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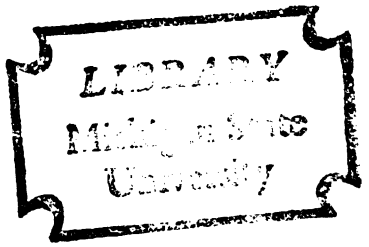

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MOLECULAR CORRELATES OF 2-ACETYLAMINOFLUORENE CARCINOGENICITY

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By

Edward Lee Schwartz

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ABSTRACT

Molecular Correlates of 2-Acetylaminofluorene Carcinogenicity

by

Edward Lee Schwartz

It has long been speculated that perturbations of the genetic material are causally related to the initiation of neoplastic changes. The purpose of this investigation was to examine the nature of the covalent binding of the hepatic carcinogen 2-acetylaminofluorene (AAF) to hepatic DNA. The following parameters were evaluated: the localization of carcinogen binding within the hepatic genome, the effect of continued carcinogen ingestion on further carcinogen binding, and the functional consequences of carcinogen-induced DNA damage on DNA transcriptional capacities.

Ingestion of AAF (0.05% w/w) for 1 or 2 weeks resulted in an apparent reduction in capacity to metabolically activate the carcinogen. An 85% decrease was observed after 2 weeks when rats were tested with [^{14}C]AAF, but only a 60% reduction occurred after injection of [^{3}H]N-hydroxy-AAF, demonstrating a reduction in both activation steps in the formation of an ultimate carcinogen from AAF.

Analysis of the initial binding of N-OH-AAF to DNA indicated that the adducts were nonrandomly distributed 2 hr after injection. Approximately 85% of the bound carcinogen was located on less than 25%

of the total nuclear DNA. The distribution was analyzed by fractionating chromatin into eu- and heterochromatin regions. Chromatin was fragmented by sonication or DNAse II digestion, and fractionated by glycerol gradient centrifugation or selective $MgCl_2$ precipitation. Initially, more carcinogen was bound to the less dense chromatin-DNA regions. Binding to DNA was also greater on expressed regions of the genome, the in vivo template-expressed DNA having 16 times the carcinogen modification of the template repressed DNA.

Rate of carcinogen loss from DNA was also not uniform among the chromatin fractions. Loss from the highly condensed pelleted heterochromatin was significantly slower than that of the less condensed chromatin regions. This may partially reflect the greater proportion of carcinogen binding to the N^2 position of guanine as compared to the C8 position of guanine on the DNA of this fraction. Carcinogen-guanine adducts were analyzed by thin layer chromatography.

The nonrandom nature of AAF-DNA interactions was also reflected in an apparent clustering of the 2 different carcinogen-guanine adducts within the genome. Fragments of DNA containing over 90% of the carcinogen at the C8 position or over 40% of the carcinogen at the N^2 position could be isolated. Furthermore, there was an inverse correlation between the extent of carcinogen modification of the DNA of a particular chromatin fraction, and the percent of that modification occurring at the N^2 position of guanine.

A "cooperative base displacement" model has been proposed as a potential explanation of the clustering effect observed. Binding of the carcinogen to the C8 position of guanine has been demonstrated to alter the double stranded conformation of the DNA molecule and cause

local regions of denaturation. These regions are more susceptible to further carcinogen attack at the C8 position, but are relatively resistant to attack at the N^2 position. A reduction in the amount of newly bound carcinogen located at the N^2 position compared to the C8 position after 2 weeks of AAF ingestion provided further support for this model.

Structural alterations of DNA and potential functional template damage was also suggested by studies of the transcription of DNA isolated from rats fed AAF (0.03%). E. coli RNA polymerase was used to transcribe the DNA under conditions in which re-initiation of RNA synthesis by the enzyme was blocked. A reduction of 40-50% in template capacity was observed after 4 days of AAF ingestion. This reduction was due to premature termination of RNA synthesis without a change in RNA synthesis initiation. Premature termination was observed on DNA purified from eu- and heterochromatin fractions obtained by sonication and glycerol gradient centrifugation. No reduction occurred on DNA of the pelleted heterochromatin fraction. This template did show a reduction in the rate of polymerization of adenosine and uridine nucleotides into the growing RNA chain. All parameters returned to control values when animals were fed a basal diet for 7 days after 4 days of AAF ingestion.

The major finding of this study was that steric factors, including the conformation of DNA in chromatin, influence the initial binding of carcinogen to DNA as well as the subsequent binding that occurs with continued carcinogen exposure. Interaction of carcinogen with DNA in vivo is neither a random nor a static phenomenon. With constant carcinogen exposure, aspects of metabolic activation, DNA binding, and possibly repair are subject to continued alterations.

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LIST OF ABBREVIATIONS

AAF	2-acetylaminofluorene
N-OH-AAF	N-OH-2-acetylaminofluorene
C8-AF	N-(guanosin-C8-yl)-2-aminofluorene
N^2-AAF	3-(guanosin-N^2-yl)-2-acetylaminofluorene
AcAAF	N-acetoxy-2-acetylaminofluorene
DMBA	dimethylbenz[a]anthracene
DMN	dimethylnitrosamine
MNS	N-methyl-N-nitrosourea
BP	benzo[a]pyrene
MMS	methylmethanesulfonate
A	Adenine
G	guanine
C	cytidine
U	uridine

INTRODUCTION

1. Rationale

The realization that some chemicals can be causally implicated in the induction of certain human cancers (146) has substantially shaped the state of cancer research today. Furthermore, rapid advances in the field of molecular biology in the last two decades have led to a multitude of speculations regarding potential mechanism(s) of these chemical carcinogens at the cellular and molecular levels. However, despite this enormous amount of research, in a recent review of the subject Heidelberger concludes that there are "very few final and definitive answers to questions about mechanisms [of chemical carcinogenesis]" (79). In fact, a series of experiments performed by Berenblum and associates (13) over 30 years ago remain the cornerstone for much of the theoretical discussions of carcinogenesis under consideration at the present time. These experiments demonstrated that carcinogenesis could be divided into two stages, initiation and promotion.

This pessimism reflects, to a large extent, the apparent complexity of the cancer problem. It has been suggested that cancer is not a single disease entity, but rather should be thought of as a heterogeneous classification of neoplastic growths (50). Likewise carcinogenesis probably results from alterations at several points in the regulation of cell proliferation and differentiation resulting in

more than one possible mechanism of carcinogen action (168,169). Different mechanisms could yield a variety of tumor phenotypes. This conclusion as much reflects a lack of complete understanding of the control of normal cell growth and differentiation as it does any definitive findings regarding the neoplastic cell.

A question of primary concern in the study of the molecular biology of cancer is the nature of the critical macromolecular target(s) for chemical carcinogenesis. Although the recent statement by the Millers that "there appears to be no firm basis at this time to decide the nature of the critical molecular change in any instance of chemical carcinogenesis" (148) echoes that of Heidelberger, alterations of DNA have received the most consideration. The interest in DNA as a molecular target has evolved directly from one of the oldest thoeries of carcinogenesis, the somatic mutation hypothesis, and has paralleled an increased understanding of the process of mutagenesis of single cells in culture.

The somatic mutation theory of carcinogenesis is usually attributed to Boveri, who in 1914 suggested that the origin of a malignant tumor is "a certain abnormal chromatin constitution, the way in which it originates having no significance (22)." Similar hypotheses were presented by Tyzzer in 1916 (198) and Bauer in 1928 (11). The concept was mostly clearly elaborated by a committee consisting of S. Bayne-Jones, R.G. Harrison, C.C. Little, J. Northop and J.B. Murphy, who stated in 1938 that "The new property of the cell appears to develop suddenly, becomes a fixed character, and is transmitted to its descendants. It gives evidence of being a somatic muatation" (quoted in

ref. 168). During this time, the first experiments demonstrating the ability of purified chemicals to induce cancer in experimental animals were performed. The first synthetic carcinogen, dibenz(a,h)anthracene, was demonstrated by Kenaway and Hieger in 1930 (99), and shortly thereafter the carcinogenic hydrocarbon benzo(a)pyrene was isolated from coal tar and identified (34). However, there was little appreciation at the time for the relationship between chemicals in the environment and human cancer (45).

Important experimental evidence and further conceptual support for the somatic mutation theory was provided by Berenblum (12,13) who, based on the work of Rous (180), proposed a two stage model of carcinogenesis. In these studies, animal skin was painted for a brief time with a subcarcinogenic dose of a chemical carcinogen (initiation) followed a variable time later with a relatively longer exposure to a second noncarcinogenic chemical (promoter). Exposure to either the initiating or promoting regimen alone did not produce tumors; nor were tumors seen when the sequence of initiation-promotion were reversed. A working hypothesis for two-stage carcinogenesis has been proposed by Boutwell (21): "the initiator results in the formation of permanent and heritable but unexpressed changes in the cell genome, and the promoter causes the phenotypic expression of these changes in genotype..." The idea that neoplastic transformation was a multistage process was a departure from the concept dominant at the time, which held that cancer was a rapid, irreversible, single step change (49).

The potential role of a somatic mutation in the initiation phase of carcinogenesis gained new life in the late 1950s mainly due to the

work of E.C. and J.A. Miller who demonstrated that many apparently nonreactive carcinogens could be metabolically activated to a reactive species capable of covalently binding to macromolecules (145). Alternatively, the possibility of a permanent and heritable change in the genome resulting from the interaction of a carcinogen with a repressor regulatory protein was proposed by Pitot and Heidelberger (166) based on the work of Monod and Jacob (151). The phenotypic changes which result in cancer can also be envisioned as occurring without any chemical modifications of DNA as the normal program of cellular differentiation has been accepted to be a process that alters the availability, but not the information content of the total DNA complement (169). The unifying concept of chemical carcinogenesis, therefore, is that the aberration that produces cancer can be traced to a crucial "malfunction" of the genetic material, a paradigm that encompasses all of the models described above. This malfunction thus can be the result of chemical, viral or epigenetic changes.

A common property of virtually all ultimate chemical carcinogens is that they are strong electrophilic reactants (146). Thus, chemical carcinogens have the potential to react with nucleophilic sites on cellular macromolecules. These nucleophilic sites are abundant in RNA, DNA and protein and include nitrogen, oxygen and carbon atoms (146). Attempts have been made to correlate a selective binding to DNA, but not RNA or protein, and carcinogenicity; these studies have (24,87,132) or have not (111) been successful in demonstrating a relationship between the extent of binding to DNA and the degree of carcinogenicity of a particular chemical. Such correlations are

probably not useful for demonstrating that DNA is the critical cellular target since the relative binding most likely reflects only the metabolic capacities of the cell; furthermore only some of the interactions of carcinogens with nucleic acids appear to be important in the initiation of carcinogenesis (185).

Other studies have shown a good correlation between *in vivo* carcinogenicity and the mutagenicity of chemicals in bacterial systems (133). Similar but less extensive results have been reported for mammalian cell culture systems (86). Correlations between mutagenesis and carcinogenesis, however, do not clarify whether mutations are the underlying cause of cancer or merely reflect the nearly universal electrophilic nature of carcinogens and the possibility that critical targets for mutagenesis and carcinogenesis might be nucleophilic centers, though not necessarily identical.

More convincing evidence for the role of mutations in cancer come from studies of cells from patients having the rare disease xeroderma pigmentosa, which is characterized by an extremely high incidence of skin cancer as a consequence of exposure to sunlight. Cells from these patients have a greatly reduced capacity to repair UV or chemical-induced damage to DNA (32). In a similar manner, when cells from *Poecilia formosa* were exposed to UV light *in vitro* and injected into suitable hosts, a high incidence of thyroid tumors resulted. The cells of this species contain a photoreactivating enzyme that cleaves the UV induced pyrimidine dimers. Exposure of the cells to visible light after UV irradiation substantially prevented the development of thyroid tumors upon subsequent innoculation of the cells (77), demonstrating that repair of DNA damage reverses malignant transformation.

Using a series of 5 criteria, it has been demonstrated that a somatic mutation, but not an epigenetic change, is the basis for malignant transformation of BHK cells by chemical carcinogens *in vitro* (20). Finally, neoplastic transformation *in vitro* has also been obtained (8) after a direct perturbation of the DNA template by 5-bromodeoxyuridine and near ultraviolet light treatment, without any concomitant alteration of any other cellular macromolecules.

The experiments cited above all provide evidence for the premise that alterations in DNA and subsequent somatic mutations are important in carcinogenesis. Interaction of carcinogens with DNA during the initiation stage is usually described as a multi-step process: 1) metabolic activation of the chemical to an electrophile; 2) covalent binding to DNA; 3) possibility of DNA repair (error free or error prone); and 4) heritably altered DNA sequence. In the skin tumor two stage system, the requirements for initiation can be satisfied with a single carcinogen exposure(154), and only a relatively short exposure is required for initiation in the liver (163). Based in part on an analogy to mutagenesis studies, it is often assumed that initiation can be described as a random single or multi "hit" phenomena. Such an analysis has been done for human retinoblastoma by Knudson *et al.* (101), who suggest that the "mutation theory assumes that carcinogenesis is related to discrete changes occurring at random and at a constant average rate."

The concept of initiation as a random mutational event has also been extended to suggest a random DNA interaction. In a hypothesis suggesting that oncogeny may be adaptive ontogeny, Nery (158) states

that "in general, chemical carcinogens and ionizing radiation randomly alter one or more bases in DNA." On the other hand, Burnet (25) reasons that there is a nonrandom component in the somatic-mutational process. Likewise, Huberman et al. (85) reported that the frequency of chemically induced transformation in vitro was 20-fold greater than that of a specific mutational event (development of ouabain resistance). As one possible explanation, they suggest that the genes for transformation may be located at hot spots within the genome, which have a higher frequency of mutation. Alternatively, the difference in frequency between mutagenesis and transformation in vitro may reflect the relative size or number of target genes for transformation. Recently, it has been reported (113) that the ratio of transformed/ouabain resistant mutants was 21 for AcAAF and only 12 for benzo(a)-pyrene. The fact that these two carcinogens had differing transformation/mutation ratios in the same cell line suggests that the relative number of target genes cannot completely explain the frequency of transformation in vitro. In a similar view, in their analysis of the two stage model of carcinogenesis, Scribner and Suss (182) suggest that "poor initiators act either through an excess number of random hits or a limited number of selective hits, whereas good initiators can achieve a high number of selective hits."

The question, then, is whether the carcinogenic process involves the expression of an aberrant phenotype superimposed on a background of random DNA damage, or whether the critical DNA lesion is more specific to carcinogens than would be predicted due to random DNA damage alone. Proceeding from the Scribner and Suss (182) model, one can ask what the nature of the selectivity of good initiators is. A

conclusion of random DNA damage would predict that initiation and promotion are independently occurring events, while a conclusion of non-random damage would suggest some chemicals are highly carcinogenic because of the inherent nature or site of the particular DNA damage they induce. In the former case, the promotional aspects of carcinogenesis would be the major determinant of frequency of tumor formation, while the latter case allows for initiation events to enter into determining potency of carcinogenesis. Presently, potential interactions between events in initiation and promotion are only poorly understood.

It is clear that gaps still exist in our understanding of the initiation process. Much work has been done on the chemical nature of carcinogen DNA interactions as well as the mutational properties of chemical carcinogens. As pointed out by Weinstein et al. (203), however, there has been a tendency to think of initiation as a single random point mutation resulting from errors in replicating the damaged DNA, a hypothesis that may not be consistent with the apparently high efficiency of initiation of some chemicals. It is important, then, to investigate further the nature of initiation, the first step in carcinogenesis. Questions which need to be answered and which can only be partially addressed in this thesis relate to the nature, location and persistence of chemical modification of DNA, the functional effects of these lesions, the specificity (if any) of these lesions in determining carcinogenic potency, and the relationship between the initiating and promoting phases of carcinogenesis.

2. Carcinogenicity of 2-Acetylaminofluorene

The aromatic amine 2-acetylaminofluorene (AAF) is a highly active experimental carcinogen that was first investigated by Wilson et al. in 1941 (201). As a class, the aromatic amines have been demonstrated to be carcinogenic in a variety of species, including rodents, rabbits, dogs and humans (107). Indications of human cancer risk was first reported in 1895 by Rehn (176), who observed a high incidence of bladder tumors in German aniline dye workers who were occupationally exposed to a variety of aromatic amines. Until World War I, more than 80% of the worldwide production of aniline and other aromatic amines was manufactured by the German chemical industry, and other investigators soon confirmed Rehn's observation. Commercial production of aniline dyes in the U.S. began during World War I; this was followed some years later by reports of increased bladder cancer among these American workers and also those in the rubber industry, where various aromatic amines were used as antioxidants (107). It has been suggested that these human bladder tumors were due to contamination of aniline with other aromatic amines, such as 2-aminonapthalene, 4-aminobiphenyl, and benzidine (201).

Besides causing bladder cancer in man, dog, rabbit and rats, AAF induces liver, mammary gland and ear duct gland carcinomas in rats (107,206). Typically, hepatocellular carcinomas are induced in rats by feeding 0.03% (w/w) AAF in the diet for approximately 6-9 months, at which time 100% of the animals will have tumors. During the initial weeks of carcinogen exposure, animals gain weight at a slower rate than controls, however, no hepatic cell death occurs for at least

7 weeks of carcinogen ingestion (measured by loss of prelabelled DNA) (3,207). Within 2 to 3 weeks of AAF ingestion, a proliferation of oval (bile duct?) cells in the liver begins (48); this is followed after 7 weeks by hepatic parenchymal cell proliferation (3). The change of the parenchymal cells from hyperplastic to neoplastic takes place slowly, with distinct stages of hyperplastic nodules, nodules with atypical cells, and small hepatocellular carcinomas observed (177). The hyperplastic nodules are thought to be the precursors of the neoplastic cells, however, most of the nodules can revert into normal hepatic structures if carcinogen exposure is stopped before a critical point is reached (193). AAF has also been reported to induce hepatic tumors after a single injection when administered to newborn (1-2 day-old) mice (60).

The metabolic activation of AAF in the liver has been worked out in some detail. Rats fed AAF convert it to a metabolite, N-hydroxy-2-acetylaminofluorene (N-OH-AAF), which is then excreted in the urine as a glucuronide conjugate (36). Unlike the ring hydroxy metabolites of AAF, the N-hydroxy metabolite is a more active and versatile carcinogen than the parent compound (144,147). The N-hydroxylation reaction is catalyzed by the endoplasmic reticulum cytochrome P-450 mixed function oxidase enzyme system (194). Although administration of N-OH-AAF to rats resulted in DNA and protein bound derivatives, the compound had little or no reactivity *in vitro*, implicating a second metabolic activation step *in vivo* (146).

