STUDIES ON THE NUCLEIC ACID BINDING SITES OF THE FLUORENAMINE CARCINOGENS

by

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Dedication

To My Wife, Karen

and

My Parents

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INTRODUCTION

A. BIRTH OF RESEARCH IN CHEMICAL CARCINOGENESIS

Cancer research, now nearly two hundred years old, may be said to have begun in 1775, the year in which the London surgeon Percival Pott published his observations relating scrotal cancer in chimney sweeps to the coal soot which they were constantly exposed (1). In those days chimney sweeps, usually small penniless orphans who had to earn their keep, actually climbed naked up the flue with brush in hand and quite literally swept out the chimney. It was the brilliant observation of Pott that the high incidence of cancer of the scrotum, then called "soot-wart" in occupational jargon, in young adults who swept chimneys as children, might be due to their former continual daily encounter with coal soot. Pott implicated physical contact with soot as a cause of this neoplasm, and stressed the long latent period required for the cancer to manifest itself clinically. Pott's now classic paper aroused little attention in his own time, other than inspiring a ruling by the Danish Chimney Sweepers' Guild that its members bathe daily (2). This practice of frequent bathing and the wearing of protective clothing, though not observed in England, did lead to the eventual disappearance of scrotal cancer as an occupational disease in chimney sweeps over the next hundred years on the European continent (3).

The publication of Percival Pott led directly to the saving of many lives and prevention of needless human suffering, while at the same time providing an impetus for investigative effort in carcinogenesis. The first breakthrough in experimental carcinogenesis came from Japan in 1915, the year Yamagiwa and Ichikawa reported the induction of epithelial cancer in rabbits and mice by repeated application of coal tar to the shaved skin of these animals (4, 5). Years of tedious, painstaking work involving thousands of distillations, fractionations, and purifications finally led to the isolation of the first pure chemical compound shown to possess carcinogenic activity, 1, 2, 5, 6-dibenzanthracene, by Kennaway and Hieger in 1930 (6). In 1933 the powerful carcinogen 3, 4-benzpyrene was isolated from coal tar by Cook, Hewett, and Hieger (7). The following decade witnessed gradually increasing interest in the field of chemical carcinogenesis, with the work of Yoshida and Kinosita on the induction of liver cancer in rats by the azo dyes (8, 9), the studies of Hueper, Wiley, and Wolfe on the induction of bladder cancer in dogs by the dye intermediate 2-naphthylamine (10, 11), and the observations of Berenblum on the initiation and promotion stages in skin carcinogenesis with the hydrocarbon carcinogens (12, 13). By 1945, both interest and research in the field of chemical carcinogenesis were mushrooming, with signs of an even more accelerating pace twenty-five years later (14).

B. THE MAJOR CLASSES OF CARCINOGENIC AGENTS

1. Physical Agents: Radiations

At present the known carcinogenic agents may be divided into three major categories: physical agents, biological agents, and chemical agents. The physical agents include both the ultraviolet and ionizing radiations. Unna (15) first correlated excessive exposure to sunlight to the development of skin cancer. It is now well established that outdoor occupation, warm climate, and high altitude all contribute to an increased risk of epithelial carcinoma, especially in fair-skinned individuals, who appear to be more susceptible to the carcinogenic effects of actinic radiation (16). Martland first recorded the tragic episode of the New Jersey luminous watch dial painters who, by licking their brushes to give them a sharp point, ingested as much as five milligrams of radium in a six month period, and later contracted osteogenic sarcoma (17). Since then, ionizing radiations have been implicated in the genesis of malignant tumors of the skin, thyroid, pharynx, larynx, lung, and blood-forming tissues (18).

2. Biological agents: Viruses

Biological agents which have been conclusively proven capable of inducing cancer include the many types of oncogenic viruses. It was Sanarelli in Uruguay who first interpreted an epidemic of multiple

colloid tumors causing death in domestic rabbits as being due to a myxomatous virus (19). Ten years later in 1908 Ellerman and Bang demonstrated the transmission of leukemia of chickens by a cell-free filtrate (20). The next fifty years witnessed the discovery of a host of oncogenic viruses, including the chicken sarcoma virus by Rous (21), the rabbit papilloma virus by Shope (22), the mouse mammary carcinoma virus by Bittner (23), the frog renal carcinoma virus by Lucké (24), the mouse leukemia viruses by Gross (25), and Friend (26), and the polyoma virus by Stewart and coworkers (27). Although viruses are suspected to play a role in the pathogenesis of certain leukemias and lymphomas in man, to date no human cancer has a proven viral etiology. There are, however, four benign tumors which affect man that have been demonstrated to be virally induced. These are the common wart, condyloma acuminatum, laryngeal papilloma, and molluscum contagiosum (28).

3. Chemical Compounds

a. Polycyclic Aromatic Hydrocarbons

The third major category of oncogenic agents comprises the class of the chemical carcinogens. Of the several groups within this category, the most widely studied is the group of the polycyclic aromatic hydrocarbons. As noted above, the first pure chemical compound shown to possess carcinogenic activity was a polycyclic

aromatic hydrocarbon, 1, 2, 5, 6-dibenzanthracene (6). Within the past forty years, several hundred polycyclic hydrocarbons have been synthesized and tested, yielding about a dozen compounds which are now known to be intensely carcinogenic in certain species. Among these are 3, 4-benzpyrene, 3-methylcholanthrene, and 9, 10-dimethyl-1, 2-benzanthracene. Several of these polycyclic aromatic hydrocarbons have been identified in cigarette tar, automobile exhaust, and domestic soot, as well as in barbequed meat, and may play a role in the pathogenesis of some human cancers (29). A very consistent theory with high predictive value, correlating carcinogenic activity with electronic structure in the polycyclic hydrocarbons, has been proposed and refined by Pullman (30).

b. Acridines and other Heterocyclic Compounds

Closely related to the polycyclic aromatic hydrocarbons are the heterocyclic acridine dyes, in which one carbon in the skeleton of the central anthracene ring is replaced by a nitrogen atom. Several of the dimethyl benzacridines are quite as potent in carcinogenic activity as their hydrocarbon counterparts. Other aromatic heterocyclic compounds such as benzocarbazoles and thiophenes possess weak carcinogenic power. Hueper and Conway have reviewed the heterocyclic carcinogens in considerable detail, attempting to correlate electronic structure with carcinogenic potency (31). Their

conclusions, with one or two notable exceptions, agree with those of Pullman (30).

c. Azo Dyes and Aromatic Amines

The azo dyes are aromatic hydrocarbon derivatives which are used in industry, and at one time in the preparation of foods. As noted earlier, Yoshida (8) in 1933 first demonstrated the hepatocarcinogenic effect of o-aminoazotoluene in rats. His observation was followed by the work of Kinosita, who showed that prolonged feeding of 4-dimethylaminoazobenzene (butter yellow) to rats caused initial degeneration and necrosis of liver tissue, followed by nodular hyperplastic regeneration which progressed to frank malignancy (32). Prior to the investigations on the azo dyes, another group of closely related compounds, the aromatic amines, were already recognized as being carcinogenic for humans. The aromatic amines are employed as chemical intermediates in the aniline dye industry, and as antioxidants in the rubber industry. Examples of such compounds include benzidine and 2-naphthylamine. As early as 1895, Rhen noted the association of tumors of the bladder with employment in the dye making industries (33). With the rise of both the dye and rubber industries in the next fifty years, cancer epidemiologists found ample material which strongly incriminated the aromatic amines as a direct cause of bladder cancer in workers exposed to these

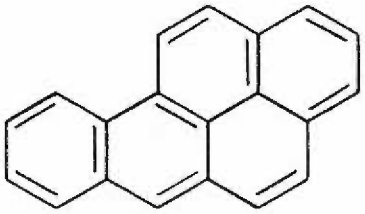
compounds for protracted periods (34). Other workers confirmed the cancer-producing properties of aromatic amines in the dog (10, 11, 35).

d. Inorganic Compounds

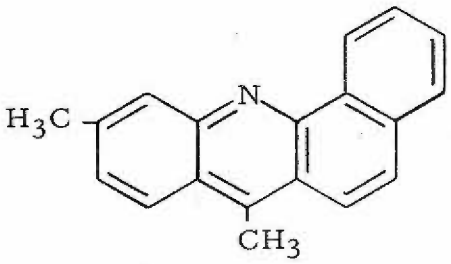
A number of inorganic substances are now known to be carcinogenic in certain situations. Thus, deep intramuscular injection of iron dextran suspension has been shown to induce sarcomas at the site of injection in the rat, mouse, and hamster (36), and there is an increased frequency of lung cancer in iron ore miners (37). Arsenic, either contacted occupationally or medicinally, is recognized as a cause of skin and lung cancer in man (38, 39). Miners of chromate and asbestos develop lung cancer at a rate of ten to eighty times the expected incidence (40, 41), and carcinoma of the nasopharynx in nickel refinery workers is 150 times the expected rate (41, 42). One very curious experimental finding is the induction of sarcomas in rodents by imbedding metal, glass, quartz, or polymer plastic films or sheets into the tissues. In this instance the actual composition of the film matters not at all; what is important is the physical form (43). Thus, the larger the surface area of the film, the more tumors are produced. Perforating, crumpling, folding, or pulverizing the sheet results in complete loss of tumorigenic capacity (44, 45).

FIGURE 1

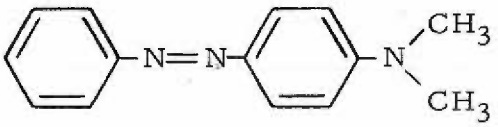
THE CLASSES OF CHEMICAL CARCINOGENS



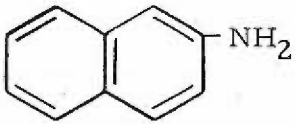
Polycyclic Hydrocarbon
(benzpyrene)



Acridine Dye
(dimethylbenzacridine)



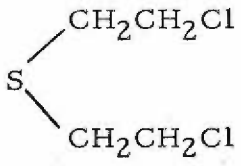
Azo Dye
("butter yellow")



Aromatic Amine
(2-naphthylamine)

Na_2CrO_4

Inorganic Compound
(sodium chromate)



Alkylating Agent
(mustard gas)

e. Alkylating agents

The alkylating agents compose the last major group of chemical carcinogens. Included are the intensely electrophilic nitrogen and sulfur mustards (2-chloroethyl amines and sulfides), the ethylenimines, and the alkyl nitrosamines. Other carcinogens presumed to be active by alkylating mechanisms are the liver carcinogens ethionine, cycasin, urethane, and aflatoxin B_1 (46-50). The polyfunctional alkylating agents, in addition to being mutagenic and carcinogenic, are also physiologically radiomimetic, and probably act by cross-linking the two strands of DNA in the double helix (51, 52). Environmental contact of the alkylating agents by man does occur. An epidemic of lung cancer in workers who were employed in a mustard gas factory in World War II has been recorded (53). Dimethylnitrosamine, a powerful carcinogen for the rat, is an important industrial solvent and intermediate in the synthesis of rocket fuels (54).

C. THE MAJOR THEORIES OF CARCINOGENESIS

1. Somatic Mutation Hypothesis

The theory of carcinogenesis which has held widest sway among oncologists over the past half-century is the somatic mutation, or nuclear gene mutation hypothesis. First formulated by Boveri in 1929, this hypothesis states that the initial event in carcinogenesis is

an irreversible alteration, or mutation, of the nuclear genome. In the words of Boveri. "The cell of a malignant tumor is accordingly. . .a cell with a definite abnormal chromatin complex" (55). A carcinogen, therefore, acts by inducing the mutation which leads to cellular autonomy; once autonomy is achieved, the mutant clone of cells replicates uncontrollably, and without further exogenous influence. Perhaps the most convincing evidence for the nuclear gene mutation theory of carcinogenesis stems from two properties which characterize the malignant change within the cancer cell, namely heritability and irreversibility (i. e., all daughter cells possess the malignant trait). Strong, one of the more vociferous supporters of the somatic mutation hypothesis, has stated that all mutagens can act as carcinogens, and implies that mutation is involved in the origin of all cancer (56). Chromosome abnormalities have been observed in some cancers, including those of man (57). Some carcinogens have demonstrable mutagenic ability (see Table 1). However, it must not be assumed that all mutagens are carcinogenic. In fact, the evidence is quite contrary to this. Burdette has written a comprehensive review on the subject of mutation in relation to the origin of tumors (66). He cites the work of numerous investigators who have tested many carcinogens for mutagenic activity, with negative results, and many mutagens for carcinogenic activity, again with negative results. While Burdette admits that somatic mutation has not been excluded as a mode of origin

TABLE 1

SOME CARCINOGENS KNOWN
TO BE MUTAGENS

Carcinogen	Demonstrated Mutagenic In	Investigator	Ref.
Ionizing Radiation	Mouse	Russell	58
Virus	Various Bacteria	Luria, et al.	59
Methylcholanthrene	Mouse	Strong	60
Dibenzanthracene	Drosophila	Demerec	61
Benzpyrene	E. coli	Scherr, et al.	62
2-Acetylaminofluorene-N-SO_4	B. subtilis	Miller	14
Urethane	Drosophila	Vogt	63
Sulfur Mustard	Drosophila	Auerbach & Robson	64
Nitrogen Mustard	Drosophila	Burdette	65

of cancer, he concludes that "a general correlation between mutagenicity and carcinogenicity cannot be proposed from present evidence" (66). If the experimental mutation of specific gene loci ever becomes a laboratory reality, future work may finally be able to confirm or reject the mutation hypothesis of carcinogenesis with certainty.

2. Irritation Hypothesis

The irritation hypothesis was introduced by Broussais in 1826, and developed by Rudolph Virchow, the founder of pathology (67, 68). Berglas, a modern proponent of this hypothesis, proposes that "the only thing common to all these factors (carcinogens) is their ability to produce a chronic irritation with all gradations of intensity, differing only in the speed by which they transform normal cells to malignant cells. This shows that the origin of cancer must have its inception in the chronic irritation of somatic cells" (69).

Berglas continues, stating that following contact with a carcinogenic stimulus, "the organism reacts in the same way as in the process of wound healing, namely, by. . . trying to heal. The cells respond by dedifferentiation and more frequent mitosis so that new tissue can be formed, but they are still controlled by the over-all regulatory system of the organism and do not continue to grow autonomously since, when healing is completed, they revert to normal

differentiated tissue cells. . . . This process fails to be consummated if the irritations continue to act incessantly on the cells engaged in the healing process. These cells become even more dedifferentiated with ever-increasing frequency of mitosis. After a latency period of many cellular generations, the dedifferentiated cells gradually lose their power of redifferentiation. These cells are then no longer subject to the regulatory mechanism of the organism but are now autonomous, that is to say, cancer cells. They have lost their specific functions and retain only the functions of nutrition and reproduction essential to self-preservation. . . . At this stage no further cellular irritation is required, the process being irreversible" (69).

The essence of the chronic irritation hypothesis, then, is a failure in the regulatory processes of healing at the cellular level, allowing the altered (cancerous) cells to reproduce new tissue without limit. To quote Schultz, "In the tumor cell, it is almost as if the chromosomes have, as their main function, their own replication" (70).

The irritation hypothesis has much, albeit circumstantial, evidence to support it (71). Cancer due to burns occurred in the Kashmiri, who carried a chracoal brazier under their robes, and in Japan where formerly beds were made on top of stoves. Pipe smoking is associated with carcinoma of the lower lip. Skin cancers are invariably preceded by inflammation, ulceration, and sometimes

scarring, often as a result of frequent sunburn to exposed parts of the body, as in farmers. Liver cancer is frequently seen after the chronic inflammatory changes of cirrhosis, and gall bladder cancer as a result of the chronic irritation of gallstones. Many industrial products such as tars and lubricating oils, which are irritants if in contact with the skin for protracted periods, can lead to epithelial carcinoma at the site of irritation.

The chronic irritation hypothesis has been criticized on the grounds that a very large proportion of tissues which had undergone irritative changes of the types mentioned above did not become malignant (71). Ross has also observed that several occupationally contacted substances with marked carcinogenic activity have no gross irritative properties (72). Nevertheless the work of Glücksmann (73), who showed that application of carcinogenic hydrocarbons to mouse skin is invariably followed by a hyperplastic, healing-type reaction histologically, prior to the onset of the actual cancer, has lent much support to this theory of carcinogenesis. To summarize, it is known that irritation is not always followed by malignant degeneration, but it is impossible to state categorically that carcinogenesis is not preceded by inflammation.

3. Warburg Hypothesis

Otto Warburg first advanced the theory which bears his name in

1931 (74). Earlier he had reported finding a higher rate of anaerobic glycolysis in cancerous tissue than in normal tissue (75). He hypothesized that a substance like urethane, which impedes respiration as well as acting as a carcinogen, initially poisons respiration, resulting in the natural selection and proliferation of those cells which derive their energy by anaerobic glycolysis. In his own words, "Cancer cells originate from normal body cells in two phases. The first phase is the irreversible injuring of respiration. Just as there are many remote causes of plague--heat, insects, rats--but only one common cause, the plague bacillus, there are a great many remote causes of cancer--tar, rays, arsenic, pressure, urethane--but there is only one common cause into which all other causes of cancer merge, the irreversible injuring of respiration.

"The irreversible injuring of respiration is followed, as the second phase of cancer formation, by a long struggle for existence by the injured cells to maintain their structure, in which a part of the cells perish from lack of energy, while another part succeed in replacing the irretrievably lost respiration energy by fermentation energy. Because of the morphological inferiority of fermentation energy, the highly differentiated body cells are converted by this into undifferentiated cells that grow wildly--the cancer cells" (76).

Warburg's theory has been severely criticized. Berenblum even questions Warburg's basic premise that tumors have increased

anaerobic glycolysis (77), since the tumor tissues were compared with normal tissues of different origin in Warburg's studies. Orr and Strickland found no evidence of an increase in the rate of anaerobic glycolysis in the liver tissue of rats during the stages of cancer induction by butter yellow prior to the development of the actual neoplasm. The transition from aerobic to anaerobic metabolism occurred abruptly with the development of the tumor (78). Warburg believed that the basic biochemical lesion of the cancer cell resided in the respiratory apparatus of the mitochondrion (76). However, recent studies have shown that tumor mitochondria are not deficient in any of the respiratory components, though the total number of mitochondria per tumor cell may be significantly reduced (79). At the present time, Warburg's hypothesis of carcinogenesis has fallen into disfavor, mainly because it is riddled with speculations and inconsistencies. However, the problem of whether a high rate of anaerobic glycolysis is a cause or a result of carcinogenesis remains unsettled (80).

4. Thiersch and Waldeyer Hypothesis

This hypothesis is included here in the interest of historical completeness. Over a hundred years ago Thiersch and Waldeyer proposed a theory of transformation of normal tissue into cancerous tissue which postulates that in normal tissue there exists an equilibrium between the connective (stroma) elements and the epithelial

elements. They propose that due "to the more rapid ageing of the connective component the tissue loses its capacity to hold the epithelium in check. Under these conditions, the epithelium now liberated from the restraining influence of the connective tissue reverts to an embryonic type of cell and reveals, or assumes, a capacity for unlimited proliferation; that is, it becomes cancerous" (81). At the time of its inception, support for this theory was derived from three facts: (a) that most cancers originate from epithelial tissue, (b) that cancer incidence rises steeply with age, and (c) that loss or shrinking of connective tissue, for example wrinkling of the face and hands, is an obvious sign of ageing (81).

