in gene 4B and require transforming DNA from the wild type cells to grow in the absence of tryptophan. If the piece of transforming DNA extends into region ①, ②, or ③ and, by chance, also carries a mutation in these regions, the transformed cells will grow in the absence of tryptophan only if supplemented with indole and will also accumulate fluorescent intermediates [anthranilic acid and 1-(carboxyphenylamino)-1-deoxyribulose].

hydroxy-AF, the glucuronide of *N*-hydroxy-AAF, and MAB yielded little, if any, inactivation or mutation of the transforming DNA.

Similar studies also showed that esters of *N*-hydroxy-4-acetylaminobiphenyl, *N*-hydroxy-4-acetylaminostilbene, and *N*-hydroxy-2-acetylaminophenanthrene caused inactivation of and mutations in transforming DNA, while the parent compounds were inactive in this test (95). In all of these cases, the chemical reactivities of the esters paralleled their inactivating and mutagenic effects on the transforming DNA.

The nature of the reactions which cause these mutational events in transforming DNA is not clear. The majority of the mutants in these studies were spontaneously revertible at rates of $1 \times 10^{-8}$ to $1 \times 10^{-9}$, and these mutations were probably due to single base-pair changes. Since the esters attack guanine most readily, GC to AT changes would be expected. However, AT to GC mutants were the type most frequently observed in the studies of Corbett, Dove, and Heidelberger (24) on reaction of *N*-acetoxy-AAF with T4 phage. Hence, the quantitatively minor attack of *N*-acetoxy-AAF on adenine (114) may sometimes be the more biologically significant reaction. On the other hand, the introduction of a bulky aryl group into the 8-position of guanine residues in DNA [as demonstrated for esters of *N*-hydroxy-AAF and of *N*-hydroxy-MAB (82)[3]] might cause some local denaturation of the double helix and inhibit or block the progress of DNA polymerase along the double helix. Indeed, Troll and his associates (156, 157) have shown that DNA which has been extensively reacted with *N*-acetoxy-AAF is a poor template for DNA and RNA polymerases. Interference with DNA polymerase would presumably lead most readily to deletion mutants. Deletion mutants were obtained by Fahmy and Fahmy (36) on treatment of *Drosophila* with esters of *N*-hydroxy-AAF and may also account for some of the nonrevertible mutants observed in our studies (95, 96). Several investigators (35, 63, 153, 164) have recently noted the persistence for many weeks of bound residues of carcinogenic aromatic amines and amides in hepatic DNA in the rat. These residues may have important genetic consequences in carcinogenesis by these agents in the rat liver.

From these studies, it is apparent that metabolically derived esters of the carcinogenic hydroxylamines and hydroxamic acids might alter DNA in a manner leading to a heritable loss of growth controls. However, it must be emphasized that the mutagenic activities of these esters are a consequence of their electrophilic reactivities and are not necessarily related to their carcinogenic activities.

**Possible Epigenetic Consequences of Reactions of *N*-Hydroxy Esters *in Vivo*.** Heritable and quasi-irreversible changes presumably occur in cellular differentiation in multicellular organisms without alterations in genic information. Carcinogenesis may consist of similar relatively permanent changes in gene expression. Thus the epigenetic consequences of reactions of the *N*-hydroxy esters of amines and amides with amino acids (*e.g.*, methionine, cysteine, tyrosine, and tryptophan) in proteins (repressors?) and with guanine (or other bases) in RNA's must also be considered as possible bases for carcinogenesis. The idea that such reactions could result in alterations in