It has been proposed (see 107) that this second step is an esterification of N-OH-AAF to a highly reactive sulfate ester. A

cytosolic enzyme which catalyzes this reaction utilizing 3'-phosphoadenosine 5'-phosphosulfate as a sulfate donor has been studied *in vitro* (38). A good correlation has been demonstrated between the activity of the hepatic sulfotransferase and the carcinogenicity of N-OH-AAF in different rodent species and sexes. Depletion of sulfate pools by p-hydroxyacetanilide reduced the protein binding of N-OH-AAF; this effect was reversed by administration of sulfate anions. Because of its high reactivity, N-acetoxy-AAF (AcAAF) is used *in vitro* in place of the sulfate ester; it yields carcinogen residues identical to those obtained after *in vivo* administration of AAF (107).

Other enzymatic pathways for the formation of electrophiles from AAF have been described. One of these is a peroxidase-catalyzed one electron oxidation of N-OH-AAF to yield a free nitroxide radical, which can then dismutate to form AcAAF (10). Alternatively, a cytosolic acetyltransferase has been reported to form the strong electrophile N-acetoxy-2-aminofluorene in the rat liver (91). N-OH-AAF can also be converted in the liver to the weakly electrophilic o-glucuronide (142), a reaction which may be important in extrahepatic carcinogenesis (89). Finally, it has been proposed that AAF-sulfate esters formed in the cytoplasm would be too unstable to result in the binding of AAF residues to nuclear DNA (107). However, recently it has been reported that activation of AAF and DNA binding can occur in isolated nuclei apparently due to nuclear envelope monoxoygenase (98) and sulfotransferase (190) enzymes.

3. Effects of 2-Acetylaminofluorene on DNA Structure and Transcription

Progress has been made in the identification of covalent adducts of carcinogen residues with DNA after AAF exposure. The identification of a reaction product of guanosine and AcAAF in vitro (108) was soon followed by several studies dealing with adduct formation in vivo (46,91,104,106,192,205). Three carcinogen-DNA adducts have been identified, all representing binding to the nucleotide guanosine. The major adduct involves binding of the amine group of the carcinogen to the C8 position of guanine. This reaction occurs either with the loss of the acetyl group from AAF (65% of total DNA binding) or retention of the acetyl group (25% of total DNA binding). The remaining 5-15% of carcinogen bound to DNA involves the binding of the ring 3 position of AAF to the nitrogen on the 2 position of guanine, with retention of the acetyl group of AAF (90,91,106,205). Binding to the C8 and N^2 positions of guanine is illustrated in Figure 1; no studies have analyzed the potential functional differences acetylated vs. deacetylated carcinogen C8-guanine modification.

Differences in carcinogen binding between RNA and DNA have been reported. Thus, while 65% of carcinogen-DNA residues have lost the acetyl group of AAF, approximately 75% of the fluorene residues bound to cytoplasmic RNA retained the acetyl group (91,92,104,105). This finding might reflect relative differences in reaction pathways generating electrophilic species capable of binding to macromolecules in the cytoplasm or in the nucleus (91). Differences in the rate of loss of carcinogen from RNA and DNA after a single injection of AAF or N-OH-AAF have also been reported. Loss of carcinogen from RNA occurs

Figure 1. Carcinogen-guanine adducts after AAF administration. Structures of DNA-bound fluorene residues after *in vivo* administration of AAF (205). Upper adduct is N-(guanin-8-yl)-2-aminofluorene, lower adduct is 3-(guanin-N^2-yl)-2-acetylaminofluorene.

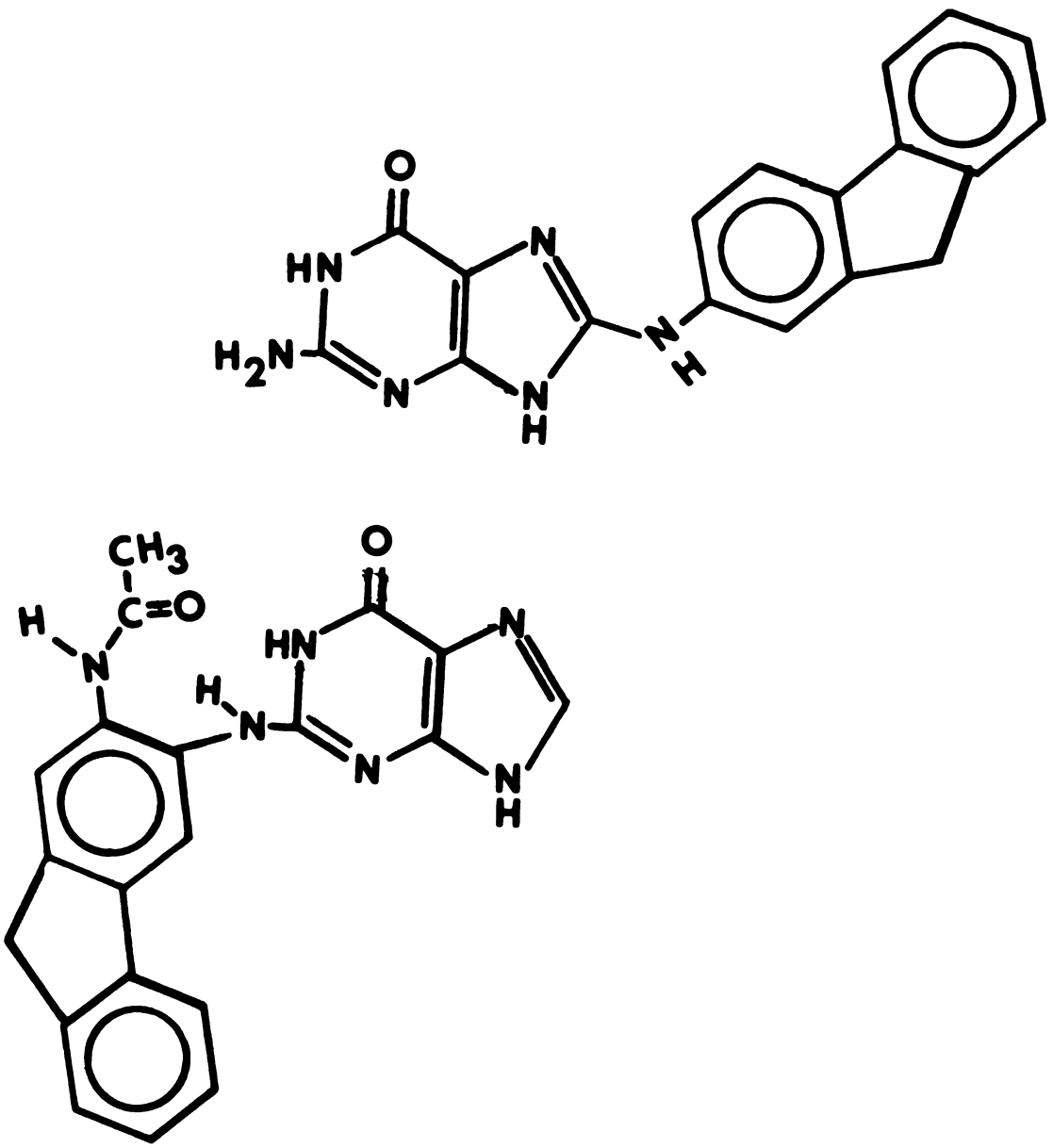


Figure 1

with a half-life of approximately 3 days, with complete loss by 4 weeks (91). Loss of carcinogen from DNA occurs with a half-life of 1-7 days, however, 10-15% of the maximal DNA-bound carcinogen remains stably bound up to 10 weeks after a single injection (90,91,106,192). The persistently bound carcinogen-DNA residue has been identified as the N^2-guanine adduct (205). Binding of AAF to the N^2 position of guanine does not occur with RNA (105), however, possibly reflecting structural differences between the 2 molecules.

The potential importance of the persistent carcinogen-DNA residue was suggested by studies of Epstein et al. (46). Rats were fed AAF for 10 weeks to induce hyperplastic nodules and were then returned to the control diet for 4 weeks. DNA isolated from cells in the hyperplastic nodules, but not from the surrounding normal hepatocytes, was found to contain distortions possibly due to persistent carcinogen-DNA moieties.

Extensive studies on the structural alterations due to modification of DNA in vitro with AcAAF have been carried out by Weinstein and associates (116, 208,209), Fuchs and Daune (56, 58,59) and Kapuler and Michelson (97). Circular dichroism (56,59) and melting curve analysis (56,59,116) suggested that carcinogen modified bases were shifted outside the double helix; formaldehyde unwinding studies (58) indicated that each bound fluorene residue gave rise to weak points in the DNA; and viscosity and light scattering findings (56) indicate that AAF introduces hinge points in the DNA molecule. Based in part on these results, a "base displacement model" has been proposed (59,116) to describe the structural effect of AAF interaction with double stranded nucleic acids: 1) AcAAF attacks the C8 position of guanine

when it is made accessible by normal DNA "breathing", 2) a disruption of the normal G-C hydrogen bond base pairing occurs, 3) there is a rotation of the guanine base around the glycosidic bond (between guanine N9 and deoxyribose C1) from the normal anti to a syn conformation, 4) the modified guanine is displaced from its normal coplanar position relative to the adjacent bases, 5) a stacking of the fluorene residue parallel to adjacent bases then occurs, 6) the large size of the fluorene molecule gives rise to distortion of the DNA helix and localized regions of denaturation.

The conformational changes resulting from either C8 or N^2 guanine modification was further examined by digestion of the DNA with a single strand-specific S1 endonuclease (55,208). Digestion of only the C8 guanine adduct, but not the N^2 adduct, occurred. This suggests that the former causes major conformational changes, while the latter does not cause major distortions of native DNA structure. Model building studies indicate that the fluorene residue of the N^2 guanine adduct could occupy the minor groove of the DNA helix with less disturbance of helical structure (209). Other studies (208) demonstrated that 5-35 base pairs of DNA were released for every gua-AAF residue released with S1 nuclease digestion, giving an approximation of the extent of denaturation due to each bound AAF residue. Using a different technique, Fuchs and Daune (58) estimated a denaturation of 7 to 23 base pairs, while exposure of human cells in culture to AcAAF induces an excision repair of approximately 140 nucleotides per AAF modification (175). The finding that the N^2 guanine adduct is not susceptible to digestion by S1 nuclease suggests that an important

factor governing DNA repair *in vivo* is the ability of repair enzymes to recognize a structurally altered template.

The base displacement model predicts that binding of AAF to C8-guanine would be sterically inhibited when the nucleotide is strictly held in a static double helical structure (55). When DNA was reacted with AcAAF in a high ionic environment, which is known to stabilize nucleic acid structure, the rate and extent of DNA modification was reduced (116). Similarly, the rate and extent of the reaction of AcAAF with denatured DNA was 2-3 times greater than that of native DNA (116). Finally reaction of AcAAF with *E. coli* tRNA, which contains both double stranded and "looped" single stranded regions, occurred primarily in the guanosine residues in the loop positions (61).

4. Biochemical Effects of 2-Acetylaminofluorene Binding to Macromolecules

Several biochemical effects of covalent binding of AAF to cellular macromolecules have been described, with the hope of correlating a biochemical action with the biologic effects of the carcinogen. Covalent binding of relatively low levels of AcAAF to *E. coli* transfer RNA's produces specific modifications in their amino acid acceptance capacities, codon recognition and ribosomal binding (51). Binding of AcAAF to ribosomal RNA impaired its ability to hybridize to homologous DNA (116). Extensive carcinogen binding also occurred on cytoplasmic and nuclear proteins both *in vivo* and *in vitro*, and there is evidence that specific proteins are particularly highly modified (107). Some of these modifications can presumably interfere with normal protein and enzyme functions, though the role, if any, of protein binding in chemical carcinogenesis is not known at present (107).

Most studies have concentrated on the effects of carcinogen modification of DNA. Exposure both in cell culture and *in vitro* to AcAAF results in macromolecular complexes between DNA and protein (52,137,152). These complexes apparently are covalent DNA-protein crosslinks, and they have been postulated to be related to DNA single strand breaks (52) and chromosomal aberrations (152). DNA reacted with AcAAF was a less effective primer-template for DNA polymerase-catalyzed DNA synthesis (127). A single injection of AAF into male rats inhibits liver regeneration following partial hepatectomy, an effect which may be due, in part, to inhibition of DNA synthesis (74). Evidence from thermal elution chromatography and pyrimidine isotich analysis suggests that damaged DNA in human cells exposed to AcAAF is faithfully restored by DNA repair synthesis to the original nucleotide sequence (117). The methods used in these studies, however, could not detect shuffled sequences or occasional DNA base substitutions (117).

One of the most striking biochemical effects of AAF is its ability to block RNA synthesis. This action may be due, in part, to alterations in RNA polymerase activity (64,66,67,82,210,211), as well as a result of direct modifications of the DNA template (2,73,96,150, 195,210,212). The inactivation of the DNA template for RNA synthesis is noteworthy in that it: 1) occurs rapidly upon carcinogen reaction with DNA (202); 2) can result in complete blockade of RNA synthesis when extensive carcinogen modification of DNA occurs (212); 3) can substantially reduce the priming activity of DNA for RNA polymerase at levels of carcinogen modification that do not affect the activity of DNA for DNA synthesis (195). The difference in sensitivity for

inhibition of DNA and RNA synthesis is approximately 20-fold, with RNA synthesis more sensitive to inhibition (195). DNA template inactivation occurs after carcinogen exposure both *in vivo* (73,195,210) and *in vitro* (64,150,208,212) and also when assayed with bacterial (150,195, 208,212) or mammalian hepatic RNA polymerases (64). *In vivo*, the greatest inhibition appears to occur in the synthesis of 45S ribosomal RNA precursor (65,96), apparently due to an effect on nucleolar DNA template function (73,210). Direct inhibition of RNA polymerase activity by AAF is dose-dependent, however, *in vivo* RNA polymerase II (nucleoplasmic) is more sensitive to inhibition than is RNA polymerase I (nucleolar) (64). A single injection of N-OH-AAF also results in an inhibition of poly-A RNA synthesis, although the inhibition of poly-A RNA was somewhat less than that of ribosomal RNA (65). Poly-A RNA is a measure of the processing of nuclear RNA to its cytoplasmic forms, a process which involves post-transcriptional events such as methylation and polyadenylation.

Using a variety of approaches, it has been demonstrated that the inactivation of the DNA template for RNA synthesis is due to a reduction of RNA elongation, but not RNA synthesis initiation (64,150,195, 212). It has been postulated that most of the AAF residues bound to the coding strand of the DNA cause premature termination of transcription, at or near the site of modification. This results in the production of shorter RNA chains; the synthesis of RNA presumably cannot continue distally to the site of the AAF-modification (150). This inhibition of DNA template capacity persists when the DNA is packaged in its native chromatin structure (208). DNA modified *in vitro* was reconstituted with unmodified chromatin proteins (208).

Carcinogen modification did not grossly affect the ability of the DNA to interact with chromosomal proteins to form normal nucleosome structures. The reconstituted chromatin, however, showed approximately a 70% reduction in template capacity for RNA synthesis.

As might be predicted for a chemical that interacts extensively with DNA, AcAAF has been shown to be highly mutagenic in a variety of systems. Maher et al. (128) reported an inactivation and an increased mutation frequency in transforming DNA of Bacillus subtilis that had been reacted with AAF esters. Similarly, Corbett et al. (35) examined the mutagenicity of AcAAF in the T4 bacteriophage-E. coli system. The types of mutants observed were large deletions, A·T to G·C transitions, and frameshift mutations, occurring with a frequency ratio of approximately 1:2:3, respectively. In the presence of a mammalian metabolic activation system, AAF induces frameshift mutations in the Salmonella typhimurium histidine revertant system (5). Mutations were also observed upon the injection of AAF, N-OH-AAF and AcAAF into Drosophila melanogaster (47). The first two compounds were only weakly mutagnic, and were selective for rRNA genes. AcAAF was highly mutagenic and in addition to the rRNA genes induced point mutations and gene eliminations in heterochromatic regions. AcAAF also produces mutations in mammalian cells in vitro (113).

5. Chromatin Structure and Fractionation

There has been an enormous amount of research in recent years into the molecular biology of chromatin, and mechanisms of gene regulation (for example, see the 1978 Cold Spring Harbor Symposium,

ref. 33). Therefore, only a selective and limited review will be attempted here. A cornerstone in molecular biology is the concept that a multicellular organism develops with every cell having the same complement of DNA. Thus, the determining factor in the specialized structure and function of each cell is the portion of DNA in the genome which is expressed at any given time. Control of gene expression is believed to occur, to a large extent, at the level of RNA synthesis such that transcription of specific genes occurs in one tissue, while being inhibited in other tissues (7). It has been estimated that 10-30% of the DNA in differentiated tissues is represented in total cellular RNA, and about one-tenth of this information is present in mRNA. This yields a value of approximately 12,000-15,000 genes actively being transcribed in the liver (7).

Many investigations have focused on the structure of chromatin as a means of elucidating its function in gene transcription. Nuclear DNA exists as a complex with RNA and proteins. The proteins include five chemically-defined histones, and large, heterogeneous group of non-histone proteins. The basic structural unit of chromatin is the nucleosome, which consists of a well defined length of DNA (about 200 base pairs) complexed with eight histone molecules, 2 each of histones H2A, H2B, H3 and H4. The DNA is wrapped around the outside of the histone octamer, forming a repeating, roughly spherical particle and giving rise to a "beads on a string" appearance (102). The non-histone proteins are then associated in a as yet undetermined manner with the DNA-histone complex.

For quite some time it has been suggested that template active and template inactive chromosomal regions can be differentiated based on the density of their staining *in situ*. Frenster *et al.* (54,122) have demonstrated that RNA transcription occurs on the more extended, template active, euchromatin, but not on the more condensed, template repressed heterochromatin. This apparent physical difference in chromatin structure has been widely used as a means of fractionating chromatin into putative eu- and heterochromatic sequences for further analysis *in vitro* (178).

Chromatin fractionation is basically a two-step process. The interspersed eu- and heterochromatin segments must first be separated, either by physical shearing, sonication, or enzymatic cleavage. The chromatin fragments are then separated based on physico-chemical differences by a variety of methods including differential centrifugation, rate-zonal sedimentation, gel-exclusion chromatography, and salt precipitation (178). The separated chromatin fractions are then characterized as either eu- or heterochromatin based on *in vitro* template capacity, association with nascent RNA, satellite DNA sequences, or endogenous RNA polymerase (178).