The greatest blow to the Thiersch-Waldeyer hypothesis was the discovery of sarcoma, or cancer of the connective tissue elements. It is obvious that a tissue which is supposed to exert a restraining influence on epithelium cannot itself be capable of malignant transformation. Another death-dealing blow came from the observation that it is possible to propagate pure cultures of epithelial cells in vitro indefinitely without malignant transformation (82).

5. Embryonic Rest Hypothesis

The first theory of cancer was put forth in 1829 by Lobstein and Récamier. They proposed that embryonic rests are the foci of tumors which develop in adult life (83). Vestiges of embryonic

structures can be found in many organs of the human body. For example, gastric mucosa cells have been found in the mucosal lining of the upper segment of the esophagus. Branchial arch remnants may be found in the roof of the mouth. The pituitary gland, a pharyngeal embryonic derivative, sometimes contains remnants of salivary glands, which also develop from pharyngeal mucous membrane. And adrenal tissue has been found in the kidney and ovary (83).

These embryonic rests can, and occasionally do give rise to either benign or malignant tumors. However, malignant change is rare. Certain childhood cancers in man, such as retinoblastoma, neuroblastoma, Wilm's tumor of the kidney, and embryonal cell carcinoma of the testis, are suspected of having their origin in dysplastic embryonic tissue remnants. But the embryonic rest theory is clearly not capable of explaining the origin of the great bulk of human and experimental cancers. The development of the techniques of experimental carcinogenesis, which led to the observation that carcinogens applied to the skin of a mouse can induce tumors at any site, provided almost conclusive disproof of the embryonic rest theory as a general mechanism of carcinogenesis (83).

6. Deletion Hypothesis

The Millers showed in 1947 that there was a high degree of correlation between the carcinogenic activity of 4-

dimethylaminoazobenzene (butter yellow) and its degree of protein binding to a given tissue (84). Thus, liver proteins were bound extensively to the dye, and it was noted that the liver was the only organ in the rat which was susceptible to the carcinogenic action of this compound. Other tissues did not contain appreciable amounts of protein-bound dye, and the livers of species not susceptible to its action likewise showed no protein binding (85). On the basis of these results, the Millers (86), and Potter (87, 88), have proposed a theory of carcinogenesis which they refer to as the protein (or enzyme) deletion hypothesis. This hypothesis visualizes carcinogenesis "as a resultant of the modification or deletion of certain cellular proteins (enzymes). Through such a process a new race of cells would be formed which differed from the parent cells in protein makeup and which possessed a new balance between their enzymatic reactions. If these cells had the requisite systems for growth but lacked the enzymatic factors which could limit that growth, they would have the properties of tumor cells" (86).

There is much circumstantial experimental evidence in support of the deletion hypothesis. Several carcinogenic hydrocarbons have been found bound to the epidermal proteins of mouse skin after painting (89, 90), and Pullman notes that carcinogenicity of a given polycyclic hydrocarbon correlates much higher with its binding to protein than to nucleic acids (30). Weisburger and coworkers found radioactivity

bound to liver proteins after administration of C^{14} labeled 2-acetylaminofluorene to rats (91). The Millers extend the deletion hypothesis to include other carcinogenic agents. Thus, radiations might act by damaging key proteins (i. e., histones or other regulator proteins) required for the control of growth, and viruses may compete for cell nutrients, causing a deficient growth control protein to be formed (86).

The deletion hypothesis has been criticized on several grounds. While it is known that certain tumors are indeed characterized by low or even absent levels of enzymes such as tryptophan pyrollase (92), it is suspected that this might simply reflect the over-all dedifferentiated state of the cancer cell. Also, mere deletion of protein in the cell by a carcinogen need not be permanent. The cell should be able to form more new protein molecules to replace those deleted by the carcinogen if the genome is left intact. Potter later modified the hypothesis to include gene mutation as a primary cause of the enzyme deletion (88, 93). Protein binding by a carcinogen might also result in the permanent deletion of an enzyme if the protein so bound were an information-handling molecule like DNA polymerase, which could, because of altered replicative specificity, introduce errors into the genome. Currently the deletion hypothesis, though not enjoying its former popularity, still arouses the attention of oncologists, for research has not conclusively demonstrated whether protein deletion

is to be regarded as an expression of the dedifferentiated state of the cancer cell, or as a possible mechanism of carcinogenesis (94).

7. Multistage Hypothesis

The idea of carcinogenesis as a discontinuous process consisting of several consecutive stages had its origin in the speculation of several independent investigators. Rous and Kidd in 1941 reported that after painting rabbit ears with subthreshold doses of tar for a time, then punching out discs of tissue for histological examination prior to tumor formation, there was initially no microscopic evidence of tumor. But later tumors developed, especially at the edge of the healing punch wound (95). Evidently the tar had initiated a stage of latent neoplastic potential, and the trauma or influence of the healing wound promoted frank malignant degeneration. Simultaneously in 1941 Berenblum reported that initial subthreshold applications of carcinogenic tar to mouse skin resulted in no tumors. If, however, the animals were subsequently painted with croton oil, a marked growth of tumors was produced (12). A remarkable feature of this experiment was that croton oil was not itself carcinogenic in the doses used. In both of the aforementioned cases, tumor formation was much greater if either the croton oil or trauma were combined with subthreshold doses of tar, than if suprathreshold doses of tar alone were employed.

The two-stage theory of initiation and promotion in chemical carcinogenesis was formulated in 1944 by Friedewald and Rous, who concluded that carcinogenesis was composed of an "initiating process" responsible for the conversion of normal cells into latent cancer cells, and a "promoting process," whereby the latent cancer cells were caused to develop into growing tumors (96). Initiation may be induced by a very small dose, even a single application, of a carcinogen. It is rapid, apparently irreversible, and determines the number of tumors that arise (97). The action of the promoting agent is gradual, and determines the latency period between initiation and tumor formation. It is sometimes reversible. The two steps are discontinuous and specific: application of a promoter prior to an initiator does not induce growing tumors (98). The effects of the initiator are very suggestive of a mutagenic mechinism, while those of the promoter may be to cause irritation and induce hyperplasia (99). It is presumed that since some compounds can induce cancer when applied alone in sufficient dosage and for prolonged periods, that they possess both initiating and promoting ability.

The idea of tumor development as a result of progressive acquisition of malignant properties was proposed by Rous and Beard (100). This theory of tumor progression was derived in part from their investigations on the transformation of virus-induced papillomas into carcinomas. Foulds later elaborated upon their theory with his

observations that breast cancers in mice appeared to acquire malignant characteristics, or progress, with each successive pregnancy, until the point where they were totally autonomous and no longer dependent on estrogen influence (101). This stepwise acquisition of malignant properties may occur, after initiation and promotion, through subsequent mutations in a rapidly growing cell population, with selection of those mutations leading to greater cellular autonomy (88, 102). Thus, the multistage mechanism in its complete form includes initiation of latent cancer by a carcinogen, promotion of the latent cancer to a growing tumor by a cocarcinogen, and progression of the growing tumor through a series of mutations leading to the cancer phenotype (99). A close cousin of the multistage hypothesis, called the multifactorial theory, has developed from the observations that two or more carcinogens (e. g., virus and x-rays) may act together simultaneously to induce cancer (103, 104).

In summary, while the multistage hypothesis may be unable to account for the genesis of cancer in every instance, there is a vast bulk of evidence that the induction of most malignant neoplasms is generally a multistage process (105).

8. Busch Theory

Harris Busch has proposed an explicit version of the multistage hypothesis which includes the first two stages of initiation and

promotion already discussed, but in addition considers a slightly different third step, the acceleration stage, in the transition of normal cells to cancer (106). Busch believes that a special segment of DNA is responsible for cancer induction which he calls cancer DNA. In normal cells the cancer DNA is attached to a suppressor protein which blocks its expression. Initiation results from the combination of a carcinogen with the cancer DNA-suppressor protein complex. Promotion represents the release of the suppressor from the cancer DNA. During the acceleration stage the cancer DNA, now derepressed, transcribes a complimentary cancer RNA. The translation of this cancer RNA produces an accelerator (cancer) protein which is able to interact with and activate the cancer DNA, causing ever-increasing acceleration of cancer RNA production.

Busch explains that "the rate-limiting factor in this cyclical process would be the active cancer DNA. It may be envisioned that in the cell there is much cancer DNA which is normally suppressed and that the formation of cancer protein proceeds at a negligible rate, if at all, in view of the fact that cancer RNA is not formed. Once the process of rapid formation of cancer protein has occurred there would be a very rapid conversion of all suppressed cancer DNA in the cancer cell to active cancer DNA regardless of whether or not suppressor was still attached. The increased availability of cancer protein would cause most or all available cancer loci in the DNA of the cell to

become converted to active loci. Competitively, this process would supersede all the pre-existing processes for biosynthesis of normal cytoplasmic protein elements and protein templates such as the ribosomes" (106).

Busch concludes that the consequences of these changes "would be reflected in altered mitosis, increase in the amount of abnormal DNA, increase in the formation of abnormally large amounts of RNA, and the formation of abnormal nuclear protein" (106). There is ample evidence that cancer cells do indeed contain abnormally large amounts of RNA (107, 108), and also synthesize abnormal nuclear protein (109, 110).

9. Viral Theory

Viruses have been recognized as a cause of cancer since 1908, the year Ellermann and Bang published the results of their experiments on the induction of leukemia in chickens by cell-free filtrates (20). During the past sixty years, research in viral carcinogenesis has yielded a wealth of information on the mechanism of action of the oncogenic viruses. There are two groups of tumor-producing viruses, those containing DNA and those containing RNA. These viruses have two types of host cells. In the permissive host cell, the virus actively multiplies and causes cell death by lytic infection. In the non-permissive host cell, the virus does not multiply, but instead

transforms the host cell to a cancer cell (111). Transformation to the cancerous state "appears to be a manifestation of infectious heredity, i. e., a modification of cellular functions consequent to the introduction of viral genes into the cells. . . " (112). That part of the total viral genome (virogene) responsible for the phenotypic characteristics of the transformed cell is called the oncogene. In most instances the transformed cells contain the viral nucleic acid, either DNA or RNA, in a functionally incomplete form. That is, part of the virogene (e. g., information needed to synthesize the viral protein coat) is apparently lost in the nonpermissive host (112).

Years of tedious research have finally elucidated the mechanism of carcinogenesis by the DNA viruses. Dulbecco, a well-known investigator in the field, explains, "Cell transformation by these (DNA) viruses includes two steps: first, the integration of the viral DNA in the cellular DNA; and secondly, the expression of viral transforming functions. If integration fails, the cells undergo abortive transformation and then return to normality. . . . The viral DNA remains in the cell. . . and in all its descendants as a true set of genes of the cell" (111). In the case of the RNA tumor viruses, Dulbecco continues, "There is no evidence of any permanent association between the viral RNA and cellular components. Apparently the virus-cell association is maintained as the cells multiply because the viral RNA also keeps multiplying independently" (111). Such may not be the case,

however. The recent exciting findings of Baltimore, Temin, and Mizutani (113, 114) have confirmed the presence of an RNA-dependent DNA polymerase in the virions of RNA tumor viruses. This discovery has not only expanded the now classic Jacob-Monod "Dogma" (115), but also allows the RNA viruses to act by the very same mechanism which Dulbecco describes for the DNA tumor viruses. The complimentary DNA synthesized by the RNA-dependent DNA polymerase is called the DNA provirus (114). Presumably it could be integrated into the host cell DNA during the process of transformation, but this has not been demonstrated as of this time.

Intensive research is currently in progress to identify viruses as possible causes of human cancer. Viral particles have been observed in electron micrographs of human acute leukemia cells, and one cancer, Burkitt's lymphoma, is strongly suspected of having a viral etiology (111). Proponents of the theory that viruses are the cause of all cancer suggest that other carcinogenic agents such as chemicals and radiations act by activating a latent oncogenic virus within the host cell. The experimental findings that both hydrocarbon and radiation induced neoplasms in experimental animals eventually become transmissible by cell-free filtrates have lent strong support to this possibility (116).

10. Extrachromosomal Mutation and Epigenetic Mechanisms

The idea that cancer may result from a mutation which affects not the nuclear genome but instead a cytoplasmic gene was derived from the studies of Woods and du Buy (117), and Haddow (118) on morphologically altered mitochondria in variegational diseases of plants, and formulated by Darlington in his paper entitled "The Plasmagene Theory of the Origin of Cancer." In it he states, "The development of unbalanced nuclei in tumours is without precedent in any living tissue. It implies a relaxation of detailed control in the nucleus. . . and this in turn argues that the nucleus is not itself directly responsible for what is going on." Darlington further postulates that "the cancer determinants arise as mutant particles in the cytoplasm--that is, as plasmagenes" (119). The tumor viruses could also serve as plasmagenes, though in this case tumorigenesis would result from infection rather than mutation.

The development of the Jacob-Monod theory of gene expression (115), with its emphasis on the central role of nuclear DNA, resulted in the waning of interest in extrachromosomal mechanisms of carcinogenesis for nearly a decade. Recently, however, Armin Braun has published an exciting book entitled The Cancer Problem: A Critical Analysis and Modern Synthesis (120), in which he explores the concept that cancer may arise as a result of a change in the expression of normal genetic determinants in a cell, rather than a

change in the integrity of the genetic material. "It is such epigenetic changes, which are concerned with persistent alterations in the expression of the genetic determinants normally present in a cell, that are believed not only to account for that aspect of cellular differentiation that is concerned with the synthesis of new and specialized products by different cell types. . . but to constitute the basic cellular mechanisms that underlie the tumorous state generally" (121). Braun musters a volume of weighty support in evidence of the epigenetic mechanism. Most convincing are the experiments of King and DiBerardino dealing with the development of free-swimming normal tadpoles following transplantation of frog renal-cell adenocarcinoma nuclei into enucleated frog ova (122), and the experiments of Mizell who demonstrated the conversion of frog renal adenocarcinoma cells into normal epithelial and connective tissue elements autoradiographically after grafting pieces of radiolabeled tumor cells onto the regenerating tails of frog tadpoles (123). These experiments apparently show that tumor cell nuclei are not always irreversibly altered, and when placed in the proper environment, may respond beautifully to epigenetic control mechanisms.

11. Pitot Hypothesis

The mechanism of carcinogenesis proposed by Pitot (124) is an epigenetic mechanism involving the endoplasmic reticulum as a

carrier of heritable information. Pitot cites numerous examples of altered gene expression in minimal deviation hepatomas (125-127). His own investigations have related these abnormalities to an alteration in the stability of the messenger RNA templates during enzyme synthesis (128). He proposes that in the cell there exists an endoplasmic reticulum-associated messenger RNA template called the MEMBRON, whose expression may be regulated by changes in the cellular environment. The MEMBRON is envisioned as a snail-like spiral of the messenger RNA molecule and its ribosomes (cochleosome) in association with structures in the mosaic of the endoplasmic reticulum. Stabilization of the MEMBRON occurs through interactions of the messenger RNA with membrane lipoproteins at specific points on the endoplasmic reticulum mosaic. MEMBRON stabilization results in the direction of ribosome aggregation by messenger, and finally enzyme synthesis.

Pitot explains, "We have shown that there is an association between the endoplasmic reticulum and the regulation of the expression of genetic information. Since this appears to be true, one may conclude that there are present in the cytoplasm of the cell functioning regulatable translating units which are stable and associated with intracellular membranes. These units are quite analogous to the operon unit of the gene, which had been postulated earlier by Jacob and Monod. The membrane associated RNA operon, which we have

postulated to exist in the cytoplasm of mammalian liver, we would designate in modern shorthand as the MEMBRON. The MEMBRON is directly analogous to the genetic operon and consists of a molecule of messenger RNA associated with an area of membrane. In conjunction with ribosomes and transfer RNA required for translation, the MEMBRON becomes essentially a cytoplasmic operon" (124).

Pitot postulates that carcinogenesis may result from the interaction of a carcinogen with the MEMBRON, leading to the altered mRNA template stability he believes is characteristic of the neoplastic state (128). ". . . Neoplasia thus becomes a disease of the MEMBRON, a disease of differentiation which is itself dependent on the MEMBRON population. Thus, the altered regulatory mechanisms which reflect the characteristic altered biology of the neoplastic cell may well result from the altered regulation of genetic expression as a consequence of environmental-induced changes in the mosaic structure of intracellular membranes" (124). The maintenance of the altered mosaic structure, Pitot believes, could occur by the same type of epigenetic inheritance which Sonneborn has shown exists for the cortical membrane of the paramecium (129, 130). Thus, Pitot's hypothesis explains carcinogenesis without any reference to a mutation in cellular nucleic acids.

12. Immunological Theories

Green has been the chief proponent of the immunological concept of carcinogenesis. His theory developed as an outgrowth of the self-marker hypothesis of Burnet and Fenner (131), who proposed that all somatic cells in an organism carry identity or self-marker proteins which enable the antibody-forming cells of the reticuloendothelial system to recognize them as self. Green postulates that "one or more of the cytoplasmic specific protein complexes which confer identity on the tissue are modified by carcinogen binding, the modified complex being self-replicating. Antibody is elicited to it and in due course adaptation, by loss of the modified identity protein, occurs. The new race of cells, now lacking in some degree their tissue-specific pattern, and presumably the genocopy of this, fail in the same degree to be recognized by the growth-regulating mechanisms. . ." (132). The outcome is loss of control over these cells, and their consequent proliferation without regard for tissue boundaries. Thus, in Green's view, the "loss of identity proteins (self-markers) in itself comprises malignancy" (132). He considers viruses and radiation to induce cancer likewise by loss of immunological identity. Green and coworkers have recently written a comprehensive volume listing at least twenty-eight experimental investigations in which tumor tissue was shown to lack one or more antigenic qualities detectable in the tissue of its origin (133).

Burnet has very recently expounded a radically different immunologic mechanism of cancer called defective immunological surveillance. He explains, "Immunological surveillance is the concept that a major function of the immunological mechanisms in mammals is to recognize and eliminate foreign patterns arising. . . by somatic mutation or some equivalent process. From the point of view of survival, this is important primarily as providing a means by which the appearance of malignant disease may be effectively cut short" (134). Surveillance is mediated solely by the thymus-dependent lymphocytes, and is a local cellular phenomenon, with circulating antibodies (immunoglobulins) playing a negligible role. It is the same mechanism responsible for the rejection of homografts (134). Burnet proposes that cancer cells arise in the body almost continually by somatic mutation, but are effectively rejected by immunological surveillance. However, a defective immunological mechanism could allow a mutant cell to pass unrejected and proliferate into clinical cancer. Burnet cites the work of Prehn (135), who demonstrated that the immunodepressive action of methylcholanthrene plays a role in the selection of mutant cells carrying new antigens, as evidence that impaired immunological surveillance may operate during chemical carinogenesis. He also notes the high propensity of patients suffering from primary immunological deficiencies, or those undergoing intensive therapeutic immunosuppression, to develop cancer (134).