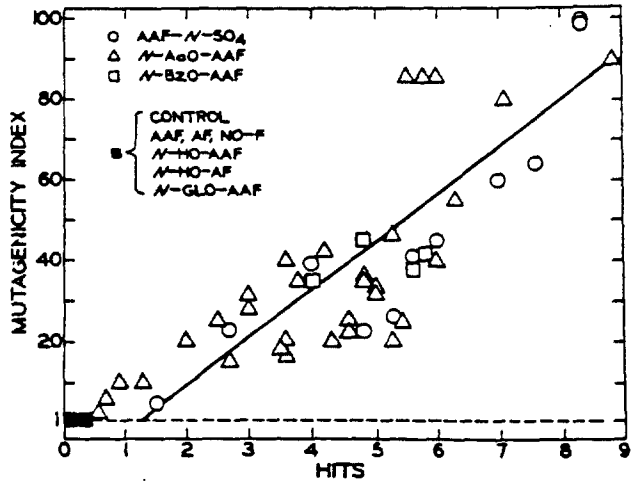



Chart 13. The mutagenic action of various esters of *N*-hydroxy-AAF on transforming DNA for *B. subtilis* (96). The mutagenicity index is the ratio of the mutation rate for the colonies derived from cells transformed with the reacted DNA to that of the control rate (*i.e.*, $1 \times 10^{-8}$). The mutation rate for *N*-acetoxy-AAF parallels the degree of inactivation (lethal hits) of the DNA and the extent of reaction of *N*-acetoxy-AAF-9-$^{14}$C with the DNA.

gene expression which might be early, and possibly reversible, events in carcinogenesis was proposed by Jacob and Monod (69, 117). The theoretical basis of this suggestion was developed in greater detail by Pitot and Heidelberger (123). At about the same time, Loeb and Gelboin (90, 91) provided experimental evidence for rapid epigenetic effects of methylcholanthrene in rat liver; Gelboin (44) later discussed these and other data in detail. More recently, Weinstein (165) has studied the biochemical effects of reaction of tRNA's with *N*-acetoxy-AAF. From these and other studies, he has proposed that tumors may result from the effects of potentially reversible aberrations in differentiation which arise through carcinogen-induced modifications of tRNA's.

## ULTIMATE CHEMICAL CARCINOGENS AS STRONG ELECTROPHILIC REACTANTS

The foregoing studies on the reactivity of the esters of *N*-hydroxy amines and amides and a consideration of the literature on the known or postulated reactive forms of other chemical carcinogens suggests that most, and perhaps all, of the chemical carcinogens either are strong electrophilic reactants as administered or are converted *in vivo* into potent electrophilic reactants. It is presumed that these electrophilic reactants then initiate the carcinogenic process through certain of their reactions with nucleophiles in crucial tissue components such as the nucleic acids and proteins. Knowledge of the structures of the reactive forms of carcinogens is still in a preliminary stage of investigation in many cases, but the data summarized below lend strong support to this generalization.

The data for a variety of aliphatic carcinogens are summarized in Chart 14. The alkylating agents are recognized as electrophilic reactants *per se*, and numerous studies have documented their nonenzymatic reactivity under physiological conditions with nucleophilic sites in nucleic acids and proteins; similar reactions also occur *in vivo* (8, 11, 12, 21, 22, 83, 85, 127, 129, 134, 141, 167). In addition, chemical carcinogens of a variety of types are converted *in vivo* into alkylating agents through enzymatic or nonenzymatic means. These potential alkylating agents include the carcinogenic nitrosamines and dialkylaryltriazenes which are activated by enzymatic dealkylation and the nitrosamides which are activated nonenzymatically through reaction with sulfhydryl groups (31, 32, 86, 87, 94, 126, 128, 139). As Laqueur has shown (84), the naturally occurring carcinogen cycasin is hydrolyzed by bacterial β-glucosidase in the intestinal tract to the aglycone methylazoxymethanol. This compound methylates nucleic acids *in vivo* (119, 139) and *in vitro* (98). Ethylation of RNA and proteins in the rat liver occurs as a consequence of the administration of ethionine (37, 39, 122, 147, 148). The demonstration that the alkylating agent *S*-adenosylethionine is formed in the liver of the rat after administration of ethionine led to the assumption that this compound was the intermediate responsible for the ethylation of proteins and nucleic acids. However, the more recent studies of Ortwerth and Novelli (122) have led to the suggestion that some other alkylating agent is also formed from ethionine in rat liver. While the highly carcinogenic pyrrolizidine alkaloids have weak alkylating activity *per se*, Mattocks (101) and Culvenor *et al.* (26) have shown that these compounds are converted *in vivo* to much stronger alkylating agents through enzymatic dehydrogenation to pyrrole derivatives. The versatile carcinogen urethan or its *N*-hydroxy metabolite apparently yield several kinds of reactive electrophiles including free radicals (70, 116, 120) which may react with cysteine (Chart 14) and with cytosine in RNA *in vivo* (10). Evidence for the formation of alkylating derivatives of carbon tetrachloride comes from the demonstration of protein- and, possibly, of nucleic acid-bound forms in rat liver *in vivo* by Reynolds (132); possible intermediates are a free radical or a carbonium ion (15, 40, 144).