Although these parameters are logically based on current knowledge of chromatin function, criticisms of some of the techniques used have suggested that the separation of different fractions could have been due to the formation of artifacts (178). These studies indicate that physical shearing of chromatin may cause important alterations due to effects on chromatin proteins (43) or RNA (183). Similarly, other studies have concluded that although physically sheared and fractionated chromatin retains many of the structural distinctions

between eu- and heterochromatin known to exist *in vivo*, these chromatins fail to demonstrate any selective fractionation of globin or keratin genes (103), actively transcribed viral nucleic acid sequences (84), or actively transcribed mRNA sequences (93). On the other hand, one study has successfully demonstrated a fractionation of ribosomal genes in sonicated chromatin fractionated on a sucrose gradient (83).

In an attempt to avoid potential artifacts induced by random physical shearing, DNA endonucleases have been used to fragment chromatin into template active and inactive fractions. It was hoped that these enzymatic probes could at least partially recognize salient features of chromatin structure as does RNA polymerase *in vivo* (162). Besides demonstrating a selective distribution of nascent RNA with the euchromatin (15), studies with DNAse I (63,162, 204) and DNAse II more importantly reveal a fractionation of a variety of gene sequences in various differentiated tissues. Several of the studies with DNAse II are listed in Table 1. Thus, the nuclease digestion procedures demonstrate chromatin fractionation into eu- and heterochromatin as judged by a more stringent criteria, a localization of actively transcribed or repressed segments of the genome in chromatin derived from differentiated tissues.

6. Intragenomal Distribution of DNA Damage and Repair

Interest has been increasingly focused in the past few years on the influence of chromatin structure on the interactions of carcinogens and DNA. Several approaches have been used to analyze the distribution of carcinogen damage and repair, including: 1) Staphylococcal nuclease digestion of chromatin. Nuclease-sensitive regions

TABLE 1

Evidence for Gene Localization After DNAse II - $MgCl_2$ Chromatin Fractionation

"Pro"	"Con"
1) S2 contains specific subset of of whole genomal DNA. S2 DNA codes for 60% of total cellular RNA (72).	1) No selective globin gene localization in Friend erythroleukemia cell S2 DNA (114).
2) S2 DNA of liver differs from S2 DNA of brain (70).	2) No selective globin gene localization in chick reticulocyte S2 DNA (75).
3) S2 enhanced for globin gene DNA in friend erythroleukemia cells (131).	
4) S2 enhanced for globin gene in reticulocytes, but not in liver (81).	
5) Nucleolar S2 enriched in rDNA (131).	
6) Thyroid hormone nuclear receptor enriched in S2 of rat pituitary cells (181).	
7) Estrogen nuclear receptor enriched in S2 of hen oviduct chromatin (80).	

S2 = $MgCl_2$-soluble euchromatin fraction, as described in Methods.

rDNA = DNA sequence which codes for ribosomal RNA.

are primarily the linker DNA, the 60 DNA base pairs found between the 140 base pairs that are intimately associated with spherical histone particle ("core DNA") (123). 2) DNAse I digestion. Nuclease-sensitive regions after a short digestion are primarily the template active euchromatin DNA (63,204). 3) Sheared chromatin fractionated by gradient sedimentation. Chromatin is fractionated based on apparent differences in physical structure. 4) Separation of repetitive vs. unique DNA sequences by DNA-DNA hybridization. Approximately 15-40% of eukaryotic DNA is composed of reiterated sequences which may play a role in the regulation of the structural genes, which consist of unique sequences (37). 5) Separation of satellite DNA from mainband DNA by Ag^{+}-$CsSO_4$ centrifugation. Satellite DNA comprises 8-9% of the total DNA and is comprised of a highly specialized set of heterochromatic, highly reiterated sequences (119). 6) In situ evaluation of DNA repair after carcinogen exposure by electron microscopy and autoradiography.

These studies indicate that carcinogen binding is initially greatest in euchromatin regions determined by sucrose gradient sedimentation (153, 155) or by DNAse I digestion (40,94,139,171,172) after exposure to BP (94), AAF (17,139,172), DMN (140,171) or UV light (187). This region of the genome also demonstrates a greater extent of DNA repair as revealed by loss of bound carcinogen from chromatin fractionated by DNAse I digestion (171,172) or sucrose gradient fractionation (155). Similarly, the extent of incorporation of ^{3}H-thymidine during DNA repair synthesis is also greater in the DNAse I

susceptible chromatin (18) and in electron microscopically-identified euchromatin (75).

In contrast, no selectivity of binding of MNU (17), MMS (17), AcAAF (119), or UV-induced damage (119) between mainband and satellite DNA sequences could be detected. Similarly, there was no difference in the amount of 7-methyl-guanine damage or repair between repetitive and unique DNA sequences after in vivo exposure to DMN (62). An equal distribution of bound adducts between repetitive and unique DNA sequences was also reported to result from exposure of human fibroblasts to AcAAF (118) or murine skin cells to a high concentration of DMBA (184). At low carcinogen doses, however, a concentration-dependent preferential binding to reiterated DNA sequences occurred (184). These investigators also demonstrated an inverse linear relationship between the enrichment of hydrocarbon adducts in reiterated DNA sequences and the logarithm of the amount of total hydrocarbon bound to DNA.

Intriguing results have also been reported in the distribution of DNA damage and repair within each nucleosome, as revealed by Staphylococcal nuclease digestion. Early studies demonstrated an enhanced binding of BP (94), AAF (139,172) and alkylating agents (140,171) to the "linker" regions of the nucleosome. However, conflicting results were obtained when chromatin was isolated at various times after carcinogen exposure. More recent studies have carefully examined this phenomenon using pulse-chase incorporation of ^{3}H-thymidine during DNA repair synthesis (18,186,187,195). It appears that from 70-100% of the DNA repair initially occurs in the linker DNA region; with time

(4-12 hr), however, the chromatin within the nucleosome rearranges such that the initially repaired lesions are now located in the core DNA, while previously unrepaired damage moves into the linker DNA region. This nucleosome rearrangement repair process is nonspecific in that it occurs in either mouse mammary cells (18) or human cells (31,186,187,195) after exposure to either AcAAF (195), UV light (31,186, 187) or alkylating agents (18).

A general conclusion from the studies cited above is that chromatin structure influences carcinogen damage and DNA repair synthesis both at the nucleosome level, and also at higher levels of chromatin organization. Furthermore, a rearrangement of the DNA within the nucleosome can occur, resulting in an enhanced exposure of damaged DNA to repair enzymes with time. At the present time it is not known whether this rearrangement process is constitutive (i.e., occurring in the absence of DNA damage) or is induced by the repair process itself (195).

7. Experimental Objectives

The objectives of this investigation were to examine the nature of the interaction of the carcinogen AAF and its N-hydroxy metabolite with target organ DNA *in vivo*. These included studies of quantitative and qualitative aspects of carcinogen-DNA binding as well as an assessment of potential functional DNA damage as measured by effects on RNA synthesis. Carcinogen binding or damage was assessed shortly after a single exposure to N-OH-AAF (2 hr), and after carcinogen ingestion for 4, 7 and 14 days. Repair of carcinogen damage was

measured 4, 7 and 10 days after a single injection of N-OH-AAF, and 7 days after a 4 day exposure to AAF in the diet.

The studies were initiated to test the premise that carcinogen-DNA interactions do not occur randomly *in vivo*, and have specifically focused on potential differences occurring between DNA in template active and DNA in template repressed portions of the genome. Chromatin was fractionated by either sonication-glycerol gradient, sonication-$MgCl_2$ precipitation, or DNAse II digestion-$MgCl_2$ precipitation. Carcinogen DNA adducts were examined either as radioactivity in a 100°C-acid hydrosylate or by determining chemically identified adducts with silica gel thin layer chromatography. These studies might be useful in understanding the relationship between carcinogen-induced DNA damage and later stages (i.e., promotion) of carcinogenesis, as well as hopefully helping to pinpoint the location of critical DNA lesions responsible for initiating the progression of a normal cell to a neoplastic cell.

MATERIALS AND METHODS

1. Animals; Carcinogen Administration

Male, Sprague-Dawley rats (150-200 g), purchased from Spartan Farms (Haslett, Michigan) were used in all studies. Animals were housed 2 per cage in a room with a controlled 12 hour light cycle beginning at 7 p.m. The rats were fed either a basal diet (Carcinogenic Basal Diet; Teklad Mills, Madison, Wisconsin) or one containing 2-acetylaminofluorene (Aldrich Chemical Milwaukee, Wisconsin). When indicated rats were injected i.p. with [ring-^{3}H]-N-hydroxy-N-acetyl-2-aminofluorene (50.2 mCi/mmole, purchased from New England Nuclear), or [9-^{14}C]-2-acetylaminofluorene (10 mCi/mmole, New England Nuclear) which was dissolved in 0.9% NaCl containing 12% ethanol.

2. Isolation of Chromatin

The method of Rodriguez and Becker (179) for the isolation of chromatin was modified as follows. Livers were homogenized in 250 mM sucrose, 50 mM Tris (pH 7.9), 25 mM KCl, 5 mM $MgCl_2$ (STKM) and filtered through cheese-cloth. Nuclei were isolated by washing twice in 2% Triton X-100 STKM followed by centrifugation at 750 x g for 10 min. The nuclear pellet was washed once in STKM.

The washed nuclei were homogenized in 10 mM Tris (pH 7.9). Chromatin was purified by layering 10 ml on a sucrose-Tris (10 mM, pH

7.9) step gradient consisting of 14 ml 1.3 M sucrose and 14 ml 1.6 M sucrose, and sedimented by centrifugation (112,000 x g for 2 hours) in a Beckman SW27 rotor. The chromatin was resuspended in 8% glycerol, 10 mM Tris (pH 7.9). Chromatin prepared in this manner had a protein:DNA ratio of 3.9, and a RNA:DNA ratio of 0.27.

3. Chromatin Fractionation

Isolated chromatin was fractionated by three different methods. In the first method, chromatin was sonicated for two 10 second bursts at 70% maximal power using an Ultrasonics sonicator equipped with a microtip. Approximately 2.5 mg of purified, sonicated, chromatin DNA was layered on a 12-90% (v/v) linear glycerol gradient in 10 mM Tris (pH 7.9), and centrifuged at 72,000 x g for 15 hours in a Beckman SW27 rotor. Gradients were monitored at 260 nm and 12 fractions, 3 ml each, were collected. Fractions of fast and slow sedimenting chromatin were pooled, as indicated in Figure 2, for further analysis. The portion of the chromatin which sedimented to the bottom of the gradient (designated pelleted heterochromatin) was also analyzed. This material represents approximately 15% of the total chromatin DNA.

Alternatively, chromatin was fractionated by selective aggregation with $MgCl_2$ (6). Sonicated chromatin, prepared as described above, was centrifuged (2,000 x g for 15 min) in the presence and absence of 1.75 mM $MgCl_2$. The supernatant and pelleted portions were analyzed. Chromatin fractions were classsified as follows: "Euchromatin", chromatin in the supernatant after centrifugation in the presence of 1.75 mM $MgCl_2$; "pelleted heterochromatin", chromatin in the pellet after centrifugation without $MgCl_2$. The portion of chromatin

Figure 2. Sedimentation profile of hepatic chromatin. Rats were injected with ^{3}H-orotic acid (30 μCi/100 g) and 20 min prior to sacrifice. The isolated chromatin was fractionated by centrifugation on a 12-90% glycerol gradient. The amount of DNA (●) and nascent RNA (O) in each 3-ml fraction was determined. Fractions demonstrating enhanced associated with nascent RNA were designated euchromatin; those fractions relatively deficient in nascent RNA were designated heterochromatin. A portion of the chromatin also sedimented at the bottom of the tube and this shall be referred to as pelleted heterochromatin.

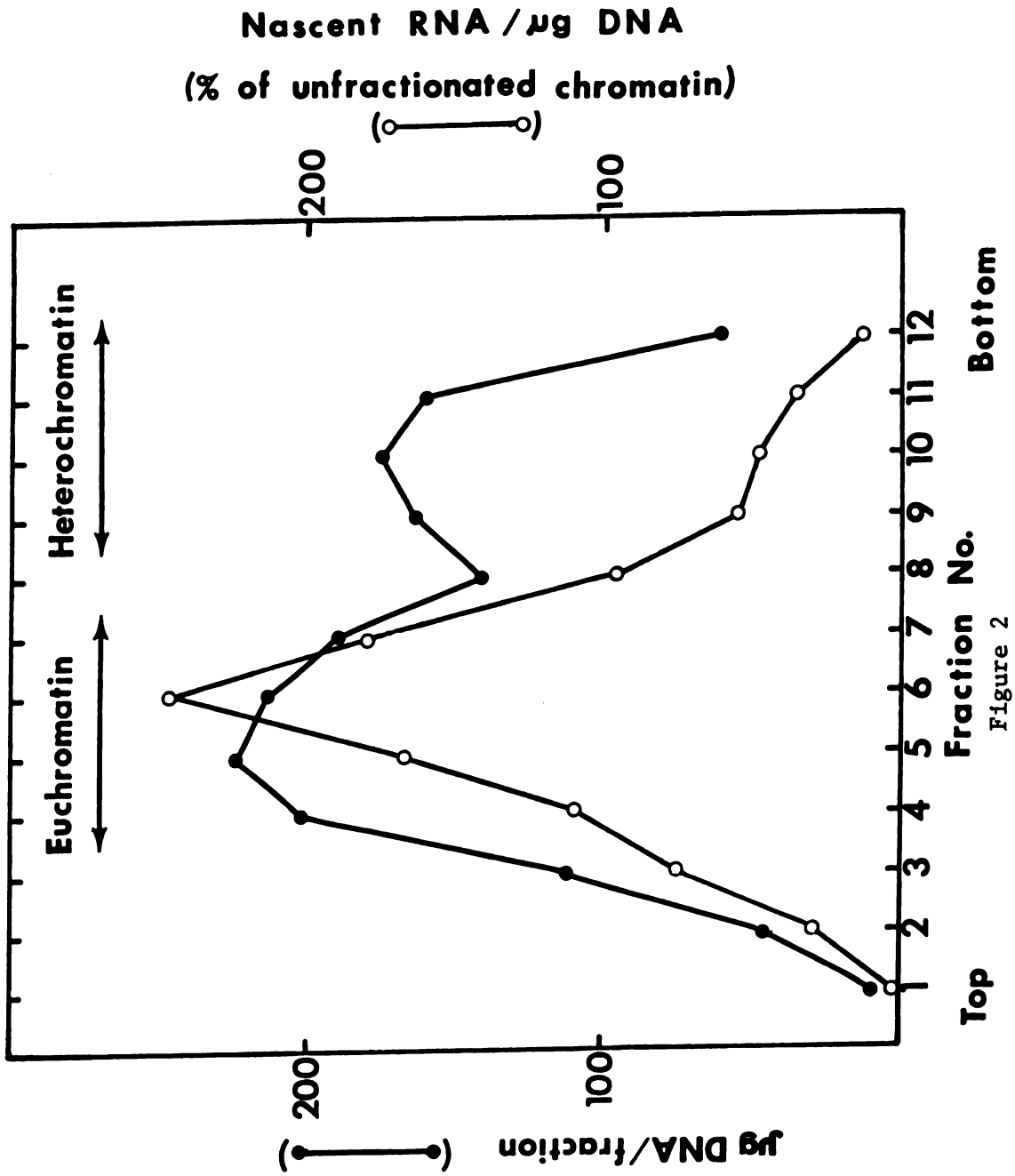

Nascent RNA / µg DNA
(% of unfractionated chromatin)
200
100
Heterochromatin
Euchromatin
Top
Bottom
Fraction No.
1 2 3 4 5 6 7 8 9 10 11 12
200
100
µg DNA/fraction

Figure 2

which was selectively precipitated by $MgCl_2$ ("heterochromatin") was determined by subtracting values for insoluble chromatin from the pelleted chromatin after centrifugation in the presence of $MgCl_2$.

The third method for chromatin fractionation was the DNAse II-$MgCl_2$ procedure of Gottesfeld et al. (128). Rat liver chromatin, purified as above, was washed once with 10 mM Tris (pH 7.9) and dialyzed overnight at 4° in a 25 mM sodium acetate buffer (pH 6.6). The volume of the dialysate was adjusted to give an $A_{260\ nm}$ of 10 (measured in 0.9 N NaOH). The solution was brought to 24°C, and DNAse II (Sigma DN-II-HP, 20,000 units per mg protein) was added to 100 units/ml. At various times (usually 30 min) the reaction was terminated by the addition of 50 mM Tris (pH 11) to pH 7.5 and cooling on ice. Nuclease resistant chromatin (P1) was removed by centrifugation (9,000 x g for 15 min). To the supernatant, one ninety-ninth volume of 0.2 M $MgCl_2$ was added (final concentration = 2 mM). After 30 min of intermittent vortexing, the suspension was centrifuged as above yielding a pellet containing the heterochromatin (P2) and a supernatant containing the euchromatin (S2). Trichloroacetic acid (TCA) was added to obtain acid-insoluble chromatin.

4. Synthesis of Nascent RNA

To label nascent RNA, animals were injected ip with [5-^{3}H]orotic acid (30 μCi/μmole/100 g body wt) 20 min prior to sacrifice. The acid precipitable (5% TCA) radioactivity was determined in aliquots of chromatin fractions. The radioactive material was rendered acid soluble by incubation in 0.3 N KOH at 37°, for 1 hr. This verified that it represented RNA.

5. Analysis of Carcinogen Binding to Chromatin Components

Chromatin protein, RNA and DNA were separated as previously described (68). Aliquots of chromatin samples were precipitated with ice cold 5% trichloroacetic acid after the addition of 3 mg of bovine serum albumin. After washing twice with 5% TCA, the RNA was hydrolyzed by incubating with 2 ml of 0.3 M KOH at 37° for 1 hour. The hydrolysis was stopped by the addition of 4 ml of cold 5% TCA. The samples were briefly centrifuged, and the supernatant containing the hydrolyzed RNA was decanted. After washing the precipitate twice with TCA, the DNA was hydrolyzed by boiling for 15 min after the addition of 2 ml of 5% TCA. The hydrolysis was stopped by placing the samples in an ice bath. They were then centrifuged, and the hydrolyzed DNA supernatant was removed with a pasteur pipet. The DNA hydrolysis was repeated twice using 2 and 1 ml of 5% TCA, which were all combined. The remaining protein precipitate was dissolved in 0.5 ml of 88% formic acid (160) to which 10 ml of Multisol scintillation cocktail (Isolab Inc.) was added. Two ml aliquots of hydrolyzed DNA and RNA were dissolved in 18 ml of Multisol for scintillation counting. Radioactivity determined by this extraction procedure represents carcinogen covalently bound to DNA or RNA.