13. Osgood Hypothesis

In 1956 Saetren reported that if a major portion of kidney or liver is removed from an animal, the mitotic index of the remaining tissue goes up, and this increase in the mitotic activity in the kidney can be prevented by injection of fresh homogenized kidney tissue, but by no other tissue tested. Similarly, mitoses in regenerating liver are suppressed by fresh homogenized liver tissue, but not by kidney or other tissues (136). Thus the concept of tissue-specific mitotic inhibitors, now called chalones, developed. These chalones are glycoproteins, and require adrenalin and sometimes glucocorticoids as cofactors. They are always produced by the tissue on which they act (137).

A theory of carcinogenesis which implicates the chalones has been proposed by Osgood (138). Osgood assumes that in all tissues of the body there are two populations of cells, alpha cells and n cells. The n cells are the mature cells of a series (i. e., mature erythrocyte, keratinocyte, hepatocyte, etc.). They are destined to die, but while alive they produce a specific inhibitor (chalone) which feeds back on the alpha cells to prevent alpha cell division. The alpha cells are the stem cells for a given series (i. e., erythroblast, lymphoblast, fibroblast, etc.). They, while few in number, maintain the genetic continuity for a cell series. In the absence of chalone they can divide either (a), into two alpha cells, or (b), into one alpha cell

and one n cell. The alpha cells never mature, are potentially immortal, and are the only cells which can start a cell culture, or a cancer.

Osgood supposes that "All that is necessary for the production of a malignant tumor is any genetic change in the somatic alpha cell of a series that leads to death of the n cells before or during the stage of production of alpha-2 alpha inhibitor. . . . There must be also enough cells with genetic change in the same location or in alpha cells protected from diffusion so that inhibitor from adjacent normal cells does not reach these alpha cells" (138). Eventually, as tumor mass increases, there would be a complete absence of chalone in the central portion of the tumor, and the mutant alpha cells could reproduce without limit.

It has been shown that tumor cells do produce chalone, though apparently lose the ability to retain it intracellularly (139). Tumor growth is also inhibited by massive doses of chalone in vivo, and some spectacular whole-tumor sloughs resulting in total cures have been produced with transplanted melanomas in rodents (140). It seems well documented that tissue-specific mitotic regulators do exist, and do in fact play a role in the regulation of cellular regeneration at the level of the tissues. Their role in the pathogenesis of cancer, however, remains hypothetical.

14. Greenstein Hypothesis

The last theory of carcinogenesis to be discussed is the Greenstein hypothesis. In 1956 Greenstein published the results of some of his own investigations showing that in tumors there is a general convergence of enzyme patterns leading to a uniform biochemical pattern in tumor tissue (141). This was in contrast to normal tissues, each of which (liver, intestine, lung, etc.) had its own biochemically distinct pattern of enzyme activities. Greenstein remarks, "This apparent uniformity is all the more remarkable when it is considered that the biochemical evidence by which this uniformity is revealed (in various tumor tissues) has been derived from studies on tissues frequently heterogeneous as to cell type" (141). Greenstein himself did not consider his findings to be the cause of cancer, but rather the result of malignant transformation. Nevertheless, other workers have persisted in speaking of the "Greenstein Hypothesis," and implicating his findings causally as a mechanism of carcinogenesis, and even as indirect support of the Warburg hypothesis (142). It is clear that the Greenstein hypothesis has outlived its usefulness, and Potter even questions the generality of Greenstein's findings (143).

15. Conclusion

Having completed this review of the major theories of carcinogenesis, it is clear that, just as there are many widely differing

known carcinogenic agents, so too are there many widely differing mechanisms offered to explain the genesis of cancer in special circumstances. Each hypothesis has its merits, its supporting evidence, its contradictions and inconsistencies. No one hypothesis is able to explain carcinogenesis in every instance, and it may well be that the pathogenesis of most human cancer involves several mechanisms operating concurrently.

D. CARCINOGENESIS BY THE FLUORENAMINES

1. Early History

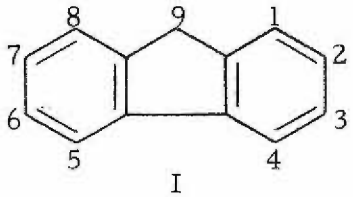
2-Aminofluorene first gained commercial and ecological importance in April of 1940, when U.S. Patent Number 2,197,249 was issued to Houston V. Claborn and Lloyd E. Smith of the U.S. Department of Agriculture for use of this compound as an agricultural insecticide. Before permission for use could be granted, however, the effectiveness and safety of the compound had to be demonstrated. There was no question as to the insecticidal effectiveness of 2-aminofluorene: a concentration of forty parts per million of water gave a 99% kill of mosquito larvae. It was also shown to be very effective against the tobacco hookworm (144).

Wilson and coworkers undertook the animal toxicity studies of the fluorenamines. In 1941 they reported that 2-acetylaminofluorene, the acetyl derivative of the compound referred to in the patent, had no

demonstrable acute toxicity in rodents. Even when fed relatively massive doses of one gram per kilogram of body weight, a temporary refusal to eat was the only deleterious symptom observed. However, rats maintained on much smaller doses (0.031% to 0.125% in diet) for prolonged periods (100 days) developed bulky nodular malignant tumors of the liver, and also tumors of the bladder, kidney, lung, stomach, colon, and breast (145). These tumors metastasized and eventually killed the rats. Wilson was able to demonstrate the presence of 2-aminofluorene in the urine of those animals fed the diet containing 2-acetylaminofluorene. His findings abruptly terminated the potential use of the fluorenamines as insecticidal agents, and provided a sobering example of the danger of predicting the chronic toxicity of a compound proposed for use in agriculture from acute toxicity data (145). In addition, his discovery of the carcinogenic properties of the fluorenamines gave investigators a new and important tool with which to study the genesis of cancer in experimental animals.

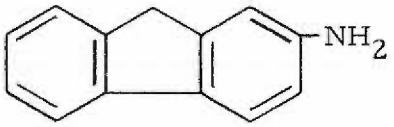
2. Chemistry of the Fluorenamines

Fluorene (I), an aromatic hydrocarbon consisting of a biphenyl with a methylene bridge, was first isolated from coal tar. It is a conjugated nonalternant polycyclic aromatic hydrocarbon possessing a planar structure (144, 146). Nitration of fluorene yields 2-



I

nitrofluorene as the major reaction product, which may be subsequently reduced by zinc and hydrochloric acid to 2-aminofluorene (II). Acetylation of this product yields 2-acetylaminofluorene (III), a light



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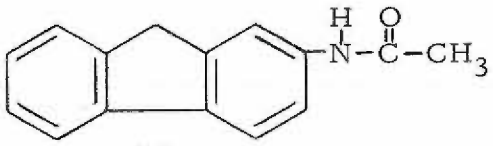


III

tan crystalline material, sparingly soluble in water, and melting at 188° C (144). Hereinafter, 2-aminofluorene will be referred to as AF, and 2-acetylaminofluorene as AAF. More concerning the chemical reactivities of AAF and its metabolites will be mentioned later.

3. Experimental Carcinogenesis by 2-Acetylaminofluorene

The two decades following the discovery of the carcinogenicity of AAF by Wilson and coworkers in 1941 produced a truly voluminous literature on experimental AAF carcinogenesis. Every position on the fluorene ring was substituted with halogen, nitro, hydroxy, acyl, alkyl, and other groups. Even heterocyclic rings were synthesized, in order to determine if the resulting derivatives were more or less

carcinogenic in vivo. Effects of species, strain, sex, route of administration, and synergistic and antagonistic effects of other agents, were studied in minute detail. Clayson remarks, "The literature on this subject is now so vast that it is not practicable to review it in detail. . ." (147). Nevertheless, Weisburger and Weisburger have written an extensive review of AAF carcinogenesis (144), in which they have surveyed the more important aspects of research in this area up to 1958. What follows is a gleaning of the more salient features of AAF carcinogenesis from their article.

Of the four isomeric acetylaminofluorenes tested (1-, 2-, 3-, and 4-AAF) only 2-AAF and some of its derivatives are active carcinogens. Polar substituents on the fluorene nucleus, which promote excretion of the AAF, reduce its carcinogenicity in vivo. Carcinogenicity is likewise reduced by large sterically-blocking substituents on the fluorene nucleus, or by replacement of the methylene bridge with a sulfur or oxygen atom. The 2-fluorenamines are readily N-acetylated and de-acetylated in vivo; substituents on the 2-amino group which are not susceptible to hydrolytic cleavage reduce the carcinogenic effect.

The most effective route of administration of AAF is by mouth, although skin painting also induces tumors. Unlike some other chemical carcinogens, AAF induces cancer in a wide variety of tissues, and at sites remote from the site of application. Different

species vary widely in their susceptibility to AAF carcinogenesis. Rats are most susceptible, rabbits and mice are less susceptible, and guinea pigs are totally resistant. The site and tissue affected are a function of the strain or species of the test animal.

Dietary factors definitely influence AAF carcinogenesis. A high protein diet generally delays carcinogenesis and reduces tumor incidence. In some cases caloric restriction has the same effect. Supplementary tryptophan, indole, or indole-3-acetic acid promotes the induction of bladder cancer in rats, and in one strain a high fat diet increased the incidence of mammary carcinoma, while simultaneously lowering the incidence of eye and ear tumors.

Hormonal influence may also affect tumor production by AAF. Male rats are much more susceptible to liver cancer, while female rats show a higher rate of mammary tumors. Removal of the thyroid or pituitary impedes liver carcinogenesis by AAF, but its metabolism appears unchanged.

The tissue levels of a number of enzymes are altered during AAF carcinogenesis. The level of riboflavin is decreased six-fold in the rat. AAF also interferes with the function of pyridoxine in the metabolism of tryptophan. Since supplementary tryptophan promotes bladder carcinogenesis in rats fed AAF, this alteration may be important in the pathogenesis of bladder tumors induced by AAF.

Studies on the metabolism of AAF by a variety of techniques have

demonstrated that in the rat, AAF undergoes (a) reversible N-deacetylation and acetylation, (b) ring hydroxylation at the 1, 3, 5, 7, and 8 positions, (c) conjugation of the hydroxylated metabolites with glucuronic and sulfuric acids, and (d) combination of some metabolites with tissue proteins. The amino nitrogen is not removed from the fluorene ring during metabolism. In the non-susceptible guinea pig, ortho-hydroxylation at the 1- and 3- positions is low or absent, a significant difference when compared to the susceptible rat. It was this observation which led to the idea that metabolic activation, perhaps via hydroxylation of the fluorene ring ortho to the amino group, might be necessary for the conversion of AAF to its active form (144).

4. Concept of Metabolic Activation

a. The Metabolism of Foreign Compounds

When a xenobiotic (substance foreign to the metabolic network of an organism), such as a drug, insecticide, or carcinogen is taken into the body, a number of mechanisms operate to metabolize the substance and promote its excretion. In general, though not invariably, such mechanisms represent metabolic conversion to polar metabolites which are more water-soluble than the parent compound, and hence more easily excreted by the kidneys. The enzymes responsible for this metabolic conversion reside mainly in

the microsomes of the liver.

Mason, North, and Vanneste (148), Conney (149), and more recently Gillette (150) have written reviews on the metabolism of foreign compounds. The general pathways of xenobiotic metabolism involve four major classes of reactions: oxidations, reductions, hydrolyses, and conjugations. Most oxidations occur via the mixed-function oxidases in liver microsomes (150). These enzyme require NADPH and oxygen, and contain a prosthetic heme (cytochrome P-450) which binds carbon monoxide and is inactivated by it. They catalyze a wide variety of reactions, including aromatic and aliphatic hydroxylations, deaminations, N-, S-, and O-dealkylations, N-hydroxylations, sulfoxidations, desulfurations, and dehalogenations. Gillette explains the currently proposed mechanism of these mixed-function oxidases: "Substrates react with the oxidized form of a microsomal cytochrome which is called cytochrome P-450. The cytochrome P-450-substrate complex is then reduced, presumably by NADPH-cytochrome c reductase either directly or indirectly through an intermediate electron carrier. The reduced cytochrome P-450-substrate complex then rapidly reacts with atmospheric oxygen and breaks down to form the oxidized drug and oxidized cytochrome P-450" (150). The mixed-function oxidases are also involved in the metabolism of some naturally occurring constituents of the body, such as fatty acids and steroid hormones (148). Other oxidations not involving the mixed-function

oxidase system include dehydrogenations (e. g., of alcohols) and direct oxidations (e. g., monamine oxidase).

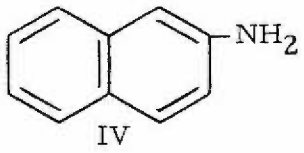
Reduction reactions include azo- and nitro-reduction in microsomes, and carbonyl reductions. Hydrolysis reactions involve cleavages of bonds by de-esterification or deamidation. Conjugation reactions include glucuronide and sulfate formation, methylation, acylation, and mercapturic acid formation (150).

b. Remote and Proximate Carcinogens

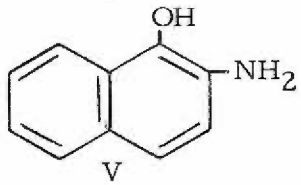
The experimental observation that compounds such as AAF and 2-naphthylamine induce tumors at various loci distant from the site of application led to the concept that the parent compound must first be metabolized to an active form, by one of the pathways discussed in the preceding section, before it is able to interact with a critical cellular target and induce cancer (147). In the case of AAF, the further observation that the guinea pig was neither susceptible to carcinogenesis by AAF, nor able to ortho-hydroxylate it, lent additional support to the metabolic activation concept (144). The active metabolite of AAF, possibly 1-, or 3-hydroxy-AAF, is designated as the proximate carcinogen. The parent compound, because it is presumably not active until metabolized, is called the remote carcinogen.

c. The Ortho-Hydroxylation Hypothesis

The first definitive systematic attempt to relate the carcinogenic action of an aromatic amine to its metabolism was reported by Bonser, Clayson, and Jull in 1951 (151). They showed that the proportion of the potent human bladder carcinogen 2-naphthylamine (IV) that is ortho-hydroxylated to 2-amino-1-naphthol (V) varies from



IV



V

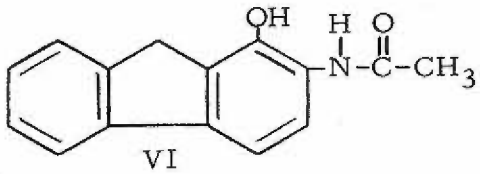
over 40% in the dog, which is extremely susceptible to its carcinogenic effect, to 15% in the mouse, which develops tumors only after a long latent period, to less than 5% in the rabbit, which is practically refractory to the carcinogenic effect of 2-naphthylamine (147). Later they were able to show that direct implantation of 2-amino-1-naphthol into the bladder wall of the mouse induced tumors at the site. Similar results were also obtained with 1-amino-2-naphthol. 2-Naphthylamine, although it induced more tumors than the controls, was much less active than the ortho-hydroxylated derivatives (152). These results led Clayson (147, 153) to suggest ortho-hydroxylation as the mechanism by which the aromatic amines are converted to proximate carcinogens. The ortho-hydroxylation hypothesis states that: "(i) Compounds which contain a hydroxyl and an amino group ortho to each other in an aromatic system of two or more rings may

be carcinogenic either in their own right or as a result of their further reaction; (ii) the reason why some aromatic amines induce tumours whereas others do not is that the former are more readily converted in the body to ortho-hydroxy amines than the latter" (153).

It is known that, for bladder carcinogenesis by 2-naphthylamine in the dog and man, ortho-hydroxylation is of central importance (154). The compound is absorbed occupationally through the skin, lungs, and possibly gastrointestinal tract. Upon reaching the liver, 2-amino-1-naphthol is produced by enzymatic hydroxylation, which is immediately rendered inactive in the liver by conjugation with glucuronic acid. The ester, being highly polar, is excreted by the kidney, resulting in a 200-fold concentration of the compound in the urine relative to the blood (147). Upon reaching the bladder, the ester is exposed to beta-glucuronidase, which has optimum activity at urinary pH levels. Enzymatic action hydrolyzes the conjugated derivative to the active proximate carcinogen, which then induces cancer in the bladder (154). Here, then, is a clear-cut example of metabolism resulting in the conversion of a remote carcinogen to a proximate carcinogen. Oxidative metabolism may also be important in the conversion of the polycyclic aromatic hydrocarbons to proximate carcinogens, possibly through epoxide formation at the reactive "K-region" (30, 155, 156).

The ortho-hydroxylation hypothesis appears to provide the

correct explanation for the metabolic activation of 2-naphthylamine. Two independent groups of investigators have even presented convincing evidence implicating abnormal tryptophan metabolism, which leads to the accumulation of ortho-hydroxyamine derivatives in the urine, in the genesis of "spontaneous" bladder cancer in man (157, 158). However, the ortho-hydroxylation hypothesis does not so easily account for carcinogenesis by the fluorenamines. Several ortho-hydroxylated derivatives of both 2-, and 3-AAF, including 2-acetylamino-1-fluorenol (VI), have been tested and found to be inactive as bladder carcinogens (147). It may be argued that, since AAF is primarily a liver carcinogen, the ortho-hydroxy metabolite reacts



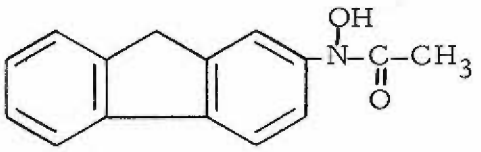
VI

with a critical intracellular target as soon as it is formed, and never reaches the bladder as an active carcinogen. Gutmann and Peters have conducted some in vitro studies showing that ring hydroxylation of AAF by liver slices results in an increase in protein binding of the hydroxylated metabolites, especially 7-hydroxy-2-AAF, but they were unable to demonstrate any protein bound ortho-hydroxy derivatives (159-161).

d. The N-Hydroxylation Hypothesis

The observations on the N-hydroxylation of AAF have tended to detract from the importance of ortho-hydroxylation as the critical metabolic pathway in the conversion of this compound to a proximate carcinogen. Miller, in reviewing the evidence, actually considers ortho-hydroxylation to be a pathway of deactivation (162). Since the first observations on the N-hydroxylation of AAF in vivo by Cramer and the Millers in 1960 (163, 164), evidence has steadily accumulated which implicates N-hydroxy-2-acetylaminofluorene (VII) as the critical proximate carcinogenic metabolite (162).