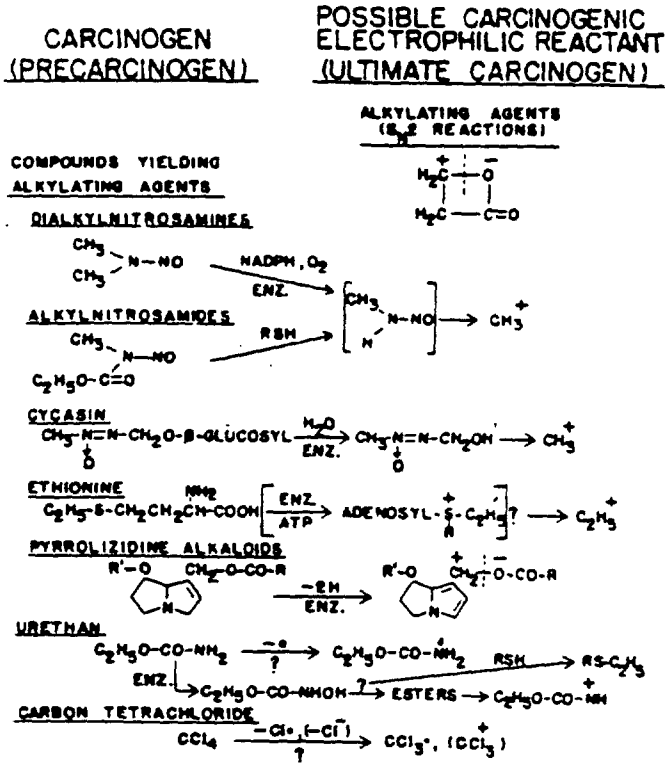



Chart 14. Ultimate carcinogenic electrophilic reactants that may be formed *in vivo* from various aliphatic carcinogens.

The known or presumed electrophilic forms of various aromatic carcinogens are similarly shown in Chart 15. The carcinogen *N*-nitroso-*N*-phenylurea decomposes in water within a few minutes to phenyl diazonium ion (126), and it seems certain this product must also be formed *in vivo*. The potent carcinogen 4-nitroquinoline-1-oxide is reduced enzymatically in rat liver and subcutaneous tissue to 4-hydroxyaminoquinoline-1-oxide (99), which is a more potent carcinogen than the parent compound (33, 142, 143). By analogy with studies on *N*-hydroxy-AAF, esterification of this *N*-hydroxyamine *in vivo* might be expected. While

no direct data are available, we have shown with Drs. Enomoto and Sato that the synthetic diacetyl derivative of 4-hydroxyaminoquinoline-1-oxide reacts with various nucleophiles at neutrality (34). Furthermore, in our studies, reaction of the diacetyl derivative with DNA *in vitro* yielded a fluorescent derivative with properties very similar to those reported by Tada *et al.* (154) and Matsushima *et al.* (100) for the DNA isolated from ascites cell tumors from rats treated with the *N*-hydroxy derivative. The carcinogenic *N*-hydroxy derivatives of various purines (151) may also undergo esterification *in vivo*. Thus, as shown recently by Stohrer and Brown (150), 8-chloro- and 8-methylmercaptoxanthine are excreted in the urine of rats treated with 3-hydroxyanthine; these same products are formed by reaction of 3-acetoxyxanthine with chloride ion or with methionine *in vitro* (172). Even aflatoxin $B_1$, which is a very strong hepatocarcinogen in the rat (171), appears to require activation for carcinogenic activity. Thus, in view of the high susceptibility of the livers of hypophysectomized rats to carcinogenesis by dimethylnitrosamine (86), the refractoriness of such rats to aflatoxin $B_1$-induced liver tumors (46, 47) suggests that they do not metabolize the aflatoxin efficiently to ultimate carcinogenic forms.