The aminoazo dye carcinogen p-dimethylaminoazobenzene (DAB) is similar to AAF in chemical structure, metabolic activation and reactivity with cellular components (121,146). DAB residues covalently bound to protein are not removed by exposure to organic solvents or trichloroacetic acid (143). While the bound DAB residues are completely hydrolyzed from protein after treatment with 5.5 N KOH for 92

hr at 80°C, only 5% of the residues are removed after treatment with 5.5 N KOH for 1 hr at 25°C, onditions for alkaline hydrolysis which are more vigorous then used in the procedure described above. In addition, adducts of DAB (121) and AAF (108) with guanine are stable under alkaline conditions. Adducts of AAF with guanine are also stable during treatment with acid (108). Therefore, it is reasonable to expect that adducts of AAF with protein, RNA and DNA are stable under the conditions described above for analysis of carcinogen binding to chromatin components.

6. Chromatography of Carcinogen-DNA Base Adducts

DNA-purines were isolated for thin layer chromatography (TLC) as follows. The chromatin samples were precipitated and the RNA was hydrolyzed as described above. After removal of the RNA supernatant, the pellet was washed once with 5 ml ice-cold 5% TCA. The DNA was hydrolyzed by incubating the pellet with 2 ml 0.3M HCl at 60°C for 40 min. After cooling on ice, the sample was centrifuged at 9,000 x g for 10 min. The supernatant was neutralized by the addition of 1 ml 0.1 M Tris (pH 11) and 1 M NaOH. The supernatant was then put on a Sephadex LH-20 column (6x95 mm). Unmodified purines and buffer salts were removed by washing the column with 15 ml H_2O. The carcinogen-modified purines were then quantitatively eluted with 5 ml ethanol, evaporated to dryness, and spotted on a Silica Gel-GF TLC plate (pre-channeled and containing a Kieselguhr pre-absorbant strip, Analtech, Inc., Newark, Del.). The TLC plates were developed with a n-butanol: acetic acid:H_2O (50:11:25) solvent (205). Portions of the silica gel

plates were removed and placed in a scintillation vial prior to the addition of 10 ml of Multisol.

7. Mass Spectral Analysis

Chemicals were analyzed by solid probe analysis on a Finnigan 3200 Gas Chromatograph-Mass Spectrometer equipped with a Systems Industry 150 data system.

8. Preparation of Purified DNA

DNA was stripped of its associated proteins and RNA as follows. Pooled glycerol gradient chromatin fractions were first concentrated with an Immersible Molecular Separator (Millipore). This is a vacuum operated Pellicon filter (10,000 MW exclusion limit). Approximately 1.2 ml samples were then incubated for 40 min with RNase (50 µg/ml at 37°C) followed by incubation for 2 hr with protease (3 mg/ml at 37°C). Both the RNAse and the protease were purchased from Sigma and were pretreated by heating for 10 min at 80°C. After completing the incubation, the samples were brought to 3 M with respect to NaCl and centrifuged at 12,000 g for 10 min. The supernatants were removed and applied to a Bio-Gel A-1.5 M agarose gel column (Bio-Rad Laboratories). The material eluting immediately after the void volume was collected and dialyzed overnight against 10 mM Tris (pH 7.9). An average of 150 µg of purified DNA per fraction was obtained; there was no difference in yield between control and AAF treated rats. The DNA thus purified was analyzed for its template activity.

9. Template Activity of DNA and Chromatin

Transcription of DNA with E. coli RNA polymerase (Miles Laboratories, Elkhart, Ind.) was performed under conditions in which reinitiation by the enzyme was inhibited (28,88). Purified DNA (200 ng) was incubated (37°) with 0.08 mM ATP and GTP, 0.02 mM ^{3}H-UTP (500 μCi/μmole), 1.2 mM $MnCl_2$, 10 mM Tris (pH 7.9) and 6.25 units E. coli RNA polymerase, in a total volume of 2.5 ml. After allowing 15 minutes for initiation to be completed, $(NH_4)_2SO_4$ was added to a final concentration of 0.4 M. The high salt concentration inhibits the reinitiation of RNA synthesis (88). RNA chain propagation was then begun by the addition of 0.08 mM CTP and 6.5 mM $MgCl_2$. At various times, duplicate 100 μl samples were removed and added to 2 ml ice-cold 5% trichloroacetic acid. After the addition of 2 mg bovine serum albumin, the precipitate was washed 3 times with ice-cold 5% TCA, dissolved in 0.5 ml 88% formic acid (160) and dissolved in 10 ml Multisol for liquid scintillation counting. In a similar manner, the template activity of chromatin was determined using 50 ng of chromatin-DNA and 0.5 units E. coli RNA polymerase.

To determine the number of initiation sites on the DNA template, increasing amounts of DNA were incubated for 10 min with 0.15 units E. coli RNA polymerase, under the conditions described above. To measure the rate of incorporation of individual nucleotides, 30 ng of DNA and 0.3 units of enzyme were incubated for 10 min with varying concentrations of one nucleotide triphosphate, with the concentrations of the other 3 nucleotide triphosphates held constant. To determine the ability of the enzyme to reinitiate RNA synthesis on the various DNA

templates, the reaction was run as described above except that the $(NH_4)_2SO_4$ was omitted.

10. Other Methods

DNA was determined by the method of Ceriotti (29). Alternatively, small amounts of DNA in individual glycerol gradient fractions were determined by the diaminobenzoic acid fluorometric assay of Kissane and Robins (100). Protein was determined by the method of Lowry et al. (124) after correction for interference by glycerol, or by the Coomasie-blue method (23). RNA was determined by the orcinol procedure of Lin and Schjeide (120). The linearity of the glycerol gradients was verified by measuring the refractive index of the gradient fractions.

RESULTS

1. Characterization of Chromatin Fractionation Procedures

Sonicated chromatin can be reproducibly fractionated by equilibrium sedimentation on a glycerol gradient into three major portions. Figure 2 illustrates the 2 peaks of chromatin-DNA at glycerol concentrations of approximately 40% and 80% (v/v); in addition, a rapidly sedimenting chromatin fraction forms a pellet at the bottom of the centrifuge tube.

To identify portions of chromatin that might represent the template active segments of the genome, rats were injected with [5-^{3}H]orotic acid (30 μCi/100 g) 20 min prior to sacrifice. Each gradient fraction was then analyzed for the presence of nascent RNA (Figure 2). When compared to the chromatin originally put on the gradient, several of the slower sedimenting fractions were relatively deficient in nascent RNA. These fractions were pooled for analysis as indicated at the top of Figure 2.

The slow sedimenting fraction represented 30% of the total chromatin DNA (Table 2), the fast sedimenting fraction contained 24% of the chromatin DNA, and the pelleted chromatin, 14% of the chromatin DNA. The *in vitro* template capacity of the slow sedimenting chromatin was significantly greater (Student-Newman-Keuls test, $p<0.05$) than that of the fast sedimenting or pelleted chromatin. As has been previously reported (202) the slow sedimenting chromatin has

TABLE 2

Composition of Chromatin Fractions[a]

	Slow Sedimenting Chromatin[b]	Fast Sedimenting Chromatin[b]	Pelleted Chromatin[c]
µg DNA/fraction	728 ±96	596±65	330±55
% of DNA	30%	24%	14%
Protein/DNA	4.88±1.2	2.80±0.4	4.12±0.5
RNA/DNA	0.31±0.03	0.18±0.04	0.24±0.07
Nascent RNA[d]	134±21	19±3.6	24±1.7
Relative purity[e]	1.94	0.28	0.35
Template activity[f]	18.2±0.4	6.1±2.0	3.7±2.0

[a]Chromatin was prepared as described in Methods, and was fractionated on a 12-90% glycerol gradient (95% of the DNA was recovered in all chromatin fractions).

[b]Pooled glycerol gradient fractions as indicated in Figure 2.

[c]Chromatin which sedimented to the bottom of the glycerol gradient.

[d]Animals were treated as described in the legend to Figure 2. The data are expressed as DPM/µg DNA.

[e]Nascent RNA relative to unfractionated chromatin = 1.00.

[f]In vitro transcription with E. coli RNA polymerase. Values are relative to naked DNA = 100 (actual value was 2340 pmole ribonucleotide per µg DNA).

proportionally more associated protein and RNA when compared to the unfractionated chromatin. Relative to the fast sedimenting chromatin, the slow sedimenting chromatin represents approximately a 6-fold purification as judged by the relative amount of the associated nascent RNA. Nevertheless, a significant portion of the nascent RNA is found in the fast sedimenting fractions, indicating that the separation is not complete.

A chromatin sample was heat denatured at 70°C for 15 min in 10 mM Tris prior to fractionation on a glycerol gradient (Figure 3). In contrast to the control sample, the denatured chromatin-DNA now sediments as a single peak of sedimentation intermediate to that of the slow and fast sedimenting chromatin. Denaturation at 70°C, which is well below the temperature for DNA strand separation in chromatin under identical ionic conditions (189) probably selectively removes some of the chromosomal proteins which act to maintain the DNA in an extended or condensed state. The nascent RNA, however, now sediments at the very top of the gradient, where little chromatin DNA is found. With more extensive denaturation (95°C for 15 min) approximately half of the DNA sediments at the top of the gradient as well, probably reflecting the transition from double-strandedness to single strandedness (data not shown).

The selective aggregation of heterochromatin by low concentrations of divalent cations is often used as a means of fractionating chromatin into eu-and heterochromatin (44). In this manner, chromatin was fractionated into three components (Table 3) analogous to those obtained with the glycerol gradient method. This method results in an

Figure 3. Physical association of nascent RNA with chromatin. Nascent RNA was labelled with ^{3}H-orotic acid as described in Figure 2. Chromatin was isolated and fractionated as described in Methods (upper panel) or partially heat denatured at 70°C for 15 min (lower panel) prior to centrifuging through a 12-90% glycerol gradient. Absorbance at 260 nm of the gradient was determined using a flow cell detector (solid line), and nascent RNA in each fraction measured (O). Fraction 1 represents the top of the gradient.

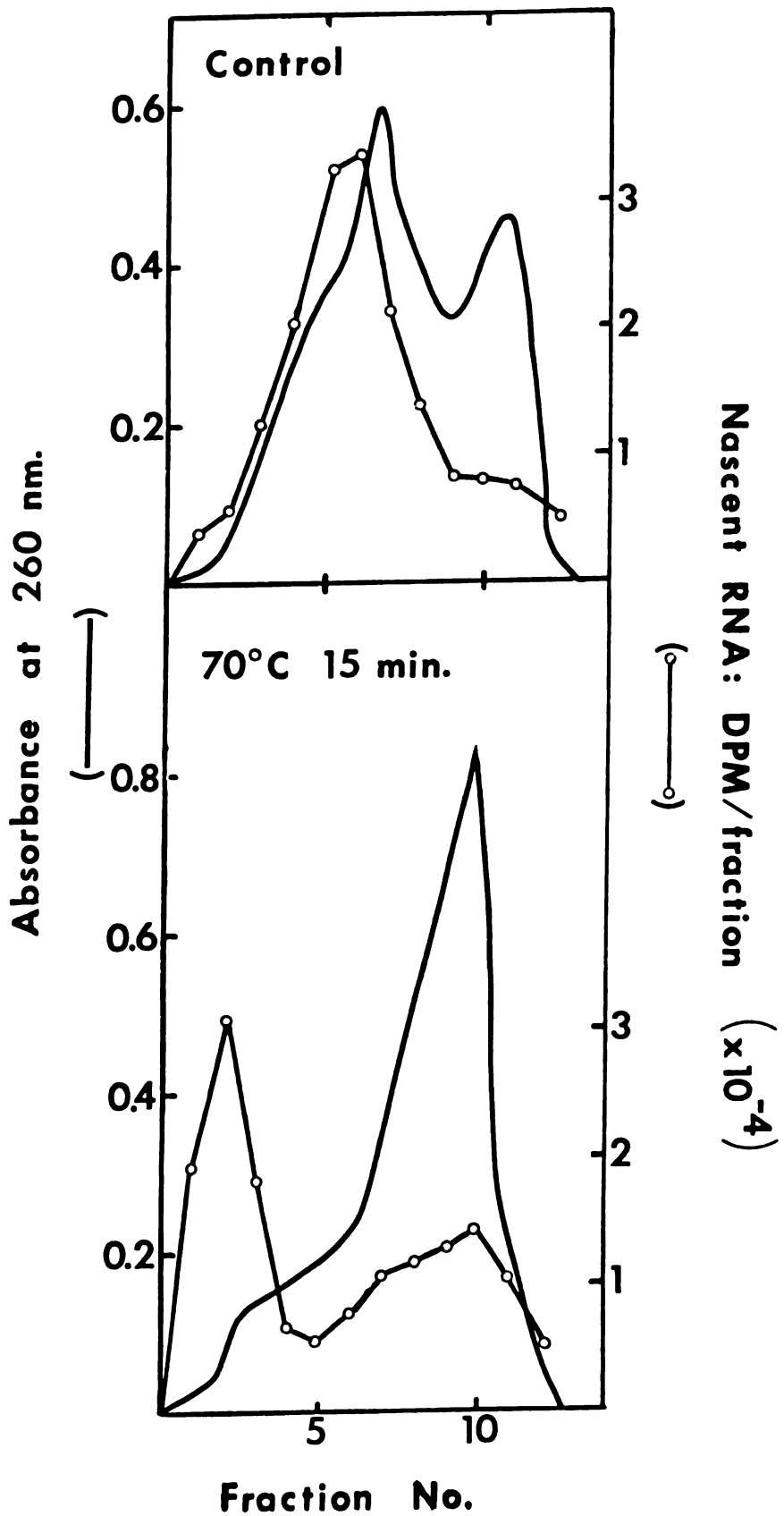

Control
0.6
0.4
0.2
3
2
1
70°C 15 min.
0.8
0.6
0.4
0.2
3
2
1
5
10
Absorbance at 260 nm.
Nascent RNA: DPM/fraction (x10⁻⁴)
Fraction No.

Figure 3

TABLE 3

Chromatin Fractionation by Selective $MgCl_2$ Precipitation

Chromatin Fraction[a]	DNA (%)	Nascent RNA (DPM/μg DNA)
I. $MgCl_2$ soluble[b]	9.4±2.5	851±206
II. $MgCl_2$ insoluble[c]	76.5±3.6	126± 45
III. Insoluble[d]	13.5±0.3	40± 1.3

[a]Sonicated chromatin was prepared as described in Methods.

[b]Supernatant after centrifugation at 2000 x g for 15 min in the presence of 1.75 mM $MgCl_2$.

[c]Precipitate after centrifugation at 2000 x g for 15 min in the presence of 1.75 mM $MgCl_2$ and after subtraction of values from III.

[d]Precipitate after centrifugation at 2000 x g for 15 min in the presence of $MgCl_2$.

apparent improved separation over that obtained with the glycerol gradient fractionation procedures. The relative purity of the $MgCl_2$ soluble chromatin, as measured by the amount of nascent RNA, is 7-20-fold when compared to the $MgCl_2$ insoluble or soluble chromatin.

Because the $MgCl_2$ and the glycerol gradient fractionation procedures take advantage of related, but not necessarily identical physicochemical properties of chromatin as the basis for fractionation, it was of interest to directly compare these two methods. Sonicated chromatin was prepared and fractionated on a glycerol gradient. Gradient fractions were pooled as indicated in Figure 2. These were then fractionated by selective precipitation with $MgCl_2$ (1.75 mM, final concentration), and the amount of chromatin DNA that was $MgCl_2$ soluble, and $MgCl_2$ insoluble was determined (Table 4).

To examine whether the pelleted chromatin represents only insufficiently sonicated chromatin, samples were resuspended, divided into five aliquots of approximately 20 μg chromatin-DNA, and resonicated for various times (Figure 4). The amount of pelletable material was then determined. Following a very brief sonication (15 sec), a large portion of the chromatin DNA is no longer pelletable. However, even with extensive sonication, 40% of the initial pelleted chromatin cannot be transformed into $MgCl_2$ soluble or $MgCl_2$ insoluble chromatin. This suggests that a portion of the pelleted chromatin exists in a different conformation than the remainder of the chromatin, not related to differences in the size of the chromatin fragments.

An attempt was made to identify particular chromatin fractions based on the presence of specific endogenous RNA polymerases (RNA

TABLE 4

Comparison of Glycerol Gradient and $MgCl_2$ Chromatin Fractionations

Glycerol Gradient Fraction[a]		$MgCl_2$ Fraction[b]		
		I	II	III
Euchromatin	DNA (%)	85.1±1.8	12.2±1.9	4.5±0.6
	Nascent RNA[c]	244±26		
Heterochromatin	DNA (%)	15.1±2.3	74.0±5.1	8.0±0.4
	Nascent RNA		45±6	
Pelleted Heterochromatin	DNA (%)	13.9±6.5	20.6±6.5	63.5±1.7
	Nascent RNA			75±14

[a]Fractions pooled as designated in Figure 2.

[b]See Table 3.

[c]Rats pretreated as described in legend to Figure 2; all values are mean ± S.E.M. of 3 rats.

Figure 4. Effect of resonication on the solubility of pelleted chromatin. Pelleted chromatin was prepared as described (fraction III, Table 3). After resuspension in 8% glycerol, 10 mM Tris (pH 7.9), the pelleted chromatin was divided into 5 portions (each containing approximately 20 μg DNA) and sonicated for the times indicated (0-90 sec). The amount of chromatin-DNA that could be repelleted (2000 g for 15 min) was then determined.