For example, N-hydroxy-2-acetylaminofluorene (hereinafter referred to as N-OHAAF), has been demonstrated as a urinary



VII

metabolite, conjugated with glucuronic acid, in those species in which AAF causes tumors (164-170). In the rat, hamster, mouse, and rabbit, N-OHAAF is more carcinogenic than AAF, and in marked contrast to AAF, especially at sites of local application, such as subcutaneous and peritoneal tissue, skin, or forestomach (168, 171-173). Liver, bladder, and the other usual tumors are also produced, as with AAF. Even more remarkable is the finding that when AAF is administered to the nonsusceptible guinea pig, no N-OHAAF can be

detected in the urine. Yet oral or subcutaneous administration of N-OHAAF to the guinea pig does indeed result in tumor induction (162, 168)! 7-Fluoro-AAF, which is readily N-hydroxylated in the rat, is a potent carcinogen for this species; the N-hydroxy derivative is an even stronger carcinogen (174). Gutmann and coworkers found no evidence that either of the non-carcinogenic fluorene derivatives, 7-hydroxy-AAF or N-benzoyl-2-aminofluorene, is N-hydroxylated in the rat. Yet the synthetically prepared N-hydroxy derivatives of both of these compounds are strong carcinogens (175). It must not be assumed that introduction of an N-hydroxy group onto any aromatic amine or amide is sufficient to convert it to a carcinogen. As shown by both Miller's group (174), and Gutmann and coworkers (175), the aryl group is a very important determinant.

In vivo evidence of N-hydroxylation of AAF is obtained by detecting N-OHAAF, conjugated with glucuronic acid, in the urine of the test animal fed AAF (163-165, 176, 177). Irving has carried out some in vitro experiments in which he demonstrated the N-hydroxylation of AAF in a test system containing rabbit liver microsomes, AAF, KCl, nicotinamide, and NADPH in a phosphate buffer, pH 7.8, shaken in air (166, 178). He was also able to show that the N-OHAAF formed was further metabolized in his test system to a great variety of compounds, including 2-nitrosofluorene, azoxyfluorene, AAF, AF, various ring hydroxylated derivatives and their

glucuronides, and that this further metabolism was partially inhibited by fluoride ion (166). N-Hydroxylation of AAF by either guinea pig or human liver microsomes was not detected by Irving, but subsequent experiments by Enomoto and Sato have clearly demonstrated that human liver microsomes do in fact perform the hydroxylation (179). N-Hydroxy derivatives of AAF have also been observed in the urine of human volunteers fed trace amounts of AAF (180). To date, microsomal P-450 has not been unequivocally implicated in the N-hydroxylation of AAF, although the in vitro systems employed to demonstrate the hydroxylation require both oxygen and NADPH. Lotlikar and coworkers have also noted that pretreatment of rats or hamsters with methylcholanthrene, an inducer of microsomal P-450 (149, 181), increases the N-hydroxylation of AAF by the treated animals (167, 182).

Recently Gutmann and Erickson have demonstrated that rat liver catalyzes the isomerization of N-OHAAF to both the 1- and 3-ortho-amidophenols of AAF (183). The reaction is inducible by methylcholanthrene, is dependent on the synergistic action of both the microsomal and soluble liver fractions, and requires no cofactors. Gutmann and Erickson propose that N-OHAAF is first enzymatically dehydroxylated to yield a positively charged amidonium ion. Then a hydroxyl ion adds to either of the 1- or 3-carbon atoms of the resonance forms of the amidonium ion to yield the corresponding 1-

or 3-ortho-amidofluorenols. A more detailed discussion of this mechanism will follow in a later Section. This observation raises again the question of the importance of ortho-hydroxylation in the conversion of AAF to a proximate carcinogen. Irving was unable to detect the ortho-hydroxy derivatives in the urine of rabbits fed either AAF or N-OHAAF (165, 166). And it has been shown that the ortho-hydroxylated derivatives of AAF are completely inactive carcinogens when tested by bladder wall implantation (147). However, this may be because the hydroxylated derivatives cannot enter the cell, due to their greater polarity. It cannot be categorically stated that an ortho-hydroxylated AAF formed intracellularly is inactive as a proximate carcinogen. But it does appear that, at the present time, there is more evidence in support of N-hydroxylation as the critical reaction responsible for the metabolic activation of AAF.

e. Reactivity of N-OHAAF with Cellular Components

Binding of N-hydroxylated AAF to cellular macromolecules has been demonstrated presumptively in vivo. Thus, greater yields of protein-bound (184), RNA-bound (185), and DNA-bound (186) fluorenyl derivatives are found in various tissues of rats given N-OHAAF, than result from administration of AAF. This binding is covalent, although it has not been conclusively shown that it is the N-hydroxylated derivative which is bound (162, 187, 188).

Kriek was first to show that N-OHAF, the deacetylated congener of N-OHAAF, reacts readily in vitro in a simple non-enzymatic anaerobic buffer system at sufficiently acid pH with the guanine in either DNA or RNA to form a very strong, presumably covalent bond. Reaction was rapid at pH 4.0, and barely detectable above pH 6.0 (189). The Millers have shown similar acid-catalyzed reactions between N-OHAF and methionine and guanosine. Again, at physiological pH, reaction was barely detectable (162, 190). In a variety of similar studies in which N-OHAAF was incubated with DNA, RNA, and protein, very little reactivity was noted at physiological pH with these macromolecules. In fact, in most cases the reactivity of N-OHAAF was just barely greater than that of AAF, in a non-enzymatic test system (14, 162, 191-195).

f. Esterification and Deacetylation of N-OHAAF

The in vitro observations that N-OHAAF had only slightly greater reactivity than AAF toward proteins and nucleic acids under physiological conditions seemed to indicate that some further step may be required to convert N-OHAAF to its active form. The glucuronide of N-OHAAF was suspected as being the final reactive metabolite of AAF, or the ultimate carcinogen, in view of the large amount of this substance which is formed in vivo (163-165, 176, 177, 196). The glucuronide was also found to be more reactive in vitro at pH 7-8 with

nucleic acids, guanosine, methionine, and tryptophan, than was N-OHAAF (190, 193), and the glucuronide of N-OHAF displayed even more marked reactivity (14). The role of the glucuronide as an ultimate carcinogenic metabolite is not supported by the inability to induce tumors in rats by repeated subcutaneous injections of the compound (190). But again, because of its extreme polarity, it may not enter the cell when experimentally applied to a tissue, yet easily react with cellular macromolecules at its intracellular formation site.

Recently Irving and coworkers (197) have shown that when N-OHAAF is administered intraperitoneally to rats, a proportion of the fluorene moieties bound to nucleic acids have lost their acetyl groups. The N-acetyl group was retained on 75% of the fluorene residues bound to ribosomal RNA, whereas only 35% of the fluorene moieties found bound to DNA contained the N-acetyl group. When the glucuronide of N-OHAAF was reacted in vitro at pH 7.4 and 37° C with yeast tRNA and calf thymus DNA, only a minor fraction (20-35%) of the fluorene moieties bound had retained the N-acetyl group. The loss of the N-acetyl group in this non-enzymatic reaction was found to be pH dependent, increasing with the pH of the reaction mixture (197). The products of reaction of the glucuronide of N-OHAAF with guanosine in vitro had previously been identified by Irving as N-(guanosin-8-yl)-2-acetylaminofluorene and N-(guanosin-8-yl)-2-aminofluorene, which were formed in the ratio of 1:2 (190). The results of Irving and

coworkers have been confirmed by Kriek (198), and King and Phillips (199), who showed in addition that enzymatic deacetylation of N-OHAAF is mediated by the soluble enzymes in rat liver, and that the N-OHAF thus formed reacts non-enzymatically with guanosine at pH 7.4 to form N-(guanosin-8-yl)-2-aminofluorene.

Several synthetic esters of N-OHAAF including N-acetoxy-AAF, N-benzoyloxy-AAF, N-propionyloxy-AAF, and N-butyryloxy-AAF, have been tested for non-enzymatic reaction at neutral pH with nucleic acids and protein. The reactivities of all of these synthetic esters are much greater than for either N-OHAF, N-OHAAF, or its glucuronide (162, 190, 200-203). They are also more carcinogenic than the glucuronide at the site of application (162), possibly because the ester groupings on each of the synthetically-prepared esters are much more hydrophobic than is the glucuronic acid group, and cell penetration would be greater (14). The target sites of both the glucuronide and synthetic esters of N-OHAAF for the in vitro reactions described above include the 8-carbon of guanine, the sulfur atom of methionine, the 3-position of tyrosine, and the 2-position of tryptophan (204).

g. Sulfate Ester as a Possible Ultimate Carcinogen

The Millers have recently presented very convincing evidence that the sulfate ester of N-OHAAF is, in all probability, an ultimate

carcinogenic metabolite of AAF and N-OHAAF in vivo (14, 204). King and Phillips (205) and Miller's group (206) in 1968 independently reported the occurrence of a sulfotransferase for N-OHAAF in the soluble proteins of rat liver, which forms an extremely reactive ester, AAF-N-sulfate. This ester is very reactive with cellular macromolecules in vitro at neutral pH, in fact, much more reactive than any other ester tested (14, 162, 202, 204). It is also markedly mutagenic to bacterial DNA when incubated with it in vitro (14, 207, 208), in contrast to the weakly mutagenic AAF, AF, N-OHAAF and its glucuronide, and N-OHAF. The soluble rat liver enzyme system which converts N-OHAAF to AAF-N-sulfate requires 3'-phosphoadenosine-5'-phosphosulfate, a specific sulfate donor in the enzymatic sulfation of various physiological hydroxy compounds (209). Magnesium and manganous ions stimulate the system in vitro (204).

There is a wealth of in vivo evidence that the sulfate ester of N-OHAAF is an ultimate carcinogenic metabolite. DeBaun and coworkers have shown that injection of sulfate ion in rats given N-OHAAF increases the formation of fluorenyl derivatives bound to total protein, RNA, and DNA in the liver, and also increases the carcinogenicity of N-OHAAF for the rats so treated (14, 206, 210). These effects can be prevented by concurrent administration of p-hydroxyacetanilide, which is known to deplete the in vivo sulfate pool, but does not inhibit the sulfotransferase (14, 210). The liver of the

male rat is far more susceptible to the carcinogenic action of N-OHAAF than the liver of the female rat, or the livers of a number of other male species. Similarly, the male rat liver has much higher levels of sulfotransferase activity for N-OHAAF than any other livers studied (14). Ablation of the thyroid, pituitary, or gonads, plus concurrent estrogen administration in the latter case, all markedly inhibit carcinogenesis by AAF and N-OHAAF in the male rat (144, 211, 212). Each of these endocrine alterations also lowers the sulfotransferase activity for N-OHAAF (14, 204).

AAF-N-sulfate has not been isolated directly in vivo due to its extreme reactivity. The half-life of the synthetically prepared AAF-N-sulfate is less than one minute in water at 37° C (14, 162). In view of this fact, it is not surprising that the sulfate ester has induced very few tumors on application to the skin and subcutaneous tissue of the rat. Miller remarks, "Thus, while AAF-N-sulfate is strongly implicated in hepatocarcinogenesis with N-hydroxy-AAF. . . 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" (14). Miller admits that either acetylation or phosphorylation of the N-hydroxy group of N-OHAAF could lead to acetate or phosphate esters with reactivities similar to the sulfate ester. Yet he has been unable to detect appreciable amounts of a

phosphotransferase or acetyltransferase for N-OHAAF in rat liver (14, 204). On the basis of his results outlined above, he believes that AAF-N-sulfate is the major ultimately reactive metabolite of the proximate carcinogen N-OHAAF in rat liver (14).

h. Sulfate Ester as an Electrophilic Reactant

Miller has very recently proposed a theory based on firm evidence, 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" (14). Some chemical carcinogens, such as the alkylating agents, are recognized as powerful electrophilic reactants per se. What Miller proposes is that all or nearly all chemical carcinogens are converted ultimately into compounds which interact with tissue components by an alkylation-type mechanism. The ultimate chemical carcinogens, or electrophiles, are thus capable of reacting with the nucleophilic centers on nucleic acids and proteins (e. g., $-COO^-$, -OH, $-H_2PO_4^-$, -SH, etc.) spontaneously.

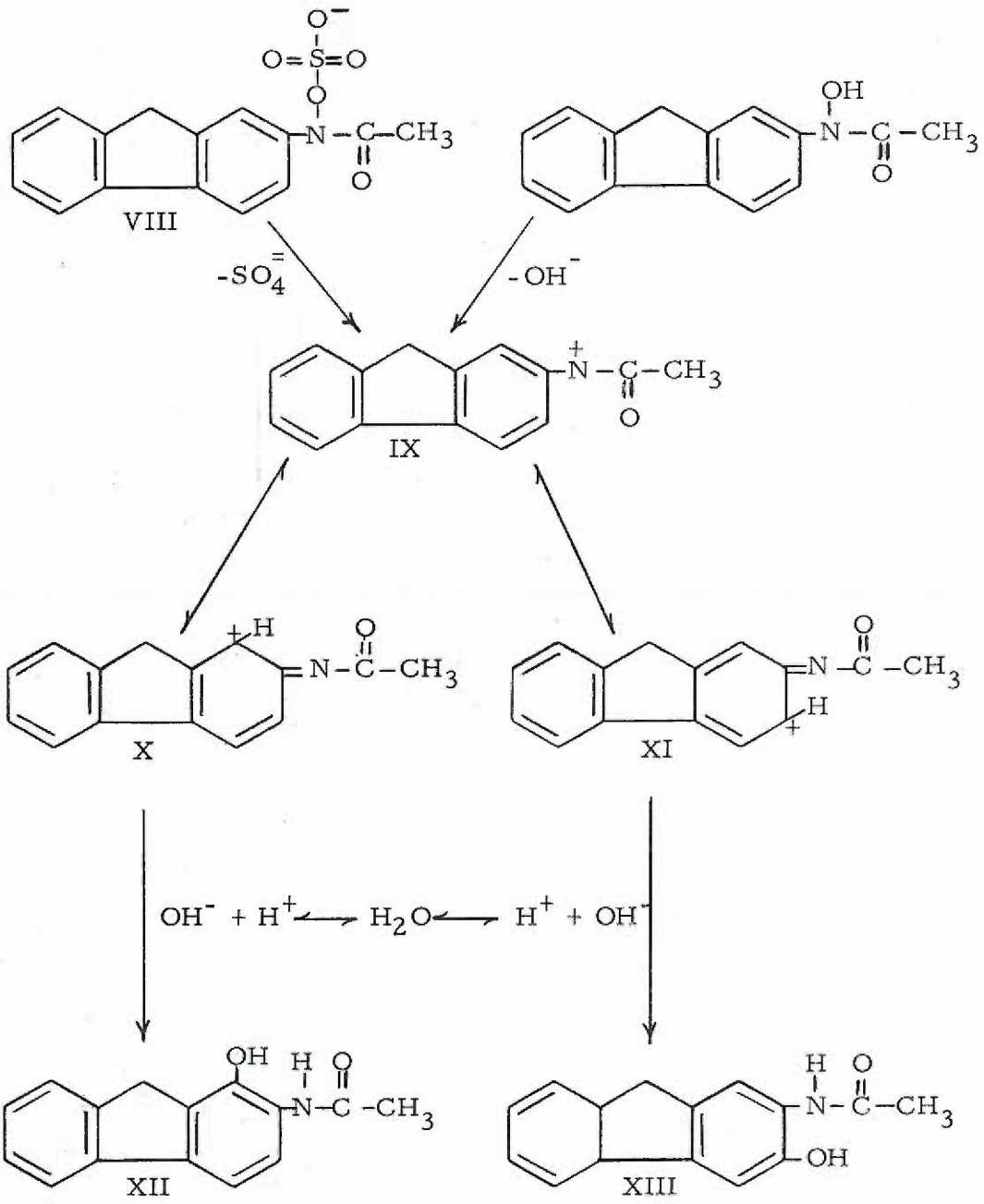
From what is known of the chemistry of the arylhydroxamic acids such as N-OHAAF, Miller has proposed a mechanism by which

esterification of the N-hydroxyl group leads to its great reactivity at neutral pH (14, 204). For the sulfate ester (VIII), ionization in aqueous solution results in the formation of the highly charged N-2-fluorenyl acetamidonium ion (IX), which is in equilibrium with both the 1- and 3-N-2-fluorenyl acetimido carbonium ions (X and XI, see Figure 2). These intensely electrophilic carbonium ion intermediates can then react with water, resulting in the formation of both the 1- and 3-ortho-amidophenols of AAF (XII and XIII), products observed by Gutmann and Erickson upon incubation of N-OHAAF with rat liver enzymes in vitro (183). The proton liberated is then free to balance the negative charge of the free sulfate ion. Stronger nucleophiles than water, such as phosphate, would compete preferentially for the carbonium ion, resulting in the greater probability of binding to intracellular macromolecules with strong nucleophilic centers. It must be strongly emphasized that binding by the ultimate carcinogen is essentially random--determined only by the degree of negative charge on the nucleophilic centers of the various intracellular molecules, and the proximity of those molecules capable of reacting in the manner described to the site of formation of the ultimate carcinogen.

The explanation of the acid-catalyzed reactions (14, 162, 189-195) between the N-hydroxy derivatives of AF and AAF, and cellular macromolecules, is obvious in the light of the mechanism described above. Clearly a low pH would facilitate removal of the N-hydroxyl,

FIGURE 2

FORMATION OF ORTHO CARBONIUM ION
FROM AAF-N-SULFATE


VIII
$-SO_4^=$
$-OH^-$
IX
X
XI
$OH^- + H^+ \rightleftharpoons H_2O \rightleftharpoons H^+ + OH^-$
XII
XIII

leading once again, via the unstable amidonium ion, to ortho carbonium ion formation. This could also be accomplished by enzymatic N-dehydroxylation of N-OHAAF (183), or by enzymatic deacetylation of this compound, via the nitronium ion, to the ortho carbonium ion (213).

DeBaun, Miller, and Miller have recently presented evidence to substantiate ortho carbonium ion formation. They have isolated both 1- and 3-methylmercapto-2-acetylaminofluorenes as degradation products of hepatic protein-bound methionyl derivatives in rats given AAF or N-OHAAF (204). These same derivatives are also the products of the in vitro reaction of free or peptide-bound methionine with various synthetic esters of N-OHAAF, including AAF-N-sulfate (206). Similar results are obtained with RNA-bound guanyl derivatives (162, 184, 204).

i. Summary and Conclusion

It seems evident that the conversion of the remote carcinogen AAF to a proximate carcinogen involves N-hydroxylation. The mechanism of activation of N-OHAAF proposed by Miller explains the presence of ortho-hydroxylated metabolites of AAF, but relegates ortho-hydroxylation to a role of deactivation rather than activation. Conversion of N-OHAAF to an ultimate carcinogen probably involves formation of an intensely electrophilic carbonium ion ortho to the

amido nitrogen. This may occur most likely in vivo via sulfate ester formation, but possibly via glucuronide ester formation, N-dehydroxylation, or N-deacetylation. Once formed, the carbonium ion may react covalently with any nucleophile in its proximity, including water, to be inactivated, or with a cellular macromolecule, possibly to induce cancer.