The reactions involved in the activation and binding of the carcinogenic polycyclic aromatic hydrocarbons to protein (56) and nucleic acids (12) *in vivo* have not been elucidated. Boyland (9) and his associates have strongly implicated electrophilic epoxide derivatives as metabolic intermediates. Grover and Sims (51) and Gelboin (45) have reported the formation of reactive metabolites of the polycyclic hydrocarbons on incubation with microsomal oxidases of rat liver; the chemical nature of these reactive metabolites (epoxides?) that bind covalently to DNA, RNA, and protein *in vitro* is not known. Another proposal is that of Fried and Schumm (42) and of Wilk and his associates (168, 169), who have implicated the formation of a radical cation by a single electron oxidant on the basis of model experiments *in vitro*. They have postulated that the radical cation reacts with a cellular nucleophile and then undergoes a final oxidation and loss of a proton to form a stable aromatic bound form. Recent data by Ts'o and his group (159) have shown that a similar oxidation of benz(a)pyrene in the presence of DNA leads to covalent linkage of the hydrocarbon to the DNA.

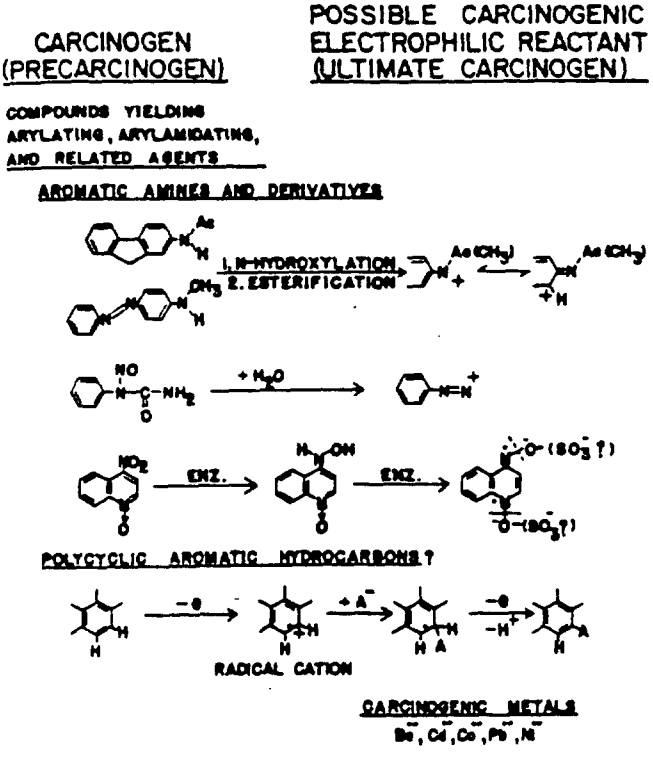



Chart 15. Ultimate carcinogenic electrophilic reactants that may be derived *in vivo* from various aromatic carcinogens, and the possible reaction of a radical cation derived from a polycyclic aromatic hydrocarbon with a tissue nucleophile. Electrophilic cations for various carcinogenic metals are listed.

Chart 15 also shows ionic electrophilic forms of the carcinogenic metals. Little is known about the mechanisms by which these metals induce tumors. However, some of these metal ions are known to react with guanine (140) or to form relatively insoluble phosphates. Thus, at least 2 means of reaction with cellular nucleic acids are evident.

It is evident that most of the bond-breaking reactions in living cells are heterolytic and generate nucleophiles and electrophiles. These reactions are almost always under tight enzymatic control, and the molecular fragments so generated are generally combined with other electrophiles and nucleophiles at the enzyme surfaces. In contrast, the foreign carcinogenic electrophiles are *strong* electrophiles which do not appear to require enzymes to facilitate their reactions with cellular nucleophiles. They can readily displace weak electrophiles such as $H^+$ from cellular components and probably enter into reactions rather indiscriminately with strong nucleophilic groups in cellular components. Some of these reactions are probably of no great consequence to cells, some must impair the functions of molecules that cells can replace, and still other reactions apparently initiate chains of molecular events which lead to losses of growth controls in cells and their progeny.