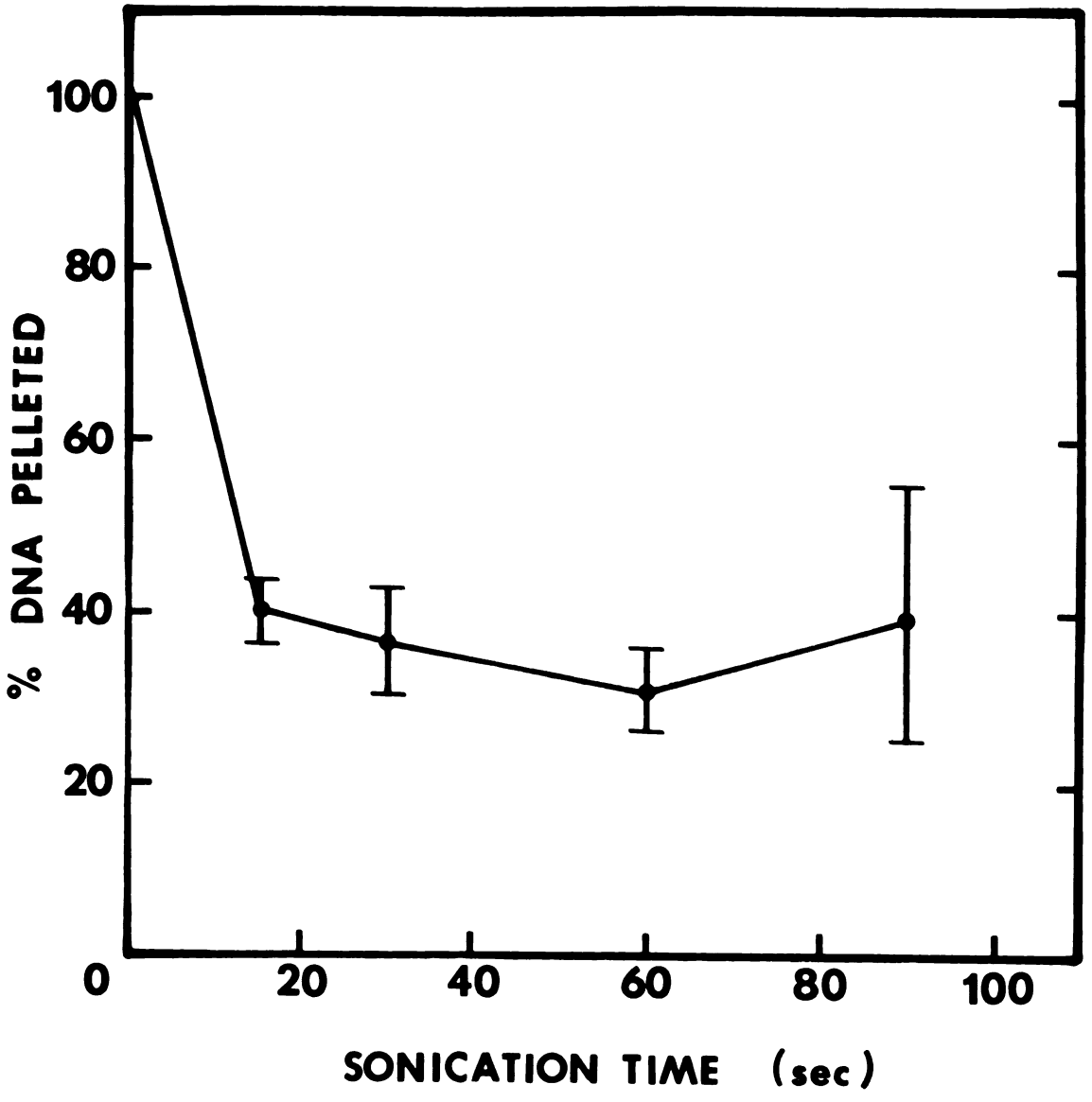

100
80
60
40
20
0
% DNA PELLETED
20
40
60
80
100
SONICATION TIME (sec)

Figure 4

polymerase I = nucleolar regions; RNA polymerase II = nucleoplasmic). No endogenous RNA polymerase activity could be detected in the isolated chromatin, however, using either an endogenous DNA template or added calf thymus DNA. A variety of assay conditions were tried, including $(NH_4)_2SO_4$ concentrations of 75 to 300 mM with either $MnCl_2$ or $MgCl_2$. It has recently been reported (41) that the endogenous RNA polymerase is extremely labile and all enzyme activity may be lost within 30 minutes of chromatin extraction, thus making its measurement incompatible with most chromatin fractionation procedures.

2. Assessment of the Purity of Radiolabelled N-OH-AAF

To assure that carcinogen residues were in fact being assayed in binding studies, the purity of the [ring-^{3}H]N-hydroxy-2-acetylaminofluorene was determined. Approximately 5 µg of material was subject to solid probe analysis on a mass spectrometer. The bulk of the material had a mass spectrum as illustrated in Figure 5. Besides the parent compound of MW 239, molecular ions consistent with the structure of N-OH-AAF were seen at m/e = 223, 197, 180 and 165. The molecular ion scan of this material is shown in Figure 6. The total ion scan (lowest panel) shows 2 peaks representing 85% and 15% of the total material. The major peak had molecular ions of 180, 223 and 239, consistent with the structure in Figure 5. The smaller peak had molecular ions at m/e of 211 and 195, and represents an unidentified contaminant.

This material was also analyzed by thin layer chromatography using 2 solvent systems designed to separate various hydroxylated fluorene residues (129). The systems used were: Polygram cellulose

Figure 5. Mass spectral analysis of [^{3}H]-N-hydroxy-2-acetylaminofluorene (New England Nuclear Lot #773-209).

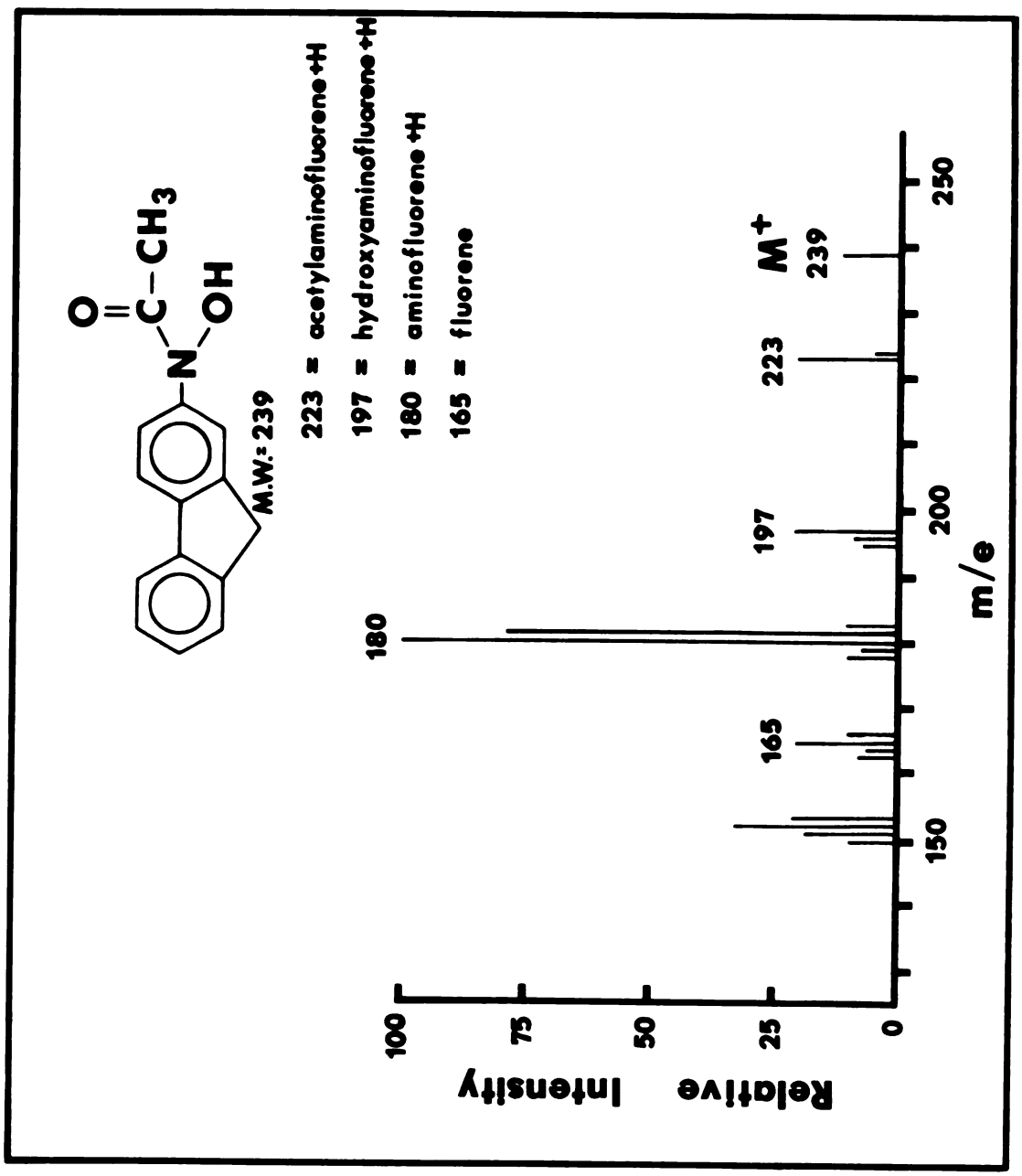

O
C
CH3
N
OH
M.W.: 239
223 = acetylaminofluorene+H
197 = hydroxyaminofluorene+H
180 = aminofluorene+H
165 = fluorene
Relative Intensity
100
75
50
25
0
180
165
150
197
223
M+
239
200
250
m/e

Figure 5

Figure 6. Molecular ion distribution of [^{3}H]-N-hydroxy-2-acetylaminofluorene. Numbers on the abscissa represent individual scans obtained at increasing probe temperature (25-200°C), the ordinate is relative intensity. The lower panel is the total ion scan; representative individual ions are also illustrated.

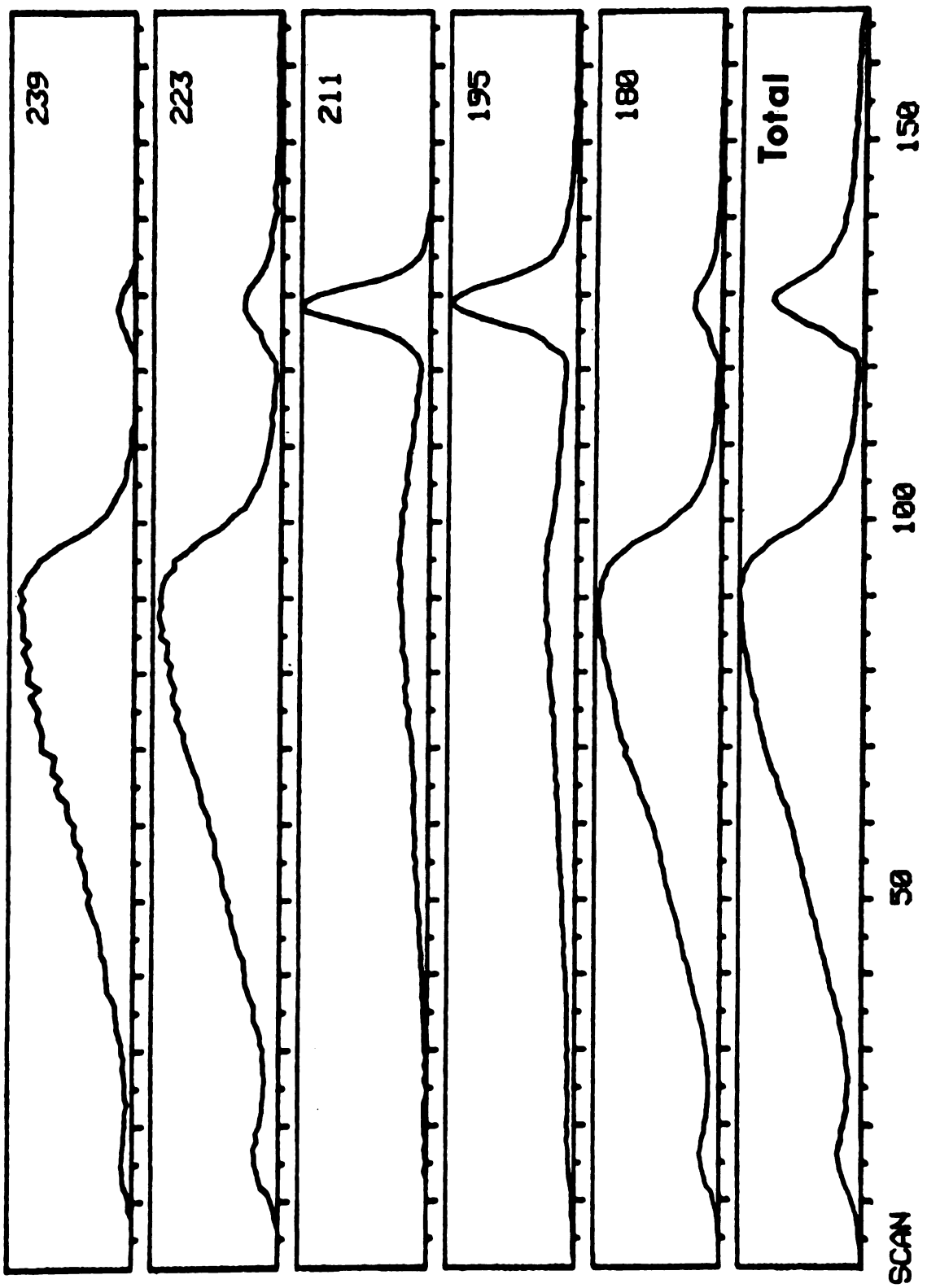


Figure 6

300 with a cyclohexane:t-butanol:acetic acid:H_2O (160:5:15:20) solvent, and Silica Gel G with a isopropranol:ammonium hydroxyde (2:1) solvent. In both cases, all the radioactivity co-chromatographed with the major UV absorbing spot (R_f = 0.5), demonstrating that the tritium label was indeed associated with N-OH-AAF.

3. Analysis of Carcinogen Binding and Loss

Carcinogen binding to cellular components was determined 2 hours after a single injection of ^{3}H-N-OH-AAF, the time of maximal binding. Approximately 2% of injected carcinogen is covalently bound to material in the liver (Table 5). Of this, less than 5% is located in the nucleus, and less than 10% of the carcinogen in the nucleus is associated with the DNA.

The time course of carcinogen binding to chromatin protein, RNA and DNA was examined after a single injection of tracer amounts (0.4 μmoles/100 g body weight) of [^{3}H]-N-hydroxy-2-acetylaminofluorene (^{3}H-N-OH-AAF). Two hours after injection, most of the carcinogen on a weight basis (i.e., per mg RNA, protein or DNA) was associated with the chromatin RNA, with smaller amounts associated with the chromatin proteins and DNA (Figure 7).

As illustrated in Figure 7, loss of the carcinogen from chromatin proteins appears to be first order over the time period examined. In contrast, no carcinogen could be detected on the chromatin RNA after 7 and 10 days. A portion of the carcinogen appears to be persistently bound to the DNA based on the 10-15% of the amounts observed at 2 hours remaining bound for 7-10 days. There were no significant

TABLE 5

Analysis of Initial Carcinogen Binding to Cellular Components[a]
(Male Sprague-Dawley Rats)

Carcinogen injected i.p. (100 μCi/0.48 mg/100 g BW)	3.9×10^8 dpm	
Hepatic Acid-Insoluble	7.8×10^6 dpm	100%
Hepatic Nuclear Acid-Insoluble	3.7×10^5 dpm	4.7%
Nuclear Protein	2.5×10^5 dpm	3.2%
Nuclear RNA	0.75×10^5 dpm	0.92%
Nuclear DNA	0.30×10^5 dpm	0.38%

[a]Animals were injected with [ring ^{3}H]-N-hydroxy-2-acetylaminofluorene (50.2 mCi/mmole) and were sacrificed after 2 hours. Cellular components were separated as described in Methods.

Figure 7. Binding of carcinogen to chromatin components. Male rats were injected with ^{3}H-OH-AAF (20 μCi/100 g) and sacrificed after 2 hours and 4, 7 and 10 days. Chromatin was isolated from hepatic nuclei and separated into component protein, RNA, and DNA as described in Methods, and the amount of radioactivity in each was determined. The rats were maintained either on a control diet (●), or one containing 0.03% (w/w) AAF (○). Each point represents the mean ± SEM of 3-7 rats.

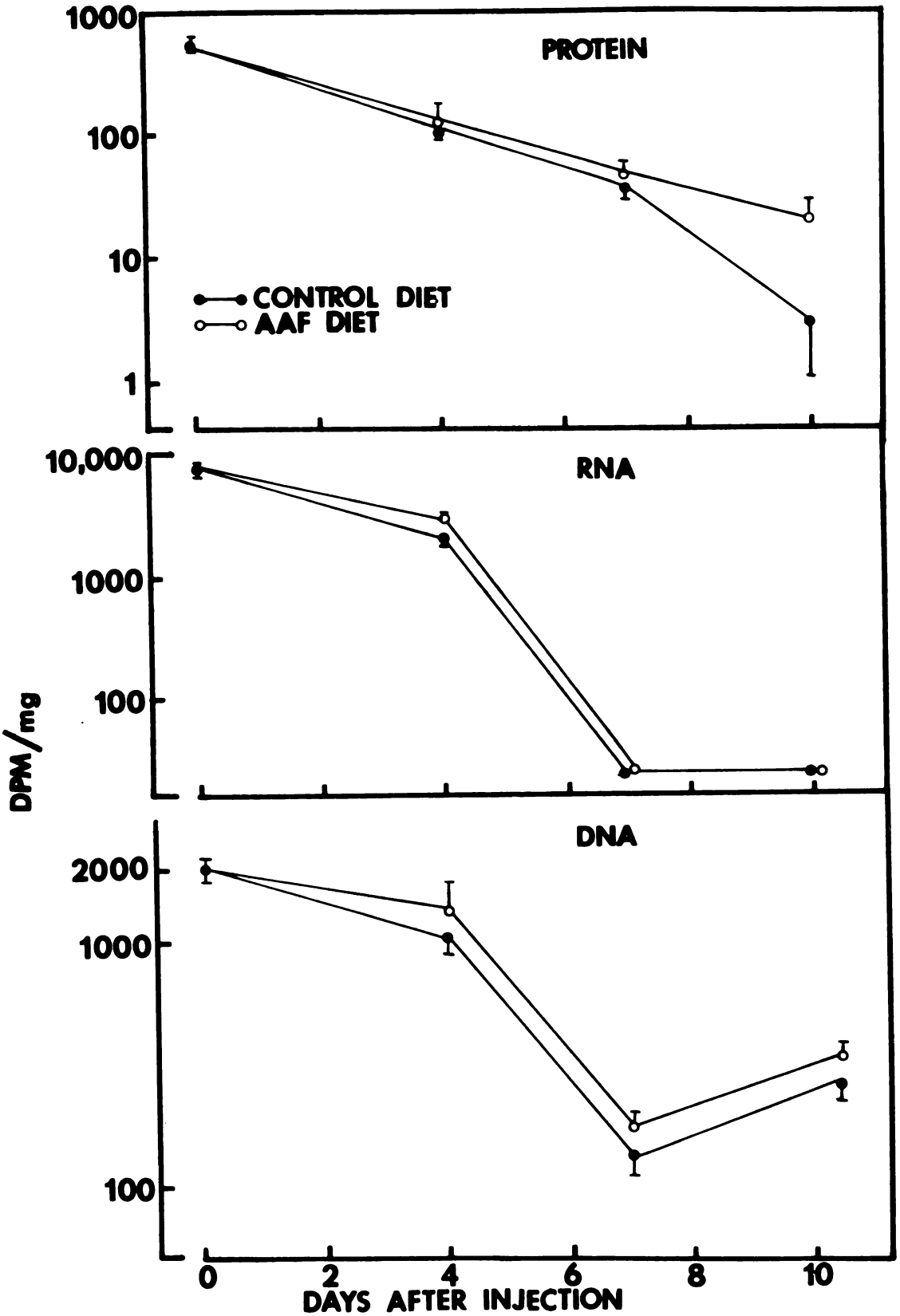

PROTEIN
1000
100
10
1
CONTROL DIET
AAF DIET
RNA
10,000
1000
100
DNA
2000
1000
100
DPM/mg
0
2
4
6
8
10
DAYS AFTER INJECTION

Figure 7

differences in the loss of carcinogen from chromatin protein, RNA or DNA between rats maintained on a control diet or one containing AAF.