E. STATEMENT OF THE PROBLEM OF THE THESIS RESEARCH

It is a fundamental assumption of research in chemical carcinogenesis that chemical carcinogens induce cancer by interacting with cellular components. Yet the fundamental question of chemical carcinogenesis research, "Where does the carcinogen act?", has not been answered with certainty for even a single carcinogen. Interactions of carcinogen and tissue components may be physical or chemical in nature, and there may be one, several, or perhaps hundreds of critical intracellular target sites. A critical "hit" by a carcinogen thus may involve either physical or covalent binding, and must be viewed in terms of probability, since there are many non-critical sites available for reaction.

To date, no systematic investigation of both the physical and chemical reactivities of a remote and proximate carcinogen with respect to the nucleic acids associated with all subcellular fractions and organelles has been performed. The experiments described in

the next section attempt to establish the in vitro reactivites of the remote carcinogen AAF and its proximate carcinogenic metabolite N-OHAAF with these nucleic acid fractions. A thorough survey of the cellular organelles, conducted by incubating each subcellular particulate fraction with carcinogen in an actively metabolizing enzyme system, extracting the nucleic acid, and assaying for bound carcinogen, may provide an answer to the question of probability, "Where might the proximate carcinogen act?".

MATERIALS AND METHODS

A. PREPARATION OF SUBCELLULAR FRACTIONS AND NUCLEIC ACIDS

1. Isolation of Rat Liver

Adult male Sprague-Dawley rats, each weighing about 0.75 kg, were maintained on Purina laboratory chow and water ad libitum. Food, but not water, was removed 18 hours prior to sacrificing. The animals were very lightly anesthetized with ether, then killed by decapitation. Exsanguination was encouraged by holding the decapitated rat in a tail-up position. The abdomen was then opened with a scalpel, and the entire liver removed with surgical scissors. Each liver weighed from 9-12 g. The livers were thoroughly perfused with 0.25 M sucrose in a cold room held at 0-4° C. Unless otherwise specified, all further preparative procedures were conducted at 0-4° C.

2. Preparation of Nuclear DNA

DNA was prepared from liver by the method of Zamenhof (214). Fifty grams of perfused liver was cut into small pieces (2-5 mm^3), and washed in five 100-ml portions of ice cold citrate buffer (0.1 M sodium citrate, pH 7.40). The diced liver was mashed on wax paper with the bottom of a 150 ml Erlenmeyer flask filled with ice, mixed

with 250 ml of citrate buffer, and thoroughly homogenized for five minutes at 500 rpm in a 50 ml Potter-Elvehjem homogenizer equipped with a motor-driven pestle and surrounded in ice. The homogenate was centrifuged in the Servall Model RC-2 refrigerated automatic preparative centrifuge for 30 minutes, using the GSA rotor at 3, 300 rpm (1800 X g).

The supernatant was carefully decanted, and the loose pellet was homogenized again for one minute in 250 ml of citrate buffer, and centrifuged as before. This step was repeated two more times, resulting finally in a pink-tan sediment, which was suspended in 250 ml of 2 M NaCl. The mixture was homogenized for five minutes at 100 rpm. During this procedure it became very viscous and slightly ropy. The mixture was allowed to stand in the cold room for 24 hours. It was then centrifuged in the Servall, using the GSA rotor at 3, 400 rpm (1900 X g), for one hour.

To the opalescent tan supernatant was added two volumes (500 ml) of 95% ethanol at the rate of 150 ml/min, with constant slow stirring (30 rpm) by a magnetic stirrer. Small brownish fibers were precipitated, and the solution lost viscosity. At this stage it was not possible to lift the precipitated DNA fibers out of the solution. The mixture was centrifuged for one hour in the Servall, using the GSA rotor at 7, 000 rpm (7970 X g).

The sediment, consisting of crude precipitated DNA, was

homogenized in 250 ml of standard buffer (0.14 M NaCl in 0.015 M sodium citrate, pH 7.1) very gently by hand. To the slightly viscous mixture was added 1/9 volume (28 ml) of 5% sodium dodecylsulfate (Eastman) in 45% aqueous ethanol. The mixture was stirred for one hour at room temperature (about 25° C), and became clear brown. Sufficient solid NaCl (11.85 g) was added to make a final concentration of 5% NaCl, and stirring was continued for 30 more minutes at room temperature. A dark brown precipitate formed, composed of protein and sodium dodecylsulfate in large discrete clumps. This was allowed to stand overnight at 4° C. It was then centrifuged for one hour in the Servall, using the SS-34 rotor at 16,250 rpm (31,000 X g).

To the clear golden supernatant was added two volumes (556 ml) of 95% ethanol, as previously described. Nearly white, long threads formed which clumped together. These were removed, washed in 75% ethanol, and redissolved in 250 ml of standard buffer with the aid of a homogenizer operated by hand. To the cloudy, slightly viscous mixture was added 1/9 volume of 5% sodium dodecylsulfate in 45% aqueous ethanol, and the mixture was stirred for one hour at room temperature. It first became clear, then opalescent with very fine white silky threads. Solid NaCl (11.85 g) was added, with stirring and standing as before. The mixture, which deposited a curdy white precipitate after standing, was centrifuged for one hour at 31,000 X g. Then all of the steps described in this paragraph were repeated once

more.

To the clear supernatant was added two volumes of 95% ethanol as previously described. Pure white threads of DNA formed. These were washed in 75% ethanol, and redissolved in 50 ml of standard buffer. This solution was extremely viscous, like honey, and perfectly clear, yet slightly light green in color.

RNA was removed from the above DNA solution by the method of Zamenhof (214). Nuchar activated charcoal was washed with distilled water for 18 hours, then once with eight volumes of 2 M NaCl, then twice with eight volumes of standard buffer. Three ml of this soggy wet charcoal was mixed with the entire 50 ml of DNA solution. The mixture was shaken at 4° C for one hour on a Vortex Jr. mixer (Scientific Industries, Inc.), then centrifuged at 31,000 X g for one hour.

The final DNA solution was a viscous, highly refractive solution with no visible trace of color. Assay for DNA by the diphenylamine procedure, to be described in a later Section, gave a final concentration of 0.734 mg DNA/ml, or a total yield of 37 mg of DNA. The orcinol and biuret tests for RNA and protein contamination respectively (also to be described later) revealed no detectable contamination. The sensitivity of these assays allows a maximum contamination of 0.005 mg RNA/ml (0.7%) and 0.1 mg protein/ml (14%). The DNA solution was stored at -15° C.

3. Preparation of RNA from Ribonucleoprotein

Rat liver RNA was prepared according to the procedure described by Schmidt (215). One hundred grams of perfused liver was shredded with a scalpel, and blended at high speed in a Waring blender for five minutes with 400 ml of ice cold 0.9% NaCl in 0.01 M sodium citrate solution. The blended mixture was then centrifuged in the Servall for 30 minutes at 2,500 rpm (1020 X g), using the GSA rotor. The cloudy supernatant was carefully decanted from the sediment and recentrifuged once again. The supernatant from this second centrifugation was adjusted to pH 4.5 with N HCl, using an Ionalyzer Model 801 digital pH meter (Scientific Products). The mixture was kept ice cold during this procedure, and became quite viscous. It was then centrifuged for 15 minutes at 2,500 rpm in the Servall as before. The supernatant was discarded. The ribonucleoprotein sediment was suspended with the aid of a hand-operated homogenizer in 0.9% NaCl to a volume of 200 ml.

To the light tan suspension was added 18 ml of 2% sodium dodecylsulfate, and the pH was raised to 7.0 with 10% NaOH. The mixture was then stirred at 100 rpm by a magnetic stirrer in the cold room for three hours. Sufficient solid NaCl was added to bring the concentration to 1 M (11.06 g used), and the mixture was centrifuged in the Servall for one hour at 14,000 rpm (23,500 X g), using the SS-34 rotor. The sediment was discarded.

To the supernatant was added two volumes (320 ml) of 95% ethanol, and the mixture was centrifuged at 7,000 rpm (7970 X g) in the Servall for 30 minutes, using the GSA rotor. The light tan RNA pellet was washed repeatedly with alcohol, then acetone, then dried in air by means of a Buchner funnel connected to a suction flask.

The crude RNA was dissolved in 200 ml of water, and 18 ml of 2% sodium dodecylsulfate was added, with stirring at room temperature for two hours. Sufficient solid NaCl (12.86 g) was then added to bring the concentration to 1 M. The mixture was centrifuged in the Servall at 14,000 rpm as before for one hour, and the pellets were discarded. Two volumes (400 ml) of 95% ethanol was then added to precipitate the RNA, which was centrifuged, washed, and dried as previously described.

The nearly white powder was dissolved in 60 ml of water. The solution was cooled to 0° C, and solid NaCl (0.540 g) was added to make a final concentration of 0.9%. The pH was adjusted with a few drops of N HCl to 4.5, and the mixture was centrifuged at 0° C in the Servall at 14,000 rpm for one hour. The brownish pellets were discarded. The supernatant was decanted into an ice cold beaker containing sufficient solid NaCl (2.967 g) to make the solution 1 M. The pH was adjusted to 7.0 with 0.1 N NaOH in the cold, and two volumes of 95% ethanol (120 ml) was added to precipitate the sodium salt of RNA. The precipitate was centrifuged, washed, and dried as before,

yielding a nearly white amorphous powder weighing 300 mg, and completely soluble in water. A solution of 1 mg/ml gave negative diphenylamine and biuret reactions. The sensitivity of these tests allows a maximum contamination of 0.001 mg DNA/ml (0.1%) and 0.1 mg protein/ml (10%). The RNA was stored in a small, tightly-capped bottle at -15° C.

4. Preparation of Transfer RNA

Transfer RNA was prepared according to the procedure of Bloemendal and coworkers (216). Two hundred grams of perfused rat liver was shredded, mashed, and added to 450 ml of an ice cold buffer containing 0.02 M potassium phosphate, 0.025 M KCl, 0.05 M tris-Cl, 0.175 M sucrose, and 0.005 M $MgCl_2$, pH 7.8. This mixture was thoroughly homogenized in a motor-driven Potter-Elvehjem homogenizer at 500 rpm for five minutes. The homogenate was centrifuged in the Servall for ten minutes at 9,750 rpm (15,000 X g), using the GSA rotor. The supernatant was carefully decanted, and recentrifuged in the Beckman Model L refrigerated ultracentrifuge, using the Type 30 rotor at 30,000 rpm (105,000 X g) for two hours.

To the red supernatant was added an equal volume (300 ml) of 90% phenol liquified with aqueous 0.001 M disodium ethylenediamine-tetraacetate (Na_2EDTA, Matheson, Coleman, and Bell). The mixture was shaken in the cold room for ten minutes, then centrifuged in the

Servall at 9, 750 rpm as before for one hour. This resulted in the formation of a top aqueous layer, a middle white layer consisting of denatured proteins, and a bottom phenolic layer. The top aqueous layer, containing the transfer RNA, was carefully aspirated with a syringe, and phenol-extracted two more times exactly as described in this paragraph. After centrifugation the second time, no white protein layer was seen at the interface of the aqueous and phenolic layers.

The clear aqueous layer was next extracted three times with three volumes of ether, to remove the phenol. The solution was then warmed to 40° C, and air was blown through it to remove ether. After cooling in ice, three volumes (about 1 liter) of 95% ethanol was added to the solution, which was placed in a freezer maintained at -15° C. After three hours, a gelatinous white precipitate formed. This was collected by centrifugation in the Servall at 9, 750 rpm (15, 000 X g) using the GSA rotor, for one hour. The white precipitate was washed with 95% ethanol, then acetone, then dried in air.

The product was 275 mg of a nearly white amorphous powder, very soluble in water. Its purity was identical to that of the RNA prepared from ribonucleoprotein.

5. Preparation of Nuclei

Nuclei were prepared according to the method described by Busch (217). Thirty grams of perfused liver was shredded, mashed,

and mixed with 300 ml of 2.4 M sucrose in 3.3 mM $CaCl_2$. The honey, thick mixture was homogenized very gently in a motor-driven Potter-Elvehjem homogenizer at 100 rpm, using only three passes of the pestle. The homogenate was then centrifuged in the Servall for one hour at 18,250 rpm (40,000 X g), using the SS-34 rotor. A nearly white pellet of nuclei separated from all other components, which floated to the top of the dense sucrose medium.

The nuclear pellets were carefully scraped out and resuspended in 30 ml of 1 M sucrose in 0.001 M $CaCl_2$ by hand homogenization. The suspension was then centrifuged for five minutes at 5,000 rpm (3000 X g) in the SS-34 Servall rotor. The nuclear pellets were collected and resuspended in 0.34 M sucrose containing 0.000 M $CaCl_2$. After determination of protein by the biuret procedure, the suspension was frozen and stored at -15° C.

This procedure yielded a nearly pure-white suspension of nuclei which was homogeneous by light microscopy. The calcium was present in the solutions used for preparation to preserve the integrity of the nucleolar membrane (218).

6. Preparation of Nucleoli

Nucleoli were isolated by the procedure described by Busch (218). Nuclei prepared by the sucrose-calcium procedure described in the previous Section, suspended in 0.34 M sucrose containing 0.001

M $CaCl_2$, were placed in the stainless steel cup of the Raytheon Model DF-101 sonic oscillator. The sonicator cup was cooled by means of circulating ice water to a temperature of 0-4° C. It was found convenient to sonicate 20 ml of nuclear suspension at a time.

The oscillator, which operates at a frequency of 10 KHz and a power of 250 watts, was tuned to maximum output (meter reading = 720 ma), and operated for intervals of 30 seconds. At the end of each 30 second interval, the sonicate was checked for degree of nuclear disintegration by mixing one drop of 0.1% toluidine blue in 0.25 M sucrose with one drop of sonicate, and examining the mixture with the light microscope at 930X magnification. The nucleoli were identified by their deep red-violet metachromatic stain, in contrast to the body of the nucleus, which does not stain at all.

It was found that a total sonication time of two minutes resulted in almost complete disintegration of nuclei, without serious loss of nucleoli (approximately one intact nucleus per 1000 nucleoli). After two minutes of sonication, the suspension became very viscous, due to the liberation of nuclear DNA into the solution.

The combined sonicate was next divided into three equal fractions, which were layered over 10 ml of ice cold 0.88 M sucrose in each of three Beckman Type SW 25.1 swinging bucket rotor tubes. The tubes were spun for 20 minutes at 3,700 rpm (2000 X g). A light pellet separated at the bottom of the tube (nucleoli) from the tan hazy

supernatant (nuclear debris).

The pellets were gently homogenized in 0.25 M sucrose, and recentrifuged in the Servall for 20 minutes at 4,500 rpm (2445 X g), using the SS-34 rotor. The lightly packed pellets which resulted were then resuspended in 0.25 M sucrose. The nucleolar suspension was assayed for protein concentration, and stored at -15° C. The final suspension was remarkably homogeneous by light microscopy; nuclear debris was virtually absent, and nuclear contamination was of the order of 0.02%.

7. Preparation of Mixed Microsomes

Mixed (i.e., heterogeneous smooth and rough) microsomes were prepared by the method of Mason and coworkers (219). To 160 g of mashed perfused liver was added 340 ml of 0.88 M sucrose. The mixture was homogenized for ten minutes in a motor-driven Potter-Elvehjem homogenizer at 200 rpm in the cold room. The homogenate was then centrifuged for 25 minutes at 7,500 rpm (9000 X g) in the Servall, using the GSA rotor. To the red supernatant, which contained the microsomes in suspension, was added 1700 ml of 0.04 M KCl, and the mixture was centrifuged for one hour at 21,000 rpm (59,000 X g), using the Beckman Type 21 rotor in the Model L ultracentrifuge. The sediment, containing heterogeneous microsomes, was scraped out and suspended in 100 ml of sucrose-KCl (one volume of

0.88 M sucrose plus five volumes of 0.04 M KCl) by gentle hand homogenation. The suspension was centrifuged again at 21,000 rpm in the Beckman ultracentrifuge. This last step was repeated one more time. The microsomal pellet was finally suspended in 16.6 mM potassium phosphate buffer, pH 7.40. After protein assay, the suspension was stored in small quantities under prepurified nitrogen at -15° C. The assay of the P-450 content of microsomes will be described later.

8. Preparation of Homogeneous Smooth and Rough Microsomes

This procedure is a continuation of the preparative process described in the previous section (219). After the first centrifugation in the Beckman ultracentrifuge at 21,000 rpm, the microsomal pellets were collected and homogenized by hand in 80 ml of sucrose-KCl. Ten ml of this homogenate was layered very carefully with a syringe over 2 ml of 1.31 M sucrose in each of eight Type 40 rotor tubes, which were then spun at 40,000 rpm (144,000X g) in the Beckman for eight hours. This density gradient centrifugation resulted in a separation of the rough-surfaced endoplasmic reticulum, which contains ribosomes, from the smooth-surfaced endoplasmic reticulum, which is free from ribosomes (219). Three distinct layers formed in the bottom of each tube. The top red layer, composed of the heme-rich smooth microsomes, was packed over a light-tan layer of rough

microsomes, which was in turn packed over a clear, jelly-like bottom layer of glycogen.

The supernatant was carefully aspirated. The smooth microsomal layer, being quite tenacious and of a semisolid character, was floated off from the rough microsomal layer with the aid of a small spatula. The rough layer was more liquid in character, and was easily separated from the tough glycogen gel by syringe aspiration. The rough and smooth microsomal fractions were homogenized separately, each in 40 ml of sucrose-KCl, layered again over 1.31 M sucrose, and recentrifuged for another eight hours as before. This second density gradient centrifugation resulted in a further separation of the rough and smooth fractions (219). Each fraction was collected as previously described, homogenized separately in 24 ml of sucrose-KCl, and centrifuged for one hour at 40,000 rpm in the Beckman. The pellets were then scraped out, suspended in 16.6 mM potassium phosphate buffer, pH 7.40, and stored in small quantities under prepurified nitrogen at -15° C following protein assay of the suspensions.

The rough microsomal fraction was tan to brown in color. The smooth fraction was a deep liver-red, and has been characterized by electron microscopy as homogeneous and uncontaminated with ribosomal granules (219).

9. Preparation of Free Ribosomes

Ribosomes were isolated according to the procedure of Moldave and Skogerson (220). Rat livers were removed and perfused with Moldave's ribosomal medium (MRM), which consists of 0.35 M sucrose, 0.035 M $KHCO_3$, 0.025 M KCl, 0.004 M $MgCl_2$, and 0.02 M K_2HPO_4, pH 7.40. They were then shredded, mashed, and 160 g of the mashed liver was mixed with 370 ml of cold MRM. The mixture was thoroughly homogenized in a motor-driven Potter - Elvehjem homogenizer at 200 rpm for five minutes. The thick red homogenate was centrifuged in the Servall for 30 minutes at 8, 750 rpm (12, 000 X g), using the GSA rotor. The cloudy red supernatant was decanted, diluted to 1 liter with more cold MRM, and centrifuged in the Beckman ultracentrifuge for 18 hours, using the Type 21 rotor at 21, 000 rpm (59, 000 X g). The clear red supernatant was discarded. The microsomal pellets were scraped out and carefully suspended in cold MRM with the aid of a homogenizer operated at 60 rpm. Approximately 20 g wet weight of microsomes was homogenized in 365 ml of MRM in this manner. Sufficient cold 0.1 M $MgCl_2$ (20 ml) was added to make the suspension 0.009 M with respect to magnesium ion. Then 36 ml of a freshly prepared 3% solution of sodium desoxycholate (Difco) was added to give a concentration of 0.26%. After stirring in the cold room for 15 minutes, the suspension became almost clear red.