The characterization of ultimate chemical carcinogens as strong electrophilic reactants brings considerable order to the confusing variety of chemical carcinogens which is now evident. From this vantage point, it is probable that there is both theoretical and practical value in research on attempts to (*a*) inhibit or prevent the generation *in vivo* of these strong electrophiles, (*b*) render these strong electrophiles harmless in reactions *in vivo* with low-molecular-weight, nontoxic nucleophiles, and (*c*) predict the carcinogenicity of foreign molecules (*e.g.*, drugs, food additives, food-water-air contaminants, etc.) from measures of their conversion *in vivo* to reactive electrophiles.

## PERSPECTIVES

From the foregoing discussions, it is apparent that studies in chemical carcinogenesis have proceeded to the point

that information is becoming available on the nature of the reactive electrophilic forms of chemical carcinogens *in vivo*. Likewise, information is accumulating on the nature of the nucleophilic targets of these ultimate carcinogens *in vivo*. However, in no case of chemical carcinogenesis do we know unequivocally the nature of the molecular target(s) critical to the induction of that carcinogenic process. Unfortunately, entirely analogous deficiencies exist in our knowledge of the molecular mechanisms of action of carcinogenic viruses and radiations and in our characterization of the molecular phenotypes of neoplasms. Lack of knowledge of the chemical nature and genetic significance of the critical molecular target(s) in all forms of carcinogenesis has severely limited progress, and only general hypotheses have been proposed for the mechanism(s) of action of carcinogens.

At the minimum, carcinogenesis evidently consists of a heritable and at least quasipermanent loss of control of cell multiplication. At present, there are at least 2 general basic hypotheses that might account relatively directly for such a change in cellular behavior (Chart 16). These hypotheses are (*a*) interactions of carcinogens with DNA resulting in alterations in the information contained in this macromolecule and (*b*) alterations in specific proteins or RNA's which result in relatively stable and heritable changes in genome expression. In addition, at least 2 indirect mechanisms have been proposed which are likely to operate through one of the previous mechanisms, *i.e.*, the activation of a latent carcinogenic virus genome and the selection of preneoplastic cells by conditions that favor the multiplication of these cells. Conceivably, combinations of these direct and indirect mechanisms may occur. Unfortunately, none of these or any other hypotheses have yet received unequivocal experimental support in any instance with any carcinogenic agent. Fortunately, however, the rapid increases in our knowledge of cellular and molecular biology in the last decade give real promise that specific carcinogenic processes will be elucidated at the cellular and molecular levels in the next few decades. The molecular oncology of the future is certain to have many exciting breakthroughs!

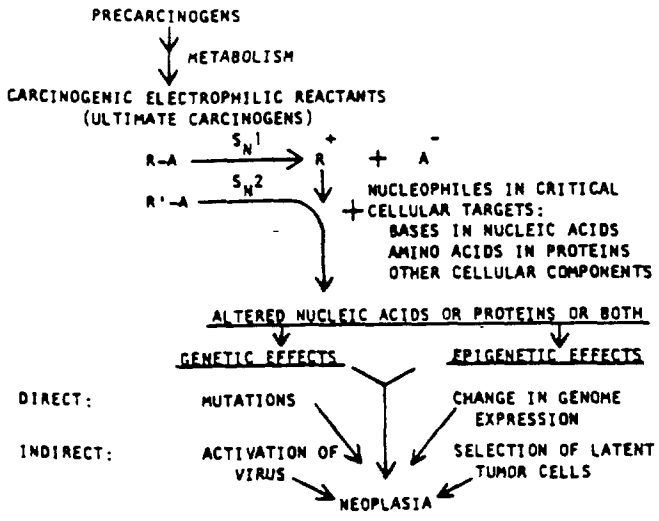



Chart 16. Some possible mechanisms of carcinogenesis by the ultimate carcinogenic electrophilic reactants derived from chemical carcinogens or precarcinogens.

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