To analyze the location of carcinogen binding within the genome, sonicated chromatin isolated 2 hrs after an injection of ^{3}H-N-OH-AAF was centrifuged on a glycerol gradient. The DNA-bound carcinogen in each fraction was then determined. As seen in Figure 8, two hours after a single injection of ^{3}H-N-OH-AAF the largest amount of carcinogen is bound to DNA in the less condensed chromatin. There is progressively less DNA-bound carcinogen in the more condensed chromatin on the glycerol gradient, while the level of binding of the carcinogen to DNA in the pelleted heterochromatin is intermediate. Although the extent of carcinogen binding appears to be inversely correlated with the density of the chromatin, several fractions within the eu- and heterochromatin (e.g., 3-6 and 9-12) appear to be uniformly labelled. Individual gradient fractions were pooled as indicated at the top of Figure 8, and the results from several experiments were compared (Table 6). When the genome was fractionated on a glycerol gradient, there was significantly more carcinogen bound to the DNA in the euchromatin, when compared to the DNA in the heterochromatin.

Chromatin can also be fractionated based on the enhanced susceptibility of the more condensed portions of the genome to precipitation by divalent cations. The euchromatin prepared by this method remains soluble in the presence of 1.75 mM $MgCl_2$, and contained significantly more DNA-bound carcinogen when compared to the heterochromatin or the pelleted heterochromatin DNA.

TABLE 6

Initial Binding of ^{3}H-OH-AAF to Chromatin DNA[a]

	DPM/mg DNA	AAF/10^6 Nucleotides[c]
Glycerol gradient fractionation[b]		
Euchromatin DNA	2719± 365[e]	7.80
Heterochromatin DNA	1592± 375	4.57
Pelleted Heterochromatin DNA	2143± 188	6.15
$MgCl_2$ fractionation[d]		
Euchromatin DNA	9080±1770[f]	26.10
Heterochromatin DNA	1350± 100	3.87
Pelleted Heterochromatin DNA	2420± 590	6.95

[a]Chromatin was fractionated from rats 2 hours after a single injection of ^{3}H-N-OH-AAF (20 μCi/100 g). The DNA in each chromatin fraction was hydrolyzed as described in Methods, and an aliquot was used for liquid scintillation counting. Values represent means ± SEM of 4-7 rats.

[b]Pooled gradient fractions as indicated in Figure 8.

[c]Calculated assuming 1.9×10^{18} nucleotides/mg DNA.

[d]Chromatin was fractionated based on selective precipitation with 1.75 mM $MgCl_2$, as described in Methods.

[e]Indicates significantly different from heterochromatin DNA, Student-Newman-Keuls test, $p<.05$.

[f]Indicates significantly different from both heterochromatin and pelleted heterochromatin DNA, Student-Newman-Keuls test, $p<.05$.

Figure 8. Distribution of DNA-bound carcinogen in glycerol gradient chromatin fractions. Rats were injected with 20 μCi/100 g of ^{3}H-OH-AAF, and sacrificed after 2 hours. Chromatin was isolated and fractionated on a glycerol gradient as described in Methods. Sedimentation was from left to right, and values for the chromatin which sedimented to the bottom of the gradient are indicated as well. The amount of DNA (●) and DNA-bound carcinogen (O) in each fraction was determined. Each point is the mean ± SEM obtained from 4 rats.

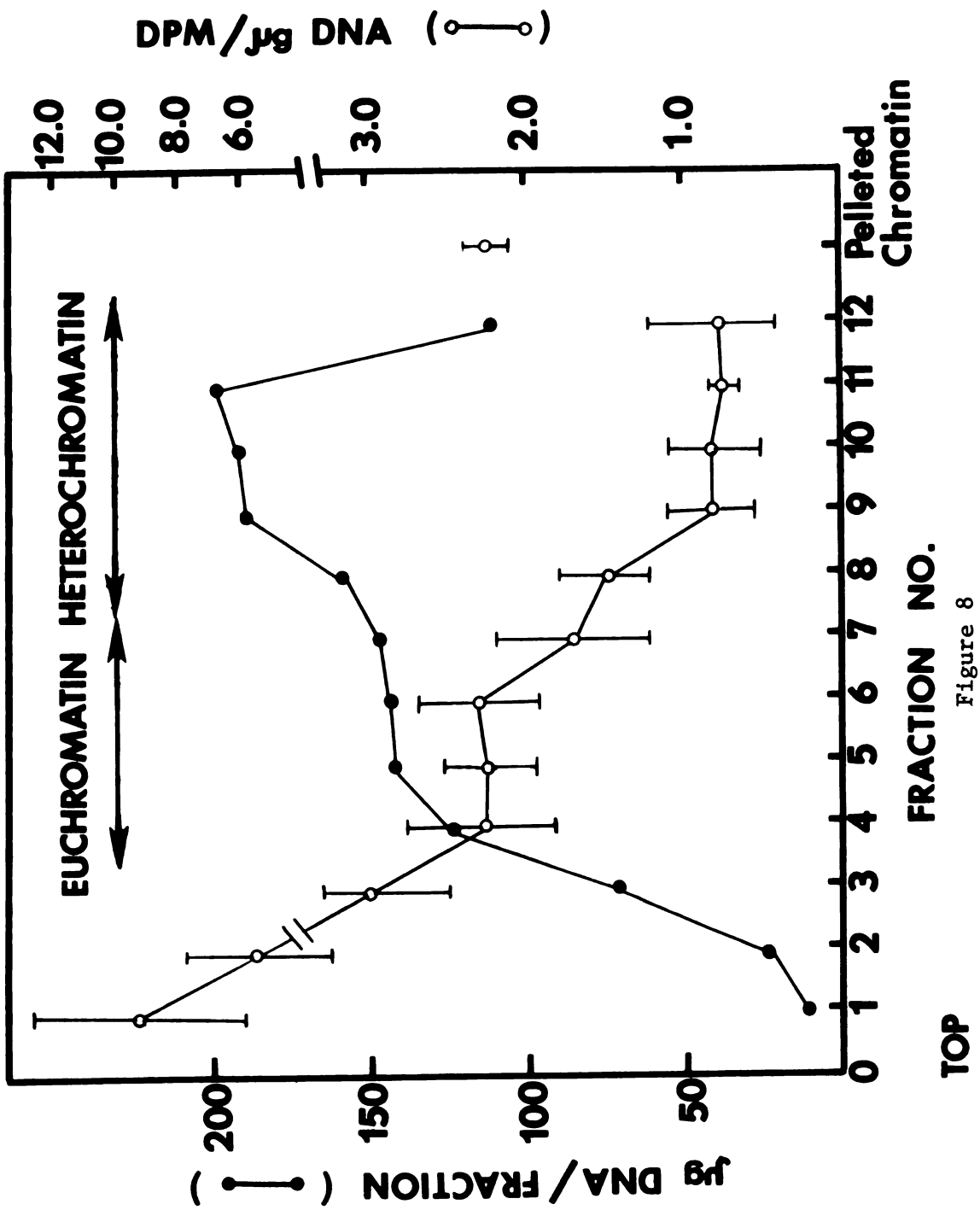

DPM/µg DNA (o—o)
12.0
10.0
8.0
6.0
3.0
2.0
1.0
EUCHROMATIN
HETEROCHROMATIN
Pelleted Chromatin
0 1 2 3 4 5 6 7 8 9 10 11 12
FRACTION NO.
TOP
200
150
100
50
µg DNA/FRACTION (•—•)

Figure 8

Loss of carcinogen from DNA and protein of the 3 chromatin fractions (Table 7) was then examined. First order rate constants were determined from the slope of a semi-logarithmic plot of the amount of carcinogen bound vs. time. Animals received a single injection of ^{3}H-N-OH-AAF and were sacrificed 2 hrs and 4, 7 and 10 days later. Animals were maintained, during this time, on a basal diet or one containing AAF (0.03% w/w). Carcinogen bound to chromatin DNA and chromatin proteins were determined. All rate constants for loss of carcinogen from DNA were calculated with values obtained from a minimum of 10 rats, the material from each rat was analyzed separately, and the correlation coefficients for these lines were statistically ($p<0.05$) significant in all instances. The loss of carcinogen from the pelleted heterochromatin DNA was significantly slower than from either the eu- or heterochromatin DNA after fractionation on a glycerol gradient. Loss of carcinogen from the pelleted heterochromatin DNA was only significantly slower than from the euchromatin DNA, when fractionated with $MgCl_2$. Despite the fact that the initial amount of carcinogen bound to the "$MgCl_2$" euchromatin DNA was larger than that bound to the "glycerol gradient" euchromatin DNA (Table 6), the rate of removal of carcinogen was approximately the same in both cases. Maintaining the rats on an AAF diet reduces the differences seen in the rate of loss of carcinogen between the chromatin fractions. This appears to result from an enhanced rate of loss from the pelleted heterochromatin DNA, and a decreased rate of loss from the euchromatin DNA. There was no apparent correlation between the loss

TABLE 7

Rate of Carcinogen Loss from Chromatin DNA and Chromatin Proteins[a]

	Glycerol Gradient[b]				$MgCl_2$[c]	
	DNA		Protein		DNA	
	Rate	r	Rate	r	Rate	r
Control Diet						
Euchromatin	35.8	0.91	23.9	0.87	32.1	0.93
Heterochromatin	30.8[e]	0.81	10.5	0.44	20.6[d]	0.89
Pelleted Heterochromatin	11.4[e]	0.64	19.9	0.82	14.7[d]	0.68
0.03% AAF Diet						
Euchromatin	26.6	0.90	16.3	0.84	25.2	0.83
Heterochromatin	34.5[d]	0.74	5.9	0.33	18.9	0.95
Pelleted Heterochromatin	17.3[d]	0.95	24.4	0.95	18.0	0.72

[a] First order rate constant of elimination (percent/day) was calculated from a semilogarithmic plot of carcinogen bound vs. time. The correlation coefficient (r) of the line was also determined. Each rate constant was calculated with values from at least 10 rats and 3-4 time points.

[b] Glycerol gradient fractions were pooled as indicated in Figure 8. Animals were sacrificed 2 hours and 4, 7 and 10 days after injection with ^{3}H-OH-AAF (20 μCi/100 g).

[c] Chromatin fractionated with 1.75 mM $MgCl_2$ as described in Methods. Animals were sacrificed 2 hours and 4 and 7 days after ^{3}H-OH-AAF.

[d] Indicates significantly different from euchromatin DNA, t-test $p<.05$.

[e] Indicates significantly different from both eu- and heterochromatin DNA, t-test, $p<.05$.

of carcinogen from the protein of a chromatin fraction when compared with the DNA of the fraction.

In Figures 9 and 10 the data regarding loss of carcinogen from DNA has been adjusted to take into account differences in the relative amounts of DNA in the 3 chromatin fractions. Thus, initially 48% of the total carcinogen bound to DNA is found on the euchromatin DNA, while 29% and 23% are found in the heterochromatin and pelleted heterochromatin DNA, respectively. Due to the low rate of carcinogen loss from the pelleted heterochromatin DNA, however, after 7-10 days the greatest amount of carcinogen remains bound to this fraction, while less than 5% of initial bound carcinogen is found on either the eu- or heterochromatin DNA. This appears to be the case for both rats maintained on a basal diet (Figure 9) and those maintained on the AAF diet (Figure 10).

4. Analysis of Carcinogen Binding to DNA by Site

To both verify the covalent nature of the carcinogen binding to DNA and to assess potential qualitative differences which might result in different rates of removal, DNA-purines were examined by silica gel thin layer chromatography. Two hours after injection with ^{3}H-OH-AAF (100 μCi/100 g), 2 peaks of radioactivity representing DNA-bound carcinogen were obtained (Figure 11). The major peak (R_f = 0.75) co-chromatographed with an authentic standard of N-(guanin-8-yl)-amino-fluorene (obtained from Dr. F. Beland, National Center for Toxicological Research). This material was analyzed by solid probe analysis on a mass spectrometer, and the spectra is illustrated in Figure 12. Besides the parent compound of MW = 152, molecular ions corresponding

Figure 9. Loss of carcinogen from chromatin DNA (control diet). Rats were injected with ^{3}H-OH-AAF (20 μCi/100 g) and maintained on a control diet. Chromatin was fractionated on a glycerol gradient in euchromatin (■), heterochromatin (●) and pelleted heterochromatin (▲). DNA was isolated from each chromatin fraction as described in Methods. The total amount of DNA-bound carcinogen in unfractionated chromatin after 2 hours was set at 100% and the relative amount of carcinogen in each chromatin-DNA fraction was determined at the various time points. Each point represents the mean ± SEM of 3-7 rats.

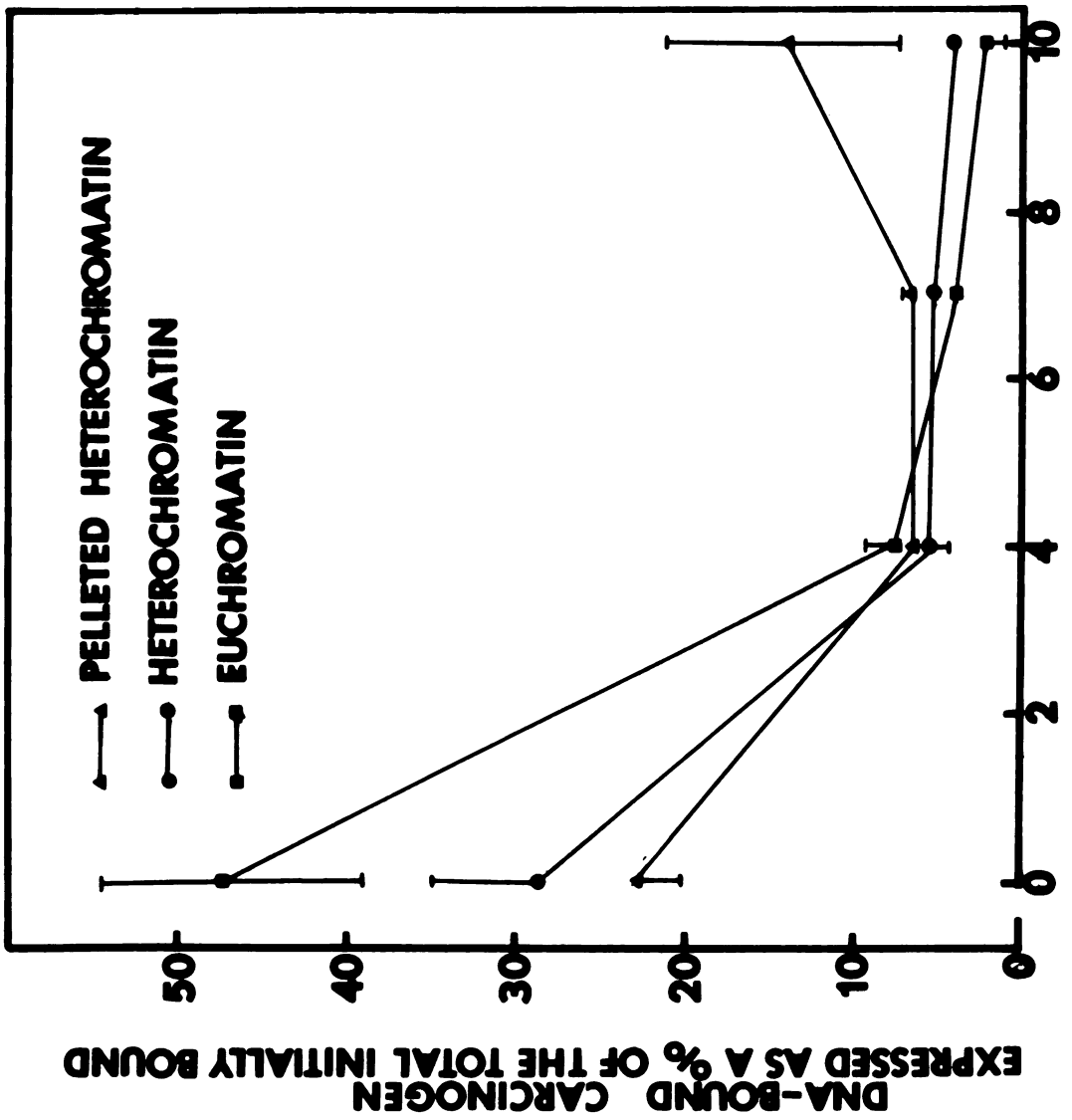

PELLETED HETEROCHROMATIN
HETEROCHROMATIN
EUCHROMATIN
DNA-BOUND CARCINOGEN
EXPRESSED AS A % OF THE TOTAL INITIALLY BOUND
0
10
20
30
40
50
0
2
4
6
8
10
DAYS AFTER INJECTION

Figure 9

Figure 10. Loss of carcinogen from chromatin DNA (AAF diet). Experiments were carried out as described in Figure 9 except that rats were maintained on a 0.03% (w/w) AAF diet.