The detergent-treated microsomal suspension was next

centrifuged in the Beckman Type 30 rotor at 30, 000 rpm (105, 000 X g) for eight hours. The clear-red firm ribosomal pellets were scraped out and homogenized gently in 100 ml of 0.001 M $MgCl_2$. Then 100 ml of 0.1 M $MgCl_2$ was added to make the mixture 0.05 M in magnesium ion. It became light tan and quite cloudy after stirring in the cold room for 30 minutes.

The suspension was centrifuged in the Servall for 30 minutes at 13, 000 rpm (27, 300 X g), using the SS-34 rotor. The clear yellow supernatant was discarded. The loosely packed ribosomal pellets were resuspended in 100 ml of 0.01 M $MgCl_2$, and recentrifuged as before. This last step was repeated two more times.

The pellets were next suspended in 100 ml of 0.05 M tris-Cl, pH 7.40, containing 0.5 M NH_4Cl and 0.01 M $MgCl_2$. The suspension was centrifuged for five minutes in the Servall, using the SS-34 rotor at 3, 500 rpm (2000 X g). The dark brown pellets were discarded. The light tan supernatant was centrifuged for 70 minutes in the Beckman Type 30 rotor at 30, 000 rpm (105, 000 X g), to sediment the ribosomes. The steps described in this paragraph were then repeated three more times with the ribosomal pellets.

The ribosomal pellets were finally suspended in 30 ml of MRM. After protein assay, the suspension was stored at -15° C.

10. Preparation of Mitochondria

Liver mitochondria were prepared as described by Johnson and Lardy (221). One hundred grams of perfused liver was shredded, mashed, and added to 250 ml of 0.25 M sucrose. The mixture was gently homogenized in a motor-driven Potter-Elvehjem homogenizer at 200 rpm in the cold room. Homogenization was continued until no intact liver tissue was visible; this required about five to seven passes of the pestle. The homogenate was diluted to 800 ml with 0.25 M sucrose, and centrifuged for ten minutes at 1,850 rpm (600 X g) in the Servall, using the GSA rotor. The supernatant was decanted and saved. The pellets were shaken with 200 ml of 0.25 M sucrose and recentrifuged as before. The combined supernatants were filtered through ten layers of cheesecloth, and centrifuged for five minutes in the Servall GSA rotor at 11,250 rpm (15,000 X g). The red supernatant was discarded.

The mitochondrial pellets were scraped out, carefully avoiding the red blood cell pellet at the bottom of the mitochondrial sediment. After suspension of the mitochondria in 100 ml of 0.25 M sucrose by gentle hand homogenation, the mixture was centrifuged at 11,250 rpm as before. The light tan mitochondrial pellets were suspended in a convenient volume of 0.25 M sucrose. After protein assay, the suspension was stored at -15° C.

B. ASSAY PROCEDURES

1. Determination of Protein

Protein was determined by the biuret method (222). The biuret reagent was prepared exactly as described by Layne (222), and standardized with LAB-TROL (Scientific Products). One ml of various concentrations of LAB-TROL was added to 4 ml of biuret reagent. A solution containing 1 ml of water and 4 ml of biuret reagent served as a blank. Thirty minutes was allowed for color development; then optical density was measured in the 1 cm quartz cuvettes at 540 mμ on the Carl Zeiss Model PMQ II spectrophotometer, and a calibration plot was drawn.

Since the heme present in microsomes, mitochondria, and other organelles may absorb light at 540 mμ, the modification of Yonetani (223) was used when assaying these suspensions by the biuret procedure. To 0.50 ml of sample was added 3 ml of 1% sodium dodecylsulfate, in order to solubilize the protein and clear turbidity. This was done in a 5 ml volumetric flask. Then 0.05 ml of 30% H_2O_2 was added before diluting to the mark. Considerable frothing occurred, so the mixture was transferred to a beaker. After foaming subsided, 1 ml of this mixture was added to 4 ml of biuret reagent, and the optical density was determined as previously described. The H_2O_2 treatment effectively bleached heme absoprtion at 540 mμ, yet

in no way interferred with the biuret color.

Typical suspensions of the various subcellular fractions prepared as described above contained from 10-50 mg protein/ml. The sensitivity of this assay is 0.1 mg protein/ml.

2. Determination of DNA

DNA was determined by the diphenylamine procedure (224). One ml of sample was mixed with 2 ml of diphenylamine reagent, prepared as described by Schneider (224), in a small test tube, which was then heated for ten minutes in a boiling water bath. After cooling to room temperature, the intensity of the blue color was measured in the Zeiss spectrophotometer by reading the absorbance at 600 mμ against a blank consisting of 1 ml of water and 2 ml of diphenylamine reagent, which was also heated for ten minutes.

For a light path of 1 cm, the DNA-phosphorus content of the sample is given by the following equation (224):

$$\mu g \text{ DNA-P/ml} = \frac{OD_{600}}{0.019}$$

Since the phosphorus content of DNA is 9.5% (225), the above value is multiplied by 100/9.5 = 10.53 to obtain the actual weight of DNA in μg DNA/ml. The sensitivity of the diphenylamine test is 1 μg DNA/ml. RNA does not interfere in this assay (224).

3. Determination of RNA

RNA was determined by the orcinol procedure (224). The orcinol reagent was prepared immediately before use as described by Schneider (224). To 0.2 ml of sample was added 1.3 ml of water and 1.5 ml of orcinol reagent. A blank containing 1.5 ml of water and 1.5 ml of orcinol reagent was also prepared. The sample and blank were both heated for 20 minutes in a boiling water bath. After cooling, the intensity of the green color was determined by measuring the absorbance at 660 mμ in the Zeiss spectrophotometer against the blank prepared with water.

Since any DNA present in the sample will react with the orcinol reagent, the equation used to calculate the RNA-phosphorus in the sample must be modified. If the sample does contain DNA, the DNA-phosphorus must first be determined by the diphenylamine procedure. For a light path of 1 cm, the RNA-phosphorus of the sample is given by the following equation (224):

$$\mu g \text{ RNA-P/0.2 ml} = \frac{(OD_{660} + 0.008) - (\mu g \text{ DNA-P/0.2 ml} \times 0.013)}{0.116}$$

Since the phosphorus content of RNA is 9.5% (225), the above value is first multiplied by 100/9.5 = 10.53 to obtain the weight of RNA/0.2 ml. Multiplication of this value by 5 then gives the amount of RNA in μg/ml. The sensitivity of this assay is 5 μg RNA/ml.

4. Determination of the P-450 Content of Microsomes

Microsomes were assayed for P-450 content essentially using the procedure of Omura and Sato (226). A suspension of 1.00 mg microsomal protein per ml of 16.6 mM potassium phosphate buffer, pH 7.40, was reduced with solid sodium dithionite. A few crystals were used to reduce 6 ml of suspension. The suspension was then divided into two equal fractions. One fraction (reduced microsomes) was carefully bubbled with prepurified nitrogen for 20 seconds; the other fraction (CO-reduced microsomes) was bubbled with purified carbon monoxide for 20 seconds. The suspensions were transferred to 1 cm quartz cuvettes, and a difference spectrum (CO-reduced minus reduced) was recorded on the Cary Model 14 recording spectrophotometer (Applied Physics Corporation). The difference in absorbance between the peak at 450 mμ and the baseline, taken at 490 mμ, was measured; using an extinction coefficient of 91 $mM^{-1}cm^{-1}$, the P-450 content of the microsomes may be calculated (226). Typical values ranged from 0.8 to 1.4 nanomoles of P-450/mg protein.

C. INCUBATION CONDITIONS

1. Preparation of Radiolabeled Carcinogen Stock Solutions

2-Acetylaminofluorene-9-C^{14}, with a radiopurity of greater than 98% specified, was obtained from Tracerlab. The compound has a

specific activity of 11.81 mC/mMole. A stock solution was prepared by dissolving 1.116 mg per ml of absolute ethanol, to give a final concentration of 5 mM AAF. The stock solution was stored in a tightly-capped vial at -15° C.

N-Hydroxy-2-acetylaminofluorene-9-C^{14}, with a specified radiopurity of greater than 98%, was likewise obtained from Tracerlab. The compound has a specific activity of 12.73 mC/mMole. A stock solution was made by dissolving 1.196 mg per ml of absolute ethanol, to give a final concentration of 5 mM N-OHAAF. It was stored at -15° C.

2. Incubation of Carcinogen with Purified Nucleic Acids

In order to determine the degree of binding of carcinogen to nucleic acid which is not enzymatically mediated, purified nucleic acid was incubated in a simple buffer system with carcinogen. In all cases, 4 mg of purified nucleic acid (DNA, RNA, and tRNA) was incubated in 10 ml of standard buffer (0.14 M NaCl in 0.015 M sodium citrate, pH 7.10), with carcinogen at a final concentration of 100 μM. Incubation was carried out under prepurified nitrogen in a 50 ml Erlenmeyer flask for 3 hours, with shaking at 37° C in a Dubnoff metabolic shaking incubator (Precision Scientific Company). Each nucleic acid was incubated with AAF and N-OHAAF separately in the manner described.

To determine the degree of physical binding (actually physical plus covalent binding, if any), 4 ml of the incubation mixture was eluted directly from a G-10 Sephadex column (procedure to be described later). Each fraction was assayed for nucleic acid content and radioactivity (also described below).

To determine the degree of covalent binding, 25 ml of absolute ethanol was added to the remaining 6 ml of incubation mixture, whereupon a white stringy (DNA) or milky (RNA) precipitate formed. The mixture was stored at -15° C for 8-12 hours, then centrifuged for one hour in the Servall at 16,250 rpm (31,000 X g), using the SS-34 rotor. The nucleic acid pellets were dissolved in 5 ml of standard buffer containing 5% sodium dodecylsulfate, and the solution was homogenized for five minutes at 500 rpm in a motor-driven Potter-Elvehjem homogenizer, which was held in a water bath maintained at 65-70° C during homogenation. To the hot solution was next added 25 ml of absolute ethanol, with cooling at -15° C, centrifugation, and detergent treatment of the nucleic acid precipitate as before. This operation was repeated until the alcoholic supernatant obtained after centrifugation showed no radioactivity above background. A total of 20-25 precipitations and detergent treatments was required. The nucleic acid pellets, presumably freed from all physically-bound carcinogen, were then dissolved in 2 ml of 0.1 M KCl in 0.01 M tris-Cl, pH 7.00, which was eluted from Sephadex in order to determine if

any radioactivity (presumably covalently bound carcinogen) was associated with the nucleic acid.

3. Incubation of Carcinogen with Nuclei

Nuclei were incubated for three hours at 37° C in air with shaking in a system containing 0.13 M sucrose, 0.375 mM $CaCl_2$, 10.37 mM potassium phosphate, pH 7.40, 100 μM carcinogen, and 6.7 mg nuclear protein/ml in a total volume of 20 ml. After incubation, the nuclei were collected by centrifugation for five minutes at 5,000 rpm (3000 X g) in the Servall SS-34 rotor. The nuclear pellets were next put through the nucleic acid extraction procedure (sodium dodecylsulfate-phenol extraction procedure described below). Analysis of the nucleic acid extract from nuclei by the diphenylamine and orcinol tests yielded a DNA'RNA ratio of 3.8-3.9:1. The extracts from nuclei incubated with both AAF and N-OHAAF were then eluted separately from a G-10 Sephadex column, and the fractions collected were assayed for nucleic acid content and radioactivity.

4. Incubation of Carcinogen with Nucleoli

Nucleoli were incubated for three hours in air at 37° C with shaking in a system containing, in a total volume of 20 ml, 0.15 M sucrose, 6.64 mM potassium phosphate, pH 7.40, carcinogen, 100 μM and nucleolar protein, 4-8 mg/ml. After incubation the nucleoli were

collected by centrifugation at 5,000 rpm (3000 X g) for 30 minutes in the Servall SS-34 rotor, and the nucleolar pellets so obtained were extracted with sodium dodecylsulfate and phenol. The nucleic acid extract resulting from this treatment contained DNA and RNA in a ratio of about 1:1 as determined by the diphenylamine and orcinol assays; similar results have been reported by Busch (218).

Part of the nucleic acid extract was eluted directly from a Sephadex column. The remainder was incubated with NaOH to hydrolyze the RNA (procedure described below). The DNA, which is not hydrolyzed by this treatment, was then precipitated, washed, and eluted from a Sephadex column. After direct determination of the amount of carcinogen bound to DNA alone, and to the sum of DNA and RNA in the extract, the amount bound to RNA may be calculated.

5. Incubation of Carcinogen with Microsomes

Microsomes were incubated with carcinogen in a system patterned after that described by Gelboin (156). The same system was used for both homogeneous and heterogeneous microsomes. Designed to support P-450 mediated enzymatic hydroxylation (149, 150, 156), the incubation system contained the following in a final volume of 20-60 ml: 16.6 mM potassium phosphate buffer, pH 7.40, 33.3 mM Na_2EDTA (to inhibit nucleases, see Ref. 156), 0.72 mM Na_4TPNH (reduced triphosphopyridine nucleotide tetrasodium salt, Sigma), 100

μM carcinogen, and 10-25 mg microsomal protein/ml. Incubation was carried out in air with vigorous shaking at 37° C in a 150 ml Erlenmeyer flask for three hours. The reaction was terminated by cooling the incubation flask in ice for 30 minutes, then centrifuging the incubation mixture at 30,000 rpm (105,000 X g) for one hour in the Beckman Type 30 rotor. The microsomal pellets were then put through the nucleic acid extraction procedure. In some experiments the nucleic acid extract was directly eluted from a Sephadex column; in others, the RNA was hydrolyzed with NaOH, and the microsomal DNA was eluted alone. DNA accounted for about 11% of the total nucleic acid in the extract from mixed microsomes.

Several incubations were carried out in the presence of carbon monoxide. Conditions were exactly as described above, except for the following changes. Incubation was conducted in a 150 ml Erlenmeyer suction flask fitted with a stopcock on its sidearm. A snugly fitting serum bottle stopper, inserted into the top of the flask, served as an anaerobic port for the admission of carcinogen. All components of the incubation mixture except carcinogen were placed in the flask, which was then stoppered and kept in an ice bath. The sidearm was connected to a vacuum pump, and evacuated to a pressure of 50 mm Hg. Prepurified nitrogen was then admitted, and the flask was shaken for a minute with nitrogen at atmospheric pressure. After evacuation to 50 mm Hg, nitrogen was again added, with shaking as before. This

operation was repeated five times. Then it was repeated five more times, this time using carbon monoxide instead of nitrogen. Finally carbon monoxide was admitted at atmospheric pressure, and the stopcock was closed. Carcinogen was added via a syringe and needle through the anaerobic port. The incubation flask was then transferred to the Dubnoff shaking incubator, where it was incubated with a flask containing the incubation mixture and open to air. After three hours, both flasks were cooled for 30 minutes in an ice bath, and their contents centrifuged to sediment the microsomal pellets as previously described. The pellet from the mixture incubated under carbon monoxide was bright red, in contrast to the liver-red pellet from the aerobic incubation flask.

6. Incubation of Carcinogen with Free Ribosomes

Free ribosomes were incubated in a simple buffer system with carcinogen. The incubation mixture, which was shaken in air for three hours at 37° C, contained 0.02 M potassium phosphate, pH 7.40, 0.35 M sucrose, 0.035 M $KHCO_3$, 0.025 M KCl, 0.004 M $MgCl_2$, 100 μM carcinogen, and 10 mg ribosomal protein/ml in a total volume of 30 ml. After incubation, the ribosomes were collected by centrifugation for one hour in the Beckman ultracentrifuge at 30,000 rpm (105,000 X g), using the Type 30 rotor. The ribosomal pellets were then extracted with dodecylsulfate and phenol, and the RNA was eluted from

a G-10 Sephadex column.

7. Incubation of Carcinogen with Mitochondria

Mitochondria were incubated with carcinogen in a medium designed to support oxidative phosphorylation continuously; the ATP formed by the mitochondria is immediately consumed by the glucose-hexokinase system present in the medium, which gives ADP and glucose-6-phosphate (227). The incubation mixture contained the following in a volume of 30 ml: 50 mM KCl, 25 mM tris-Cl, 25 mM KH_2PO_4, 5 mM $MgCl_2$, 10 mM succinic acid, 1 mM ATP (Adenosine triphosphate disodium salt, Sigma), 30 mM glucose, 0.25 M sucrose, 100 μM carcinogen, hexokinase (Sigma Type II), 50 units/ml, and mitochondrial protein, 16.7 mg/ml. The pH was adjusted to 7.50. Incubation was carried out in a 150 ml Erlenmeyer flask open to the air, with vigorous shaking at 30° C for three hours. After incubation the mixture was centrifuged in the Servall SS-34 rotor at 12,000 rpm (15,000 X g) for five minutes to sediment the mitochondria. The pellets were put through the nucleic acid extraction procedure, and the nucleic acid solution was then eluted from a G-10 Sephadex column.

It was found that DNA accounts for only 6.5% of the nucleic acid content of mitochondria, the remainder being RNA. Due to the very low nucleic acid content of mitochondria, which would have necessitated using inordinately large amounts of material, the binding of

carcinogen by DNA and RNA separately was not determined.

D. NUCLEIC ACID EXTRACTION AND PURIFICATION PROCEDURES

1. Dodecylsulfate-Phenol Extraction of Nucleic Acids

Nucleic acid was extracted from the various subcellular organelles, after incubation with carcinogen, using a procedure adapted from that described by Shapot and Pitot (228). A pellet containing 500 ml biuret protein was suspended in 5 ml of 0.01 M tris-Cl buffer, pH 7.00, containing 0.5 g sodium dodecylsulfate and preheated to 65° C. This suspension was poured into a preheated (65° C) homogenizer tube, and homogenized for five minutes at 500 rpm, all the while keeping the tube suspended in a 65° C water bath. During homogenization the suspension became quite clear. The high concentration of sodium dodecylsulfate at 65° C was employed to denature lipoproteins, thereby aiding in the release of bound DNA and RNA, and also to inhibit nucleases. After homogenation, 10 ml of tris-Cl buffer was added, and the mixture was cooled in ice.

After cooling for 30 minutes, 15 ml of ice cold tris buffer-saturated phenol was added to the homogenate, which was transferred to rubber-stoppered glass centrifuge tubes. These were shaken on the Vortex mixer at 4° C for five minutes. The milky white emulsion was centrifuged for one hour in the Servall at 15,000 4pm (27,000 X

g), using the SS-34 rotor with the rubber cushion inserts for the glass tubes. The top aqueous phase was aspirated with a syringe and saved; two-thirds volume (total of 10 ml) of tris-Cl buffer was added to each tube, which was then shaken and centrifuged as before. Both aqueous phases were combined. The phenolic phase, containing denatured protein, was discarded.

The aqueous phase, containing DNA, RNA, and some protein in solution, was treated repeatedly with 12 ml of tris buffer-saturated phenol, shaking and centrifuging each time as described above, until no more white emulsion (denatured protein) formed at the interface of the aqueous and phenolic layers. The number of phenol extractions required to achieve this varied with type of particle being treated. Twelve extractions sufficed for ribosomes, but as many as 25 were required to remove the last traces of an apparently very tightly bound protein (possibly histone) from nuclear nucleic acids.

The purified nucleic acid solution, about 20 ml in volume after phenol extraction, was next treated by shaking with five successive 200 ml aliquots of ether to remove phenol. Ether was removed by warming the nucleic acid solution to 40° C and bubbling air through it until the odor of ether could no longer be detected. The final solution gave a negative biuret reaction, and had the appearance of distilled water.

2. Ethanol Precipitation of Nucleic Acid

To the nucleic acid solution resulting from the dodecylsulfate-phenol extraction procedure was added five volumes of absolute ethanol. The solution was stored for eight hours at -15° C, then centrifuged at 16,250 rpm (31,000 X g) for one hour, using the Servall SS-34 rotor. The pure white nucleic acid pellets were washed repeatedly with cold ethanol, dissolved in 0.1 M KCl in 0.01 M tris-Cl buffer, pH 7.00, at 65° C, and five volumes of ethanol was added as before, with cooling and centrifuging. In no instance did the alcoholic supernatant from this second ethanol precipitation contain radioactivity above background. The precipitated nucleic acid was then dissolved in 2 ml of KCl-tris buffer, which was either eluted directly from a Sephadex column, or hydrolyzed with alkali to remove RNA.

3. Separation of DNA by Alkali Treatment

DNA has been found associated with a number of cellular components which also contain RNA, including nuclei (217), nucleoli (218), microsomes (229), and mitochondria (230). When these fractions are extracted by the dodecylsulfate-phenol procedure, the dqueous phase contains both DNA and RNA (229). Elution of the mixed nucleic acids from a G-10 Sephadex column gives no separation of DNA from RNA; any radioactivity associated with the single nucleic acid peak must be interpreted as being bound to the sum total of the nucleic

acid in the solution. Yet it cannot be assumed that binding by either DNA or RNA is proportional to its content in the nucleic acid extract. It is possible that a small proportion of one nucleic acid could be responsible for most or even all of the binding by carcinogen. For this reason, it was necessary to separate the nucleic acids. This was done by alkali treatment, which hydrolyzes the RNA while leaving the DNA intact. The DNA was then eluted separately from a Sephadex column. By knowing the rate of binding of carcinogen to the sum of DNA and RNA, and to DNA alone, and also the ratio of DNA to RNA in the original extract, the binding rate of carcinogen to RNA may be calculated by difference.

Hydrolysis was conducted according to the procedure of Schneider and Kuff (229). The nucleic acid extract was made 0.3 M in NaOH, and then incubated at 37° C for one hour. After cooling in ice for 30 minutes, solid trichloroacetic acid was added to the solution to give a final concentration of 10% free acid. Upon standing for one hour at 0° C, fine threads of DNA appeared in the solution. These were collected by centrifugation in the Servall for one hour at 16,250 rpm (31,000 X g), using the SS-34 rotor. The DNA pellet was repeatedly washed with 10% trichloroacetic acid, then dissolved in 0.1 M KCl in 0.01 M tris-Cl buffer, pH 7.00, for elution from Sephadex. RNA was not detectable in the DNA solution by the orcinol assay.

E. SEPHADEX CHROMATOGRAPHY

Binding of carcinogen to nucleic acid was ascertained by demonstration of coincidence of the radioactivity and nucleic acid profiles after elution of the nucleic acid solution from a G-10 Sephadex column. G-10 Sephadex was chosen because of its very low molecular exclusion (any molecule over 700 daltons), which permits easy separation of any unbound carcinogen from nucleic acid, yet allows nucleic acid molecules of varying sizes to elute as a single peak. Since aromatic compounds are strongly adsorbed to Sephadex, and retained to a greater degree than would be expected from their molecular size (231) the free carcinogen molecules are retarded on the column by virtue of their low molecular weight and their aromatic character. The large nucleic acid molecules are excluded from the Sephadex, and pass through the column unretarded.

When the nucleic acid solutions were extensively purified by detergent-phenol treatment or repeated ethanol precipitations as previously described, the nucleic acid and radioactivity profiles were exactly coincident in every instance. Prolonged elution with buffer failed to reveal any free carcinogen in the sample. However, when the nucleic acid-carcinogen incubation mixtures were eluted directly without purification (to demonstrate physical binding), the nucleic acid-associated radioactivity peak was retarded one or two fractions from the nucleic acid peak. This could result from dissociation of

weakly-associated carcinogen-nucleic acid complexes during the process of elution. The free dissociated carcinogen molecules would first travel rapidly down the column with the large nucleic acid molecules as "passengers," then become dislodged from the nucleic acid partway down the column, to travel more slowly and elute slightly after the nucleic acid peak. Prolonged elution in this latter instance always revealed a very broad free-carcinogen peak which, in contradistinction to the nucleic acid-associated radioactivity peak, was separated from the nucleic acid peak by 60-80 fractions.

Sephadex chromatography was carried out according to the following procedure. Twenty grams of G-10 Sephadex (Pharmacia Fine Chemicals) was allowed to swell in 500 ml of elution buffer (0.1 M KCl in 0.01 M tris-Cl, pH 7.00) for 24 hours. The suspension was swirled periodically, allowed to settle, then the fines were poured off and more buffer was added. The Sephadex was next poured into a cylindrical glass chromatography column, which was 2.3 cm in diameter and 40 cm high. The final gel height was 12 cm, with a bed volume of 49.80 ml. A piece of filter paper was placed on top of the gel bed to prevent disturbance during elution. A 2 ml sample of nucleic acid was then introduced onto the column and eluted from it with elution buffer. Forty fractions of 20 drops each (1.5-2.0 ml) were collected in small test tubes. The elution rate was approximately 0.75 ml/minute.

Each fraction was assayed for absorbance at 260 mμ in the Zeiss spectrophotometer, using the 1 cm quartz cuvettes. In those instances when a fraction had an absorbance greater than 2.00, appropriate dilutions were made, and the final absorbance was obtained by multiplication. It was found that absorbance and nucleic acid content vary linearly for samples with absorbances under 40 optical density units.

F. SCINTILLATION PROCEDURES

After absorbance measurements, 1 ml of each fraction from the Sephadex column was pipeted into a liquid scintillation counting vial containing 10 ml of Bray's solution (see Ref. 232). The vials were kept in the dark for 24 hours at -8° C prior to counting, which was conducted in a Packard Model 3320 Tri-Carb liquid scintillation spectrometer equipped with an automatic changer. The discriminators were set to 50 and 1000 volts, with a gain of 20.0%. Background was determined using a vial containing only buffer and Bray's solution. Efficiency measurements were made during each run, using a known amount of labeled carcinogen in buffer and Bray's solution as a standard; quenching by nucleic acid can be determined in a similar manner, using a system containing the same components plus a known amount of either DNA or RNA. Overall efficiencies were always greater than 90%. Counting time per sample was ten minutes. Each sample was counted at least three times.

G. CALCULATION OF BINDING RATIOS FROM RAW DATA

A complete calculation of the nucleic acid:carcinogen binding ratio from raw data will now be described, using the results of Experiment 8 as an illustrative example (see Figure 3). The principle of this calculation simply involves the determination of the number of moles of nucleic acid nucleotide per ml and the number of moles of carcinogen per ml of the peak fraction from the Sephadex column. The two numbers are then expressed as a ratio (e.g., 40,000:1 implies that one carcinogen molecule is bound per 40,000 nucleic acid nucleotides).

Since nucleic acids contain one phosphorus atom per nucleotide, the absorbance of a nucleic acid solution at 260 mμ can be related to phosphorus concentration, which is determined empirically by chemical methods (233). A molar absorptivity, $\epsilon(P)$, for nucleic acid, based on one gram-atom of phosphorus per liter, can then be derived (233):

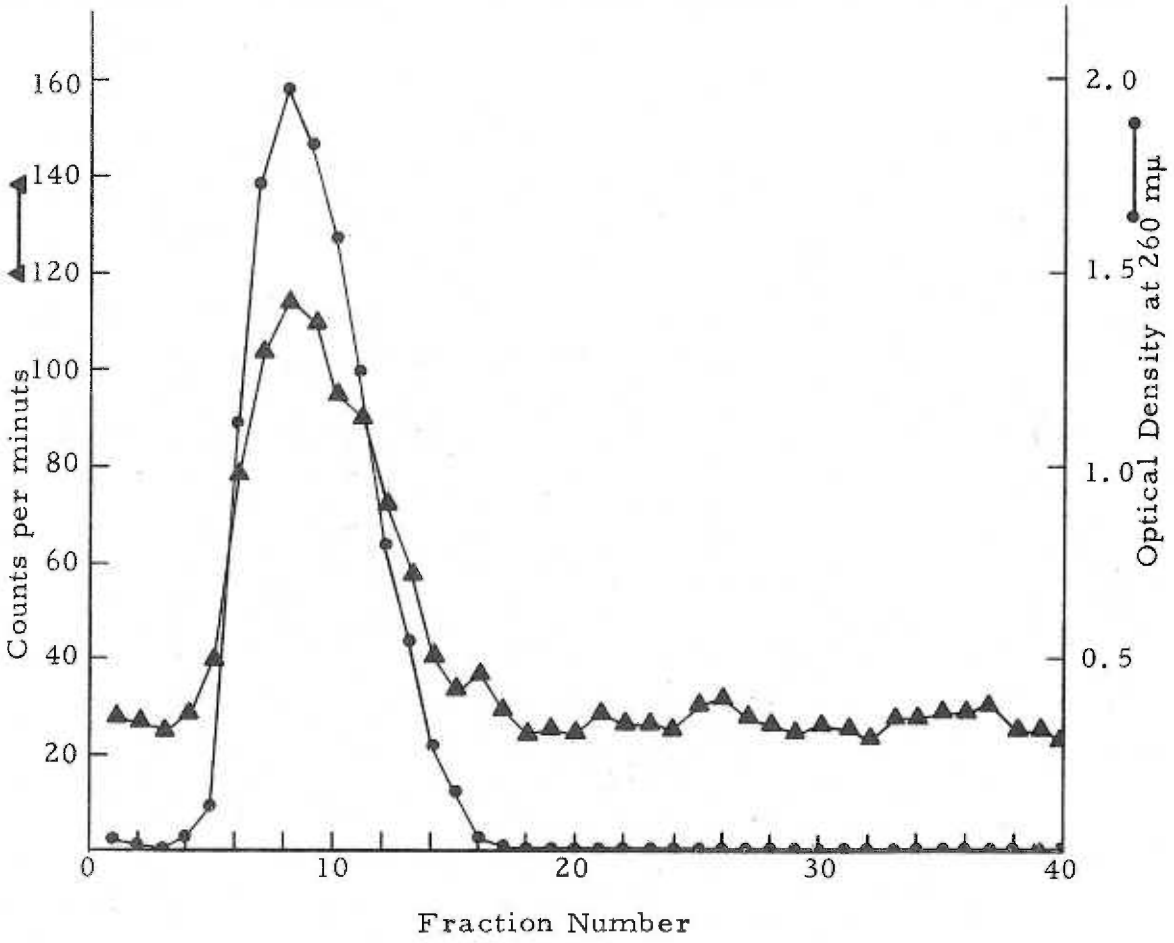
$$\epsilon(P) = \frac{A}{C \cdot d}$$

A is the absorbance of the nucleic acid solution at 260 mμ, C is the concentration of phosphorus expressed in gram-atoms of phosphorus per liter, and d is light path measured in cm. $\epsilon(P)$ is taken as 6600 for DNA (234), and 8500 for RNA (235).

FIGURE 3

ELUTION OF DNA FROM A G-10
SEPHADEX COLUMN

DNA was eluted from a G-10 Sephadex column following incubation of purified rat liver DNA with radiolabeled N-OHAAF, and repeated detergent treatment and ethanol precipitation of the nucleic acid to remove physically bound carcinogen. Details of the procedures are found in the text. This figure depicts the results of Experiment 8 (see next Section), and is used to illustrate the calculation of the nucleic acid:carcinogen binding ratio. The complete calculation is given in the text.


160
140
120
100
80
60
40
20
Counts per minuts
2.0
1.5
1.0
0.5
Optical Density at 260 mμ
0
10
20
30
40
Fraction Number

The equation above may be rearranged to the following form:

$$C = \frac{A}{\epsilon (P) \cdot d}$$

Fraction 8 represents the peak for Experiment 8. The absorbance of this fraction, using 1 cm quartz cuvettes, was 1.93 at 260 mμ. Thus $C = 1.93/(6600) \cdot (1) = 2.92 \times 10^{-4}$ M or 2.92×10^{-7} gramatoms P/ml. Since nucleic acids contain one phosphorus atom per nucleotide, this represents 2.92×10^{-7} moles of DNA nucleotide/ml.

The gross radioactivity measured from 1 ml of Fraction 8 was 114 counts per minute. Background during this run was 30 counts per minute. Overall counting efficiency was 92%. The net radioactivity of the sample is therefore $(114 - 30) \times (100/92) = 91$ disintegrations per minute. The specific activity of the radiolabeled N-OHAAF is 12.73 curies/mole. A curie is, by definition, 222×10^{10} disintegrations per minute (236). Thus,

$$\frac{91 \text{ dpm}}{1} \times \frac{1 \text{ curie}}{222 \times 10^{10} \text{ dpm}} \times \frac{1 \text{ mole N-OHAAF}}{12.73 \text{ curies}} = 3.22 \times 10^{-12} \text{ moles N-OHAAF/ml.}$$

Both concentrations are then expressed as a molar ratio:

$$\frac{\text{DNA}}{\text{N-OHAAF}} = \frac{2.92 \times 10^{-7}}{3.22 \times 10^{-12}} = 9.07 \times 10^{4},$$

or one N-OHAAF molecule bound per 91,000 DNA nucleotides. Note that the greater the numerical value of the molar ratio, the less is the degree of binding of the carcinogen to nucleic acid.

RESULTS

A. PHYSICAL BINDING OF CARCINOGEN TO NUCLEIC ACIDS

The results of the experiments demonstrating nonenzymatic physical binding of AAF and N-OHAAF to purified nucleic acids are given in Table 2. The binding ratios represent mainly physical binding, but in actuality include the sum of both physical and covalent binding. It is seen that for a given nucleic acid, the degree of physical binding is approximately the same with either AAF or N-OHAAF. Both DNA and RNA from ribonucleoprotein bind carcinogen to roughly the same degree. Transfer RNA, however, is about three times as reactive to physical binding by the carcinogens when compared to DNA or insoluble RNA.

TABLE 2

DEMONSTRATION OF NONENZYMATIC PHYSICAL BINDING OF AAF AND N-OHAAF TO PURIFIED NUCLEIC ACIDS

Purified nucleic acids were incubated with carcinogen under nitrogen as described in the text, and the incubation mixtures were eluted directly from Sephadex. The binding ratios for these experiments actually represent the total binding (i. e., physical plus covalent) of carcinogen by nucleic acid.

Experiment	Material Incubated	Carcinogen	Peak OD_{260} (OD units)	Peak Net Radioactivity (dpm)	Binding Ratio (nucleotides / carcinogen)
1	DNA	AAF	2.79	968	11,000
2	DNA	N-OHAAF	3.40	1830	7,900
3	RNA	AAF	3.92	1130	11,000
4	RNA	N-OHAAF	5.49	1500	12,000
5	tRNA	AAF	0.95	914	3,200
6	tRNA	N-OHAAF	1.53	1100	4,600

B. COVALENT BINDING OF CARCINOGEN TO NUCLEIC ACIDS

1. Purified Nucleic Acids

The results of the experiments demonstrating nonenzymatic, presumably covalent binding of AAF and N-OHAAF to purified nucleic acids are given in Table 3. For any given nucleic acid, AAF is less reactive than its hydroxylated metabolite by a factor of 2-5 times, a significant difference from the physical binding results. DNA is less reactive than insoluble RNA to both carcinogens by a factor of 2-4 times. Transfer RNA is, as in the case of physical binding, the most reactive substance tested; it is 16 times as reactive as DNA and 4 times as reactive as insoluble RNA when incubated with AAF, and 8 times as reactive as DNA and 4 times as reactive as insoluble RNA when incubated with N-OHAAF.

As can be determined by comparing the binding ratios from this section with those of the previous section, covalent binding accounts for only about 10% of the total binding in most instances. The one striking exception is N-OHAAF binding by tRNA, where covalent binding accounts for over 40% of the total binding.

TABLE 3

DEMONSTRATION OF NONENZYMATIC COVALENT BINDING OF AAF AND N-OHAAF TO PURIFIED NUCLEIC ACIDS

Purified nucleic acids were incubated with carcinogen under nitrogen as described in the text. After extensive hot detergent treatment and ethanol precipitation, the nucleic acids were eluted from Sephadex. The binding ratios for these experiments represent (presumably) covalent binding of carcinogen by nucleic acid.

Experiment	Material Incubated	Carcinogen	Peak OD_{260} (OD units)	Peak Net Radioactivity (dpm)	Binding Ratio (nucleotides / carcinogen)
7	DNA	AAF	2.05	19	410,000
8	DNA	N-OHAAF	1.93	91	91,000
9	RNA	AAF	6.05	186	100,000
10	RNA	N-OHAAF	4.20	334	41,000
11	tRNA	AAF	4.74	587	25,000
12	tRNA	N-OHAAF	4.75	1440	11,000

2. Nuclei and Nucleoli

The results of the experiments demonstrating presumably covalent binding of AAF and N-OHAAF to nuclear and nucleolar nucleic acids are given in Table 4. While AAF is less reactive than N-OHAAF to nuclear nucleic acid by a factor of 16 times, it appears to have the same reactivity as N-OHAAF to nucleolar nucleic acid (see Experiments 15, 16, and 17a). Nucleoli are more reactive than nuclei to AAF by a factor of 91 times, and to N-OHAAF by a factor of 6 times. When nucleoli are incubated with N-OHAAF and the binding ratios for total nucleic acid, DNA, and RNA are determined separately, it is found that nucleolar DNA is 11 times less reactive to carcinogen than nucleolar RNA. Thus, while DNA accounts for nearly 50% of the nucleolar nucleic acid, the N-OHAAF bound nucleolar DNA represents only 8.26% of the total N-OHAAF bound nucleolar nucleic acid (see Table 9).

Nucleolar DNA is only about half as reactive to covalent binding by N-OHAAF when compared to free purified DNA. However, nucleolar RNA is about twice as reactive as the free RNA prepared from ribonucleoprotein.