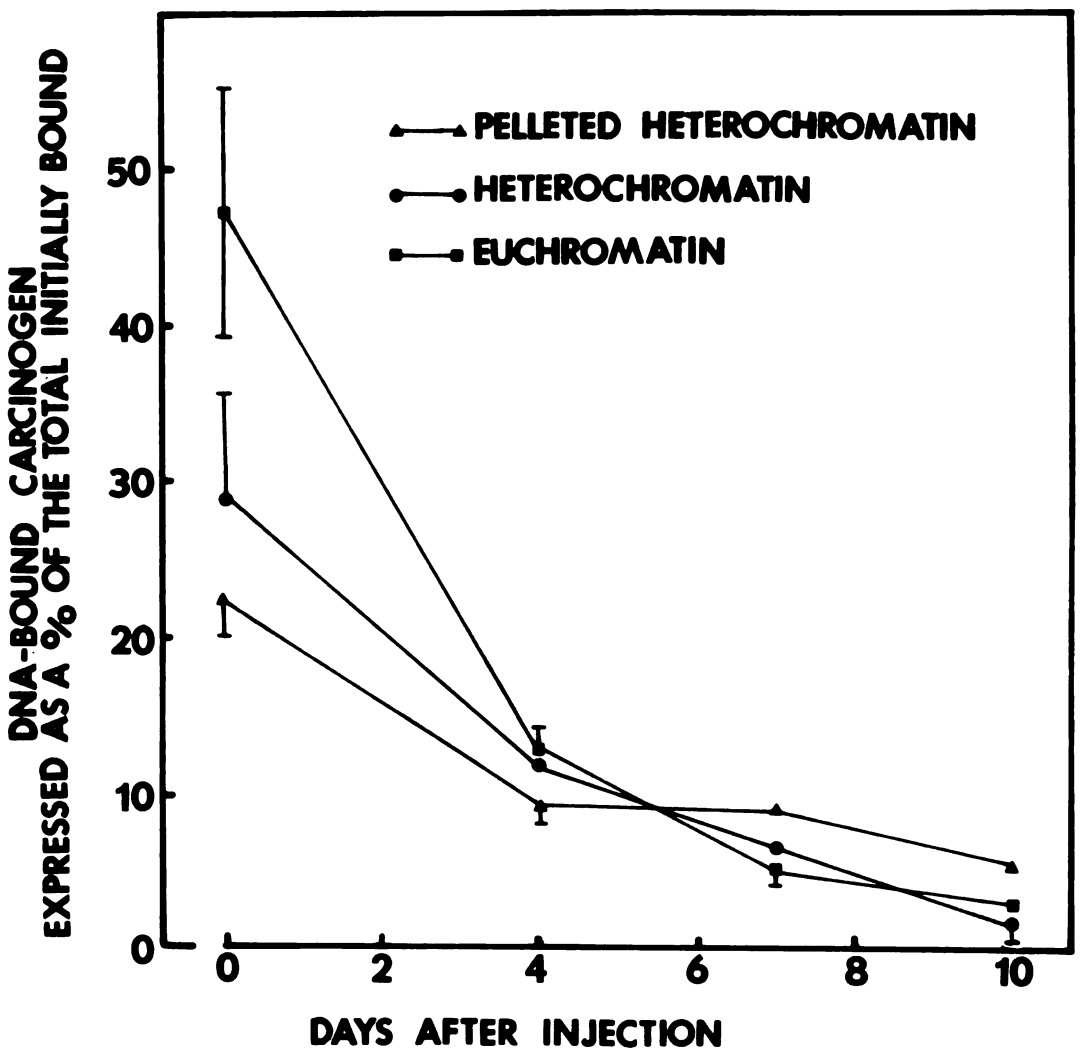

PELLETED HETEROCHROMATIN
HETEROCHROMATIN
EUCHROMATIN
DNA-BOUND CARCINOGEN
EXPRESSED AS A % OF THE TOTAL INITIALLY BOUND
0
10
20
30
40
50
0
2
4
6
8
10
DAYS AFTER INJECTION

Figure 10

Figure 11. Thin layer chromatography of carcinogen-base adducts. Rats were sacrificed 2 hours after injection with ^{3}H-OH-AAF (100 µCi/100 g). RNA-purines were hydrolyzed in 0.3 N KOH (37°C 60 min) from hepatic chromatin followed by hydrolysis of DNA-purines in 0.3 N HCl (60°C 40 min). Purification on Sephadex LH-20 was as described in Methods. Samples were spotted on Silica gel TLC plates and run with a n-butanol:acetic acid:H_2O solvent. Portions of the silica gel plate were scraped and the radioactivity determined. Bars represent the locations of guanine and adenine standards, and the C8-GUA-AF adduct.

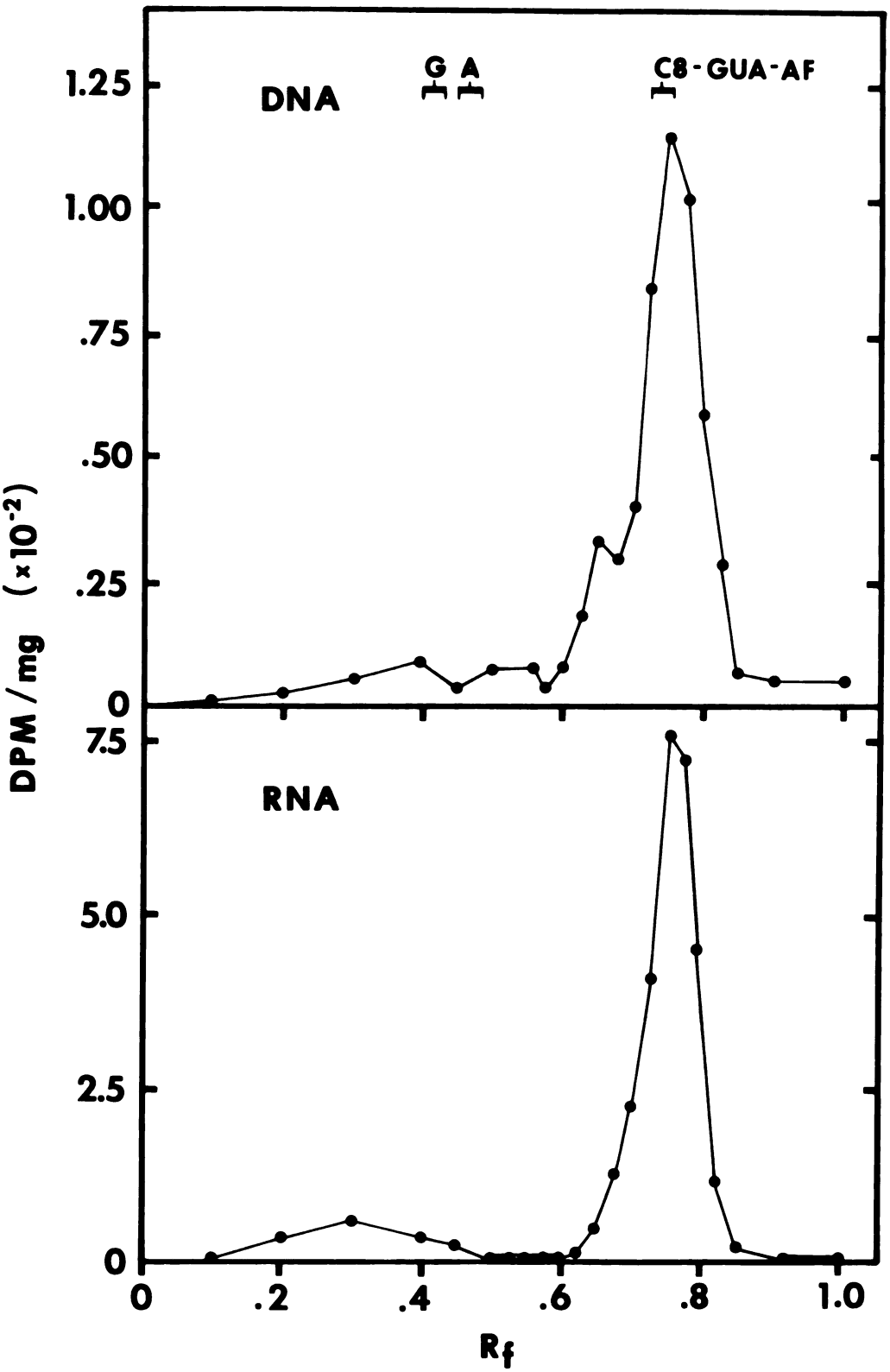

DNA
G A
C8-GUA-AF
1.25
1.00
.75
.50
.25
0
RNA
7.5
5.0
2.5
0
0
.2
.4
.6
.8
1.0
DPM/mg (x10⁻²)
Rf

Figure 11

Figure 12. Mass spectral analysis of N-(guanin-8-yl)-2-aminofluorene. Molecular ions of 330 (parent compound), 180 (aminofluorene), 165 (fluorene), and 152 (guanine) were observed. The thin layer chromatography of this material is illustrated in Figure 11 (C8-GUA-AF).

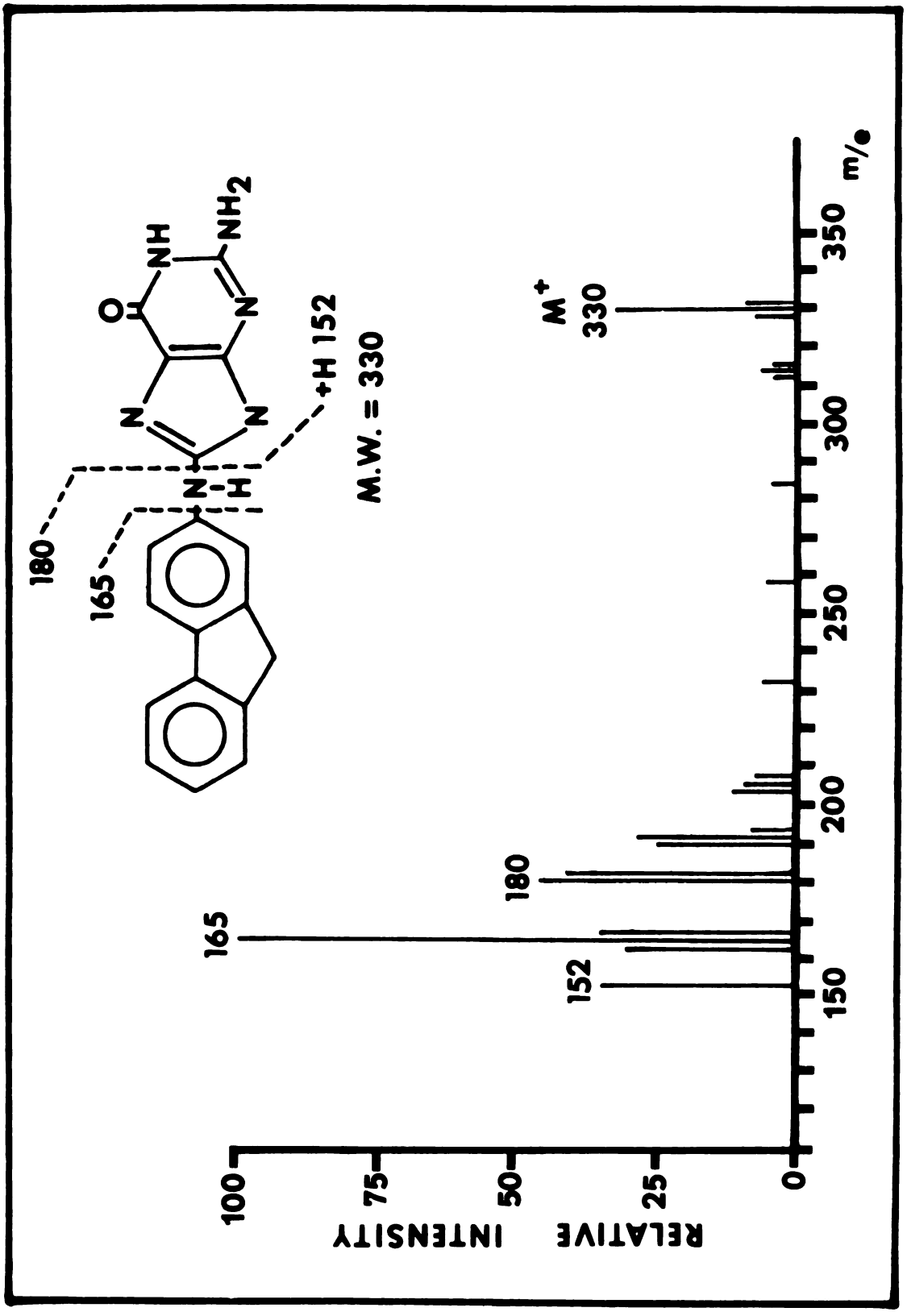

RELATIVE INTENSITY
100
75
50
25
0
150
200
250
300
350
m/e
165
180
152
M+
330
M.W. = 330
+H 152
180
165
NH
NH2
O
N
N
N
H

Figure 12

to aminofluorene (180), fluorene (165), and guanine (152) can be seen. A minor peak of radioactivity (R_f = 0.67) was also reproducibly obtained from DNA purines. The minor peak had a R_f value identical to that reported by Westra et al. (205) for 3-(guanin-N^2-yl)-acetyl-aminofluorene. Furthermore, as illustrated in the lower panel of Figure 11, this adduct could not be detected in purines isolated from RNA, in agreement with previously published reports (106). When the distribution of carcinogen bound to these two guanine sites was examined, the pelleted heterochromatin DNA (prepared by $MgCl_2$ precipitation) had a significantly greater proportion of the N^2-guanine adduct than did either the eu- or heterochromatin DNA (Table 8).

5. DNAse II:$MgCl_2$ Fractionation of Chromatin and Analysis of Carcinogen-DNA Binding

To confirm this nonrandom distribution of carcinogen adducts within the genome, a third chromatin fractionation procedure was utilized. As indicated in Figure 1, the selectivity of the DNAse II-$MgCl_2$ technique has been verified by rigorous criteria. This procedure has effected a separation of DNA gene sequences coding for specialized functions in differentiated tissue. The fractionation procedure is diagramed in Figure 13, and the time course of formation of the 3 chromatin fractions is illustrated in Figure 14. Although it has been previously demonstrated (81) that a very short DNAse II digestion (e.g., 2-6 min) results in the greatest enhancement of euchromatin genes in the S2 fraction, a digestion time of 30 min was chosen to insure a more complete separation of the heterochromatin (P2 fraction) from the nuclease resistant fraction (P1 fraction). As

TABLE 8

Thin Layer Chromatography of Carcinogen-Base Adducts[a]

Source[b]	% Total Radioactivity[c] N^2 Adduct	C8 Adduct
Unfractionated DNA	12±1.5%	88±1.5%
Euchromatin DNA	8±0.8%	92±0.8%
Heterochromatin DNA	11±2.8%[d]	89±2.8%
Pelleted Heterochromatin DNA	23±1.2%[d]	77±0.8%

[a]Animals were sacrificed 2 hours after injection of ^{3}H-N-OH-AAF (100 µCi/100 g). Silica gel thin layer chromatography and n-butanol:acetic acid: H_2O (50:11:25) were used to separate the adducts. Values are means ± SEM of 3 rats.

[b]$MgCl_2$ (1.75 mM) fractionation of chromatin as described in Methods. DNA was hydrolyzed from chromatin fractions and carcinogen modified bases were purified on a Sephadex LH-20 column as described in Methods.

[c]The radioactivity in each peak expressed as a percent of total CPM recovered on TLC plate. Average recovery was 1074±271 cpm/rat. Separation of carcinogen DNA adducts is illustrated in Figure 11.

[d]Indicates significantly different from both eu- and hetero-chromatin, Student-Newman-Keuls test, $p<.05$.

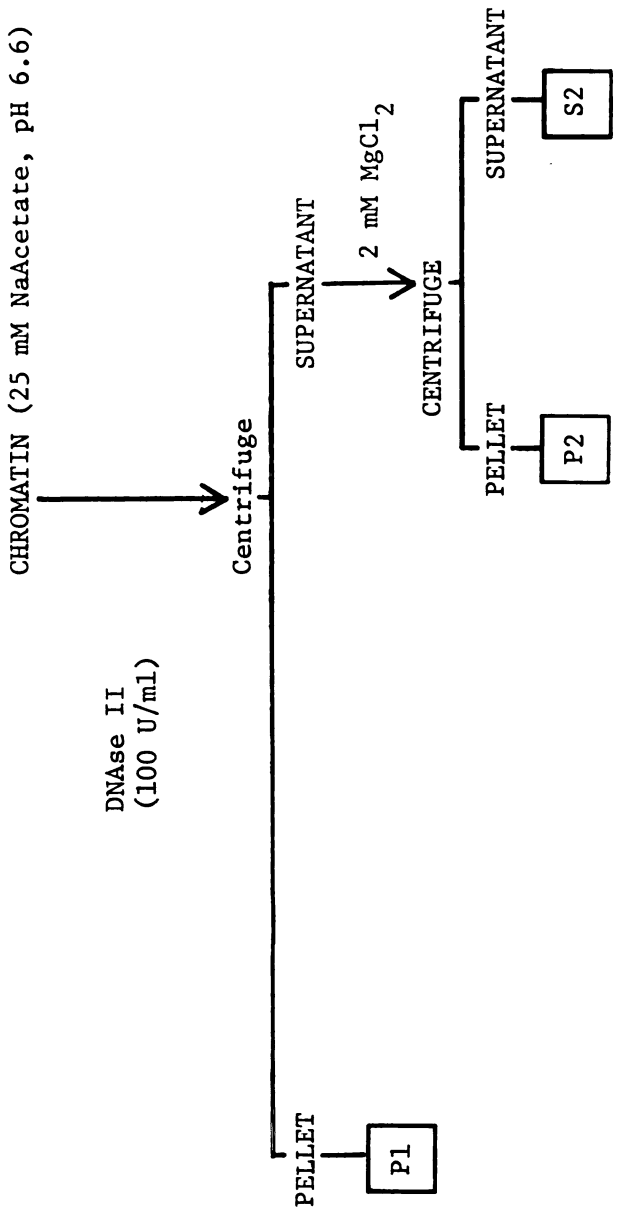


Figure 13. Diagramatic representation of chromatin fractionation by DNAse II-$MgCl_2$.

Figure 14. Time course of chromatin fractionation by DNAse II-$MgCl_2$. Chromatin was fractionated as illustrated in Figure 13 into P2, S2 and P1 fractions. The relative amount of DNA in each fraction was determined. The total amount of the original DNA which was acid insoluble (5% trichloroacetic acid) is indicated in the upper panel.

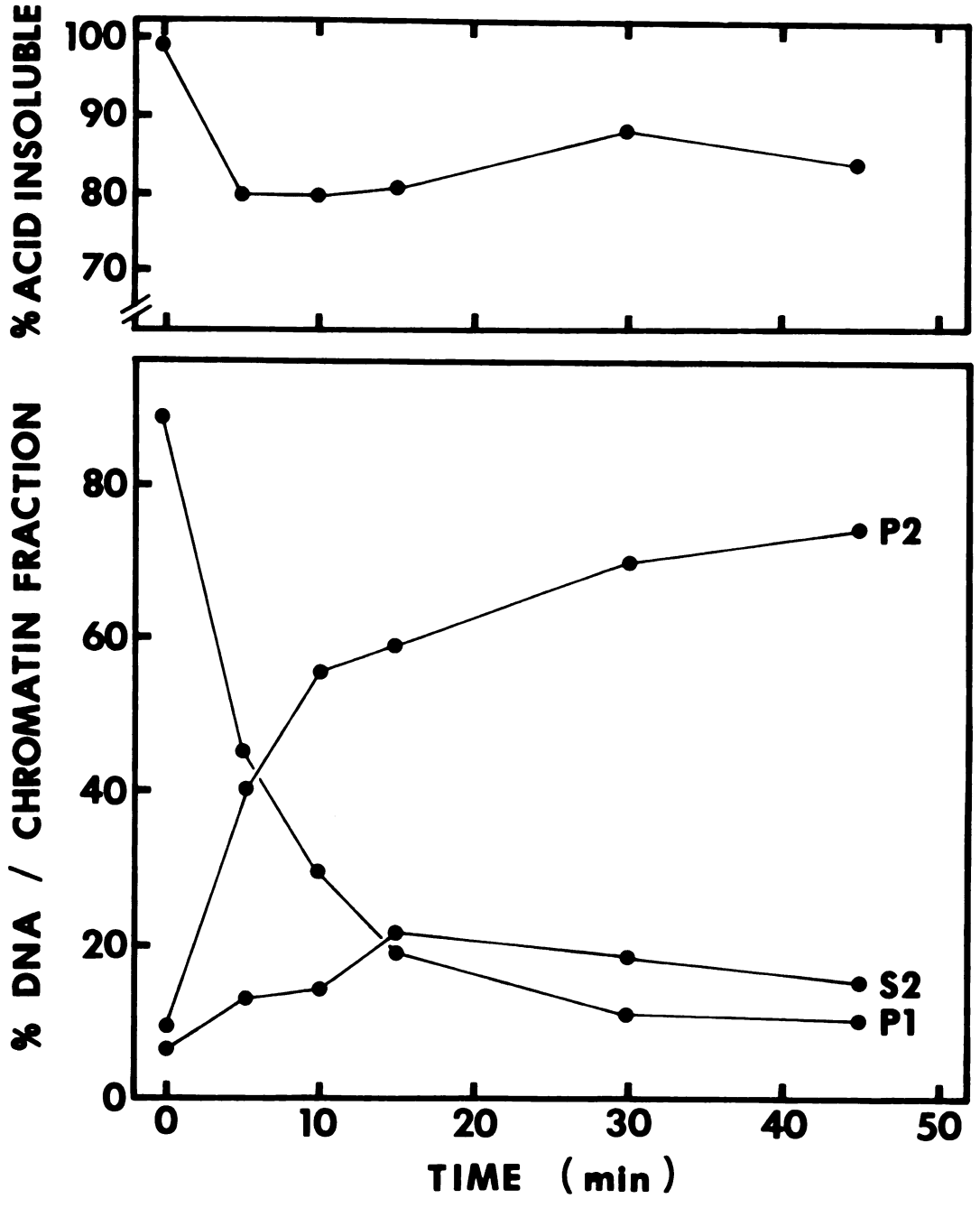

% ACID INSOLUBLE
100
90
80
70
% DNA / CHROMATIN FRACTION
80
60
40
20
0
P2
S2
P1
0
10
20
30
40
50
TIME (min)

Figure 14

indicated in the upper panel of Figure 14, DNAse II digestion is limited in that less than 20% of the DNA is rendered acid soluble; this action does not appear to be time dependent.

The binding of [^{3}H]-N-OH-AAF to DNA in the three DNAse II-$MgCl_2$ chromatin fractions is indicated in Table 9. In confirmation of the data presented in Table 6, carcinogen binding is greater in the DNA derived from euchromatin (S2) when compared to the DNA of the heterochromatin (P2). The DNA from the nuclease resistant chromatin fraction also had a greater extent of carcinogen bound when compared to the DNA of the P2 fraction. Analysis of the site of carcinogen binding showed there were significant differences in the relative amount of the N^2 adduct formed between all three chromatin fractions. It appeared that the chromatin fraction having the lowest total carcinogen bound (P2) had the greatest percent of carcinogen bound to the N^2 position of guanine. The reverse appeared to be true for the fraction containing the highest total carcinogen bound, fraction P1.

To examine this relationship more closely, data from chromatin fractions of each rat were plotted to compare the total extent of modification with the percent of that modification occurring on the N^2 position (Figure 15). Each point represents one DNA fragment obtained from chromatin which was fractionated with $MgCl_2$ after either sonication (closed circles) or DNAse II digestion (open circles). It appears that there is a clustering effect in terms of the location of the 2 guanine adducts; DNA fragments containing over 90% C8 and over 40% N^2 adducts were obtained. There also was a statistically significant ($p<0.01$) inverse correlation between the extent of modification

TABLE 9

Analysis of Carcinogen Binding to DNA: DNAse II-$MgCl_2$ Fractionation of Chromatin

	DNAse II Fraction		
	S2	P2	P1
DNA/Fraction (mg)	1.7±0.16	9.7±0.69	1.3±0.08
%	14±1.0%	76±1.2%	10±0.82%
DPM/mg DNA	1230±260	77±22[a]	1630±300
% N^2 Adduct	23±1.8%[b]	35±3.1%[b]	13±2.4%[b]
% C8 Adduct	77±1.8%	65±3.1%	87±2.4%
N^2 Adduct/Base Pair ($x10^6$)	1.57±0.38	0.24±0.06	1.50±0.38
C8 Adduct/Base Pair ($x10^6$)	5.31±1.20	0.43±0.05	10.4±3.7
N^2/C8 Ratio	0.30	0.54	0.15

Chromatin was digested with DNAse II (30 min) and fractionated with $MgCl_2$ as illustrated in Figure 13. Purines were isolated from S2, P2 and P1 chromatin by acid hydrolysis and purified on Sephadex LH-20 as described in Methods. Carcinogen-guanine adducts were separated as indicated in Figure 11. Values are means ± S.E.M. of 4 rats.

[a] Significantly different from P1 and S2, $p<.01$.

[b] Significant difference between all 3 chromatin fractions, $p<.01$.

Figure 15. Correlation between extent of carcinogen binding to DNA and percent as N^2 adduct. Purines were hydrolyzed from chromatin fractions and carcinogen-base adducts were analyzed as in Figure 11. Chromatin was fractionated by $MgCl_2$ after either DNAse II digestion (O) or sonication (●). Each point represents one chromatin fraction. The correlation coefficient ($r = 0.65$) was statistically significant ($p<.01$).

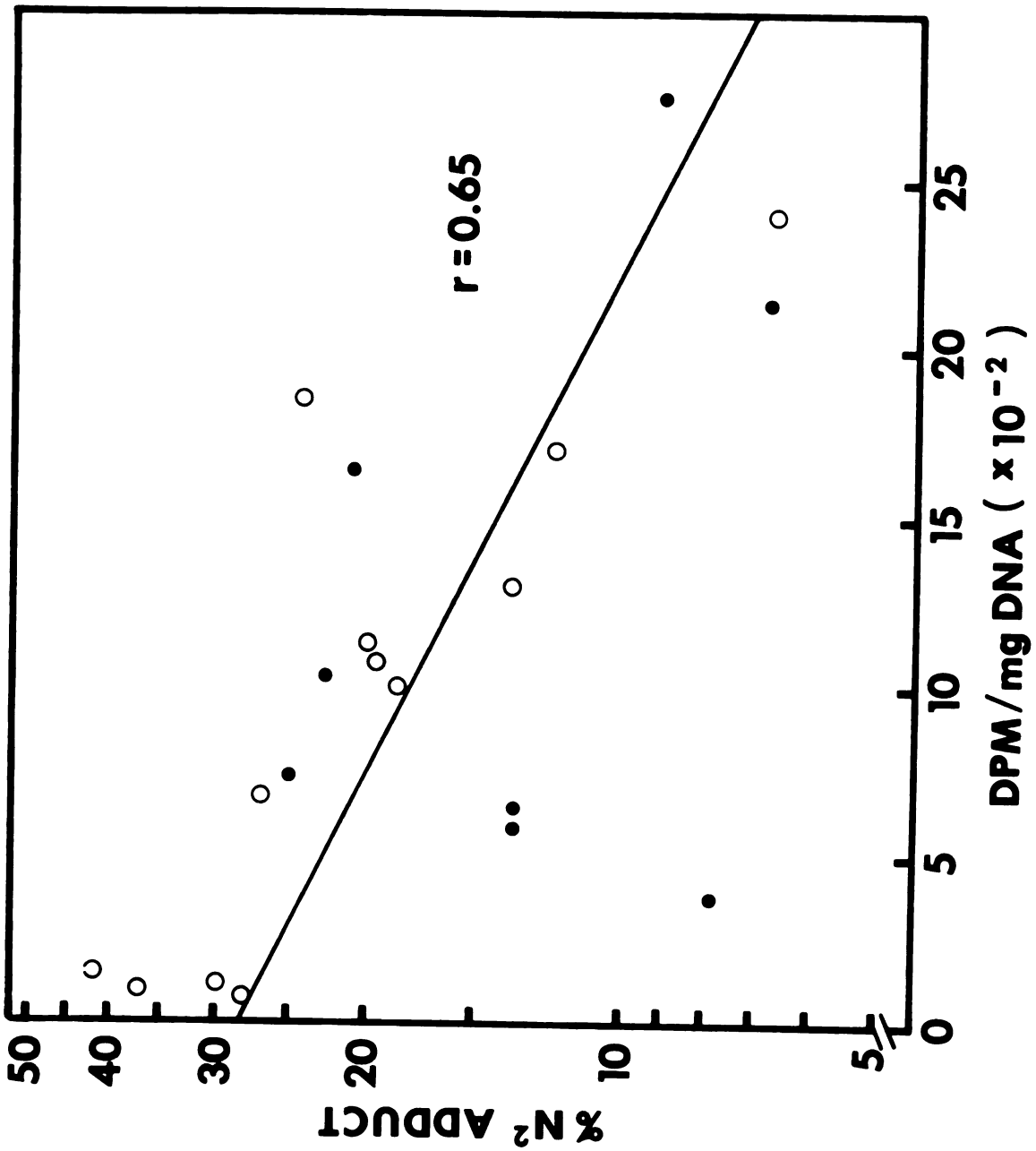


Figure 15

of DNA fragment and the percent of that modification occurring at the N^2 position.

6. Effect of Continued AAF Ingestion on Carcinogen Binding to DNA

Because induction of hepatic tumors in adult male rats occurs only after continuous carcinogen exposure, it was important to evaluate carcinogen-DNA interactions under these conditions. Rats fed 0.05% AAF for 0, 1, or 2 weeks were fasted for 24 hr and then injected with either ^{14}C-AAF or ^{3}H-N-OH-AAF (Table 10). The amount of newly bound carcinogen in total acid-insoluble material and in the DNA was determined. In all cases, there was a time dependent decrease in the amount of newly bound carcinogen; this decrease was statistically significantly in all groups after 2 weeks of AAF pretreatment. A significant difference was also observed in the extent of the decrease that occurred with AAF binding vs. N-OH-AAF binding. Thus, it appears that part of the inhibition of metabolic activation is due to the first metabolic activation step, the N-hydroxylation of AAF, while most of the reduction is due to the second step, the esterification of N-OH-AAF.

The effect of carcinogen ingestion on the site of binding was also examined in these experiments. As seen in Table 11, there was a progressive decrease in the relative amount of carcinogen binding to the N^2 position of guanine compared to the C8 position of guanine. The N^2/C8 ratio of newly bound carcinogen was significantly less than controls after 2 weeks of AAF ingestion.

TABLE 10

Effect of Continuous Carcinogen Ingestion on Metabolic Activation Capacities

I. Total acid-insoluble carciongen (nmoles carcinogen/g liver)

Pretreatment	AAF		N-OH-AAF	
Control	2.58±0.68	(100%)	8.45±0.42	(100%)
1 week 0.05% AAF	1.75±0.34	(68%)	5.03±0.64[a]	(60%)[b]
2 weeks 0.05% AAF	0.372±0.090[a]	(14%)	3.48±0.44[a]	(41%)[b]

II. DNA-bound carcinogen (pmoles carcinogen/mg DNA)

Pretreatment	AAF		N-OH-AAF	
Control	20.0±4.31	(100%)	21.4±4.14	(100%)
1 week 0.05% AAF	11.5±1.20	(58%)	11.3±1.92	(53%)[b]
2 weeks 0.05% AAF	3.72±0.50[a]	(19%)	7.68±1.60[a]	(36%)[b]

Animals were fed diets containing 0.05% AAF for 0, 1 and 2 weeks, fasted for 24 hours, and then injected with radiolabelled carcinogen (AAF: [9-^{14}C], 1 µmole/100 g B.W.; N-OH-AAF[ring-^{3}H], 2 µmoles/100 g B.W.). Animals were sacrificed after 16 hrs (AAF) or 2 hrs (N-OH-AAF) and the total acid-insoluble and DNA-bound radioactivity determined.

[a] Indicates significantly different from control, $p<.05$.

[b] Indicates significant difference between AAF and N-OH-AAF treatment, $p<.05$.

TABLE 11

Effect of Carcinogen Ingestion on the Site of Newly-Bound Carcinogen[a]

Pretreatment	N^2 Adduct[b]	C8 Adduct[b]	N^2/C8 Ratio[c]
Control	0.94±0.17	3.5±0.80	0.25 ±0.017
1 week 0.05% AAF	0.50±0.019	2.9±0.63	0.20[d]±0.035
2 weeks 0.05% AAF	0.22±0.063	1.5±0.25	0.14[d]±0.22

[a]Male rats were fed 0.05% AAF for 0, 1 or 2 weeks, fasted for 24 hrs, and injected with [^{3}H]-N-OH-AAF (100 μCi/100 g BW). Animals were sacrificed after 2 hrs and the DNA-carcinogen adducts were isolated and measured as illustrated in Figure 11.

[b]pmoles/mg DNA.

[c]Ratio calculated for each individual rat. Values are means ± S.E.M. of 4-6 animals.

[d]Indicates significantly different from control, Student-Newman-Keuls test, $p<.05$.

7. Effect of AAF Ingestion on DNA Transcriptional Capacity

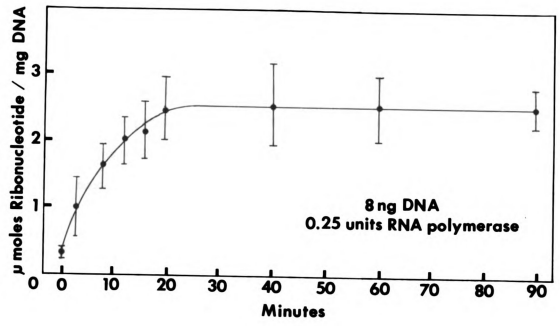
A typical analysis of the synthesis of RNA on a purified DNA template is shown in Figure 16. In the upper panel, 200 ng of DNA was incubated with a saturating amount of E. coli RNA polymerase for various times up to 90 min. The small amount of RNA synthesis indicated at 0 min represents incorporation of radioactivity occurring during the 15 min initiation period (in the absence of CTP and $MgCl_2$). RNA synthesis is essentially complete by 20 min; no further significant incorporation occurred when the reaction was carried out to 90 min. In the presence of 50 μM actinomycin-D, 95% (±0.6) of the transcription of the isolated DNA template was inhibited. This indicates that the transcription measured in vitro was dependent on a double stranded nucleic acid template.

To determine the number of initiation sites on the DNA template, a fixed amount of enzyme (0.15 units) was titrated with an increasing amount of DNA (Figure 16, lower panel). The end point of the titration occurs when the number of initiation sites and the number of molecules of RNA polymerase are equal; addition of DNA above this point will not result in further RNA synthesis. The end point was estimated by determining the intercept of a regression line of the initial linear phase (including the origin after subtraction of values for 0 min incorporation) and the maximal amount of RNA synthesized with an excess of DNA. In the example illustrated in Figure 16 (lower panel), the end point is approximately 44 ng DNA.

Prior to the isolation of the DNA for transcriptional studies, the hepatic chromatin was fractionated on a glycerol gradient into

Figure 16. Analysis of DNA template capacity. Chromatin fractions were stripped of protein and RNA and the template capacity of the purified DNA was determined. Under the assay conditions used [0.4 M $(NH_4)_2SO_4$] reinitiation by the enzyme was blocked. Purified DNA (200 ng) was incubated with 6.25 units E. coli RNA polymerase in a total volume of 2.5 ml containing 80 μM ATP, GTP, and CTP, 20 μM ^{3}H-UTP, 1.2 mM $MnCl_2$, and 6.5 mM $MgCl_2$ (upper panel). At various time points, duplicate 0.1 ml samples (containing 8 ng DNA and 0.25 units enzyme) were removed, and the acid precipitable radioactivity determined. The amount of RNA synthesis indicated at 0 min represents the incorporation of radioactivity which occcurred during the initiation period (in the absence of CTP and $MgCl_2$). This small amount of incorporation was not different when experiments were performed using DNA obtained from either control or AAF-treated rats. Each point represents the mean ± SEM of 4 rats.

In the lower panel, increasing amounts of DNA were incubated