TABLE 4

DEMONSTRATION OF COVALENT BINDING OF AAF AND N-OHAAF TO NUCLEAR AND NUCLEOLAR NUCLEIC ACIDS

Nuclei and nucleoli were incubated with carcinogen in air as described in the text. After extensive dodecylsulfate-phenol extraction and ethanol precipitation, the nucleic acids were eluted from Sephadex. In Experiment 17, part of the nucleic acid solution was hydrolyzed with alkali to remove RNA, and the DNA was eluted separately. The binding ratios for these experiments represent (presumably) covalent binding of carcinogen by nucleic acid.

Experiment	Material Incubated	Carcinogen	Nucleic Acid	Peak OD_{260} (OD units)	Peak Net Radioactivity (dpm)	Binding Ratio (nucleotides / carcinogen)
13	Nuclei	AAF	Total	10.3	13	3,000,000
14	Nuclei	N-OHAAF	Total	13.7	315	190,000
15	Nucleoli	AAF	Total	5.88	624	33,000
16	Nucleoli	N-OHAAF	Total	4.51	572	30,000
17a	Nucleoli	N-OHAAF	Total	8.28	856	37,000
17b	Nucleoli	N-OHAAF	DNA	2.75	54	220,000
17c	Nucleoli	N-OHAAF	RNA	*	*	20,000

*calculated indirectly

3. Microsomes

The results of the experiments demonstrating presumably covalent binding of AAF and N-OHAAF to microsomal nucleic acids are given in Tables 5 and 6. AAF is about 25 times less reactive to covalent binding by total microsomal nucleic acids than its hydroxylated derivative. When the mixed microsomal nucleic acids are separated and the binding ratios determined separately, DNA is found to be 8.2 times less reactive than RNA. While DNA accounts for about 11% of the total mixed microsomal nucleic acid, N-OHAAF bound DNA represents only 1.50% of the total N-OHAAF bound nucleic acid (see Table 9). Note that whenever microsomes are incubated with N-OHAAF, the binding ratio for either the total nucleic acid or RNA (which comprises almost 90% of the total mixed microsomal nucleic acid) always centers around 40,000. Both homogeneous rough and homogeneous smooth microsomes give approximately the same ratio as mixed microsomes. This ratio of about 40,000 is identical to the covalent binding ratio observed when N-OHAAF is incubated in a nonenzymatic system with RNA prepared from ribonucleoprotein (Table 3, Experiment 10).

It is noteworthy that carbon monoxide failed to inhibit significantly the covalent binding of both AAF and N-OHAAF to microsomal nucleic acids (Table 6). The possible consequences of these findings will be discussed later.

TABLE 5

DEMONSTRATION OF COVALENT BINDING OF AAF AND N-OHAAF TO MICROSOMAL NUCLEIC ACIDS

Microsomes were incubated with carcinogen and TPNH in air as described in the text. After extensive dodecylsulfate-phenol extraction and ethanol precipitation, the nucleic acids were eluted from Sephadex. In Experiment 20, part of the nucleic acid solution was hydrolyzed with alkali to remove RNA, and the DNA was eluted separately. The binding ratios for these experiments represent (presumably) covalent binding of carcinogen by nucleic acid.

Experiment	Material Incubated	Carcinogen	Nucleic Acid	Peak OD_{260} (OD units)	Peak Net Radioactivity (dpm)	Binding Ratio (nucleotides / carcinogen)
18	Mixed Microsomes	AAF	Total	8.83	21	1,300,000
19	Mixed Microsomes	N-OHAAF	Total	5.20	320	53,000
20a	Mixed Microsomes	N-OHAAF	Total	2.60	196	43,000
20b	Mixed Microsomes	N-OHAAF	DNA	1.70	23	320,000
20c	Mixed Microsomes	N-OHAAF	RNA	*	*	39,000
21	Rough Microsomes	N-OHAAF	Total	4.29	374	38,000
22	Smooth Microsomes	N-OHAAF	Total	17.7	1430	41,000

*calculated indirectly

TABLE 6

DEMONSTRATION OF THE FAILURE OF CARBON MONOXIDE TO INHIBIT COVALENT BINDING OF AAF AND N-OHAAF TO MICROSOMAL NUCLEIC ACIDS

Microsomes were incubated with carcinogen and TPNH in either air or carbon monoxide as described in the text. After extensive dodecylsulfate-phenol extraction and ethanol precipitation, the nucleic acids were eluted from Sephadex. The binding ratios for these experiments were calculated on the basis of total microsomal nucleic acid, and represent (presumably) covalent binding of carcinogen by nucleic acid. Binding is not inhibited to a significant degree by carbon monoxide.

Experiment	Material Incubated	Carcinogen	Incubated Under	Peak OD_{260} (OD units)	Peak Net Radioactivity (dpm)	Binding Ratio (nucleotides / carcinogen)
23	Mixed Microsomes	AAF	Air	23.2	86	830,000
24	Mixed Microsomes	AAF	Carbon Monoxide	15.2	51	920,000
25	Mixed Microsomes	N-OHAAF	Air	21.5	1620	44,000
26	Mixed Microsomes	N-OHAAF	Carbon Monoxide	17.6	1150	50,000

4. Ribosomes and Mitochondria

The results of the experiments demonstrating presumably covalent binding of AAF and N-OHAAF to ribosomal RNA and mitochondrial nucleic acids are given in Table 7. Both ribosomal RNA and mitochondrial nucleic acid (6.5% DNA and 93.5% RNA, see Table 8) are quite unreactive to covalent binding by either AAF or N-OHAAF. Since free ribosomes bind N-OHAAF to such a low degree, most of the N-OHAAF bound to mixed microsomal nucleic acid is apparently associated with the membrane bound (possibly messenger) RNA. Microsomal DNA is very unreactive to N-OHAAF, as noted previously.

TABLE 7

DEMONSTRATION OF COVALENT BINDING OF AAF AND N-OHAAF TO RIBOSOMAL AND MITOCHONDRIAL NUCLEIC ACIDS

Ribosomes and mitochondria were incubated with carcinogen in air as described in the text. After extensive dodecylsulfate-phenol extraction and ethanol precipitation, the nucleic acids were eluted from Sephadex. The binding ratios for these experiments represent (presumably) covalent binding of carcinogen by nucleic acid.

Experiment	Material Incubated	Carcinogen	Nucleic Acid	Peak OD_{260} (OD units)	Peak Net Radioactivity (dpm)	Binding Ratio (nucleotides / carcinogen)
27	Free Ribosomes	AAF	RNA	2.83	2.3	3,800,000
28	Free Ribosomes	N-OHAAF	RNA	3.02	7.2	1,400,000
29	Mitochondria	AAF	Total	3.06	11	860,000
30	Mitochondria	N-OHAAF	Total	2.65	8.4	1,000,000

TABLE 8

COMPOSITION OF SEVERAL NUCLEIC ACID EXTRACTS OBTAINED BY THE DODECYLSULFATE-PHENOL EXTRACTION PROCEDURE

TABLE 9

N-OHAAF BINDING BY DNA EXPRESSED AS A PERCENTAGE OF THE TOTAL N-OHAAF BOUND NUCLEIC ACIDS FROM NUCLEOLI AND MICROSOMES

Nucleic Acid	%DNA	%RNA	DNA:RNA Ratio
Nuclear	79.50	20.50	3.871
Nucleolar	49.11	50.89	0.965
Mixed Microsomal	11.18	88.82	0.126
Mitochondrial	6.50	93.50	0.069

Nucleic Acid	%DNA	N-OHAAF Bound DNA as a % of Total N-OHAAF Bound Nucleic Acid
Nucleolar	49.11	8.26
Mixed Microsomal	11.18	1.50

DISCUSSION

It is evident from the results presented in Tables 2 and 3 that nonenzymatic physical binding of both AAF and N-OHAAF to purified nucleic acids does occur. In every instance the degree of physical interaction is greater than the degree of presumably covalent interaction, by a factor of from 1.4 to 37 times. Physical binding ratios for N-OHAAF are not significantly different from the corresponding ratios observed for AAF. The structure of the side chain probably plays a minor role in the physical interaction of the fluorenamine carcinogens with nucleic acids; most of the effect is likely due to the fluorene nucleus. As long ago as 1946, Weil-Malherbe demonstrated solubilization of polycyclic aromatic hydrocarbons by purines (237). Other investigators have confirmed the weak physical interaction of the polycyclic hydrocarbons with DNA, RNA, and nucleic acid bases (238-240). Such interactions may result from hydrophobic or surface adsorption effects. It is also possible that polycyclic hydrocarbons become intercalated within the nucleic acid coil (240). Intercalation models for the binding of hydrocarbons to DNA involve the postulate of hydrocarbon-induced local uncoiling of the DNA double helix. Such uncoiling has recently been demonstrated to result in the removal and reversal of the supercoils of closed circular DNA (241).

The incubation of purified nucleic acids with AAF and N-OHAAF in a nonenzymatic system at physiological pH also results in a small

but definitely measurable degree of strong, presumably covalent binding (Table 3). The rather drastic purification procedures employed to remove physically bound carcinogen from nucleic acid support the conclusion that any remaining carcinogen is covalently bonded to the nucleic acid. It must be emphasized that such binding is presumably covalent; in no instance has covalent binding been conclusively demonstrated in these experiments. It is significant that, with respect to this nonenzymatic, presumably covalent binding, the proximate carcinogen N-OHAAF is 2-5 times more reactive to a given purified nucleic acid than the remote carcinogen AAF. This finding supports the concept that AAF interacts with cellular molecules via its N-hydroxylated metabolite, either directly to a low degree, or by ester formation to a much higher degree (14, 162).

When the various subcellular fractions are incubated with AAF and N-OHAAF, presumably covalent binding of both carcinogens to the nucleic acids associated with these fractions is observed in every instance (Tables 4-7). N-OHAAF is significantly more reactive than AAF in all but two instances. Mitochondrial nucleic acid is decidedly unreactive to both AAF and N-OHAAF; binding by AAF is slightly higher, but not significantly so. Nucleolar nucleic acid reacts to a high degree about equally with both AAF and N-OHAAF. No organelle shows such high reactivity of its nucleic acid to AAF as the nucleolus. This raises the question of the occurrence of N-hydroxylating

enzymes for AAF within the nucleolus.

Marked differences are observed when the various subcellular fractions are incubated with N-OHAAF, and the binding ratios for their associated nucleic acids are compared. Ribosomal RNA reacts with N-OHAAF to an extent barely detectable, and mitochondrial nucleic acid is just slightly more reactive than ribosomal RNA. In both cases, the reactivity is of the order of 25-35 times less than that observed for purified RNA in a nonenzymatic test system. Nuclear nucleic acid is 5-7 times more reactive than mitochondrial and ribosomal nucleic acid, but only about one-fifth as reactive as microsomal nucleic acid; this probably reflects the large proportion of the less reactive DNA in the nuclear nucleic acid. Microsomal RNA shows a covalent binding ratio approximately identical to that found for purified RNA in a nonenzymatic system; and nucleolar RNA is about twice as reactive to presumably covalent binding by N-OHAAF as purified RNA.

Incubation of microsomes with N-OHAAF results in a binding ratio for microsomal nucleic acid which invariably centers around 40,000 (range = 38,000-53,000, see Tables 5 and 6). With AAF, however, binding is approximately 30 times less, despite the fact that the incubation system is designed to support P-450 mediated enzymatic hydroxylation. Incubation of microsomes with both AAF and N-OHAAF separately in the presence of pure carbon monoxide

instead of air has no significant effect on the binding of either compound to microsomal nucleic acid (see Table 6). These results may be interpreted in three ways: (a) N-Hydroxylation of AAF does not occur to a significant degree in the system employed; (b) N-Hydroxylation of AAF does occur, but the N-OHAAF formed is immediately metabolized to inactive compounds by the microsomes, so that a significant concentration of N-OHAAF is never available to react with microsomal nucleic acid. Irving (166, 178) has shown that microsomes do in fact metabolize enzymatically-generated N-OHAAF, which rapidly disappears from the incubation system at nearly the same rate as it is formed; (c) Microsomal P-450 is not involved in the N-hydroxylation of AAF. Hlavica and Kiese have shown that N-hydroxylation of N-ethylaniline by rabbit liver microsomes is not inhibited by carbon monoxide. They also found that the N-hydroxylating system has a very low affinity for oxygen, and is not inducible by phenobarbital (242, 243). Such results imply that a system other than the cytochrome P-450 mixed-function oxidase system is involved in the N-hydroxylation of N-ethylaniline.

When compared with DNA for presumably covalent binding to carcinogen, RNA is found to be more reactive in every instance, especially with N-OHAAF. This greater reactivity of RNA is observed both for purified nucleic acids and for membrane associated nucleic acids. Several explanations may account for the observed

difference. RNA associates with itself to a lesser degree than does DNA, and hence more reactive sites may be open to attack by carcinogen in RNA. Also, for a given number of nucleotides, there are more molecules of RNA available for reaction than molecules of the larger DNA. Transfer RNA is more reactive than any other nucleic acid tested. This may be due to its very low molecular weight, which allows many more molecules for reaction per given number of nucleic acid nucleotides than exist for DNA or insoluble RNA. Transfer RNA also contains several minor bases which may be more reactive to attack by carcinogen than the regular RNA bases.

One question which may be raised with an in vitro study of the reactivity of tissue components with chemical carcinogens is whether any binding observed actually reflects the in vivo situation in the host tissue. A serious limitation in this regard, but one which is inescapable in a study of this type, is the possibility of an extraneous reaction occurring during the rather drastic conditions employed to extract nucleic acid from tissue. Another limitation involves the intracellular localization of both carcinogen and the enzyme system(s) which might activate the carcinogen. Thus, ribosomal RNA is found to be very unreactive when free ribosomes are incubated with AAF and N-OHAAF. Yet intracellular generation of N-OHAAF at the surface of the endoplasmic reticulum, in the presence of the soluble sulfotransferase system, could result in a local concentration of the

highly reactive AAF-N-sulfate in the immediate proximity of ribosomes bound to the endoplasmic reticulum. This might result in a high degree of binding by ribosomal RNA when AAF is administered in vivo. Similarly, nucleolar RNA is quite reactive with both AAF and N-OHAAF in vitro. But neither of these compounds may ever reach the nucleolus when administered in vivo; enzymatic conversion to highly reactive metabolites in the cytoplasm may result in the binding of all of the carcinogen to cytoplasmic constituents. A third consideration involves the distribution of carcinogen in the cell. The large hydrophobic aromatic ring system of the fluorenamine carcinogens would tend to favor their distribution in those organelles having a high lipid content, such as microsomes and mitochondria, over those organelles having a low lipid content, such as ribosomes and nuclei.

My findings lend strongest support to those theories which explain carcinogenesis by a mutation, either of the nuclear genome, or of an extrachromosomal cytoplasmic gene, for example, of the type proposed by Pitot (124). From the results presented in this thesis, it is evident that the thesis question, "Where might the proximate carcinogen act?", can only be answered for the fluorenamine carcinogens in terms of probability, since they react with a wide variety of information-handling molecules. Both AAF and N-OHAAF react by presumably covalent binding with the nucleic acids

of all subcellular fractions tested. The fact that N-OHAAF shows greater reactivity is consistent with the generally-accepted belief that this compound is the proximate carcinogenic metabolite of AAF (14, 162). Presumably AAF-N-sulfate, the ultimate carcinogenic metabolite proposed by Miller (14), would react to an even greater degree. Although there are wide differences observed in the binding ratios for N-OHAAF and the nucleic acids associated with the various subcellular fractions tested, it cannot be inferred that any one reaction is more important in carcinogenesis than another on the basis of a greater degree of binding. A critical "hit" of one N-OHAAF molecule on the oncogenic locus of DNA might initiate malignant transformation, whereas a hundred sites bound on RNA or other DNA loci may be without carcinogenic effect. Even the weak physical interaction of the fluorenamines with nucleic acids may be important in the induction of cancer. Though not the subject of this study, DeBaun, Miller, and Miller (204) have clearly demonstrated covalent binding of N-OHAAF to rat liver protein--still another reaction with an information-handling molecule that may be critical in the initiation or promotion stages of carcinogenesis.

It seems probable that the proximate and ultimate carcinogenic metabolites of AAF are chemical reactants which have a rather low degree of reaction specificity for intracellular information-handling molecules. Hopefully, future work in the molecular biology of cancer

will determine the location of the specific molecular mutations or alterations which confer malignant characteristics on the cancer cell, and disclose those reactions with cellular components which are critical in the induction of cancer by the fluorenamines.

SUMMARY AND CONCLUSIONS

The remote carcinogen 2-acetylaminofluorene (AAF) and its N-hydroxylated metabolite (N-OHAAF) were incubated with purified nucleic acids and a variety of subcellular organelles in vitro. Physical and presumably covalent binding of both AAF and N-OHAAF to purified nucleic acids was demonstrated in a nonenzymatic test system. When the carcinogens were incubated with the various subcellular fractions in vitro, presumably covalent binding of both AAF and N-OHAAF to the nucleic acids associated with each of the subcellular fractions was demonstrated in every case. Regarding the presumably covalent binding, DNA was found to be less reactive than RNA; purified tRNA was the most reactive nucleic acid tested. In general, N-OHAAF displayed a significantly greater degree of covalent binding to any given nucleic acid than AAF. The one notable exception was nucleolar nucleic acid, where binding of AAF and N-OHAAF occurred to approximately the same extent. This result raises the possibility of the presence of an N-hydroxylase for AAF in the nucleolus. The limited degree of presumably covalent AAF binding observed to microsomal nucleic acid, and the failure of carbon monoxide to inhibit this binding, may mean that either AAF was not hydroxylated in the system employed, or that any N-OHAAF formed was removed from the system by further metabolism to inactive compounds, or that the P-450 mixed-function oxidase system is not involved in the

N-hydroxylation of AAF.

The results of this study support the conclusion that N-OHAAF is the proximate carcinogenic metabolite of AAF. It is evident that the proximate carcinogen can react with a wide variety of intracellular information-handling molecules, but it is not possible to determine from this study which of these reactions is critical in carcinogenesis. Further investigative effort in the molecular basis of neoplasia may one day determine the location of the specific molecular mutations or alterations which confer malignant properties on the cell. Until then, the central question of research in chemical carcinogenesis will remain unanswered.

ADDENDUM

The binding results presented in this thesis are subject to a limitation imposed by the radiochemical purity of the carcinogens employed. The radiochemical purities of both carcinogens were specified by Tracerlab as greater than 98%. However, in all experiments the amount of radiolabeled carcinogen present in the incubation mixture was of the order of 10^4 to 10^6 times the amount of radioactivity bound to nucleic acid. It is conceivable that a radioactive impurity present in the proportion of one part per 100 to 10,000 parts of carcinogen could account for most or even all of the nucleic acid-associated radioactivity observed in the experiments. Thus, the experimental procedure employed places a rather stringent requirement on the radiochemical purity of the carcinogens tested, and the results presented herein must be interpreted with this rather significant limitation in mind.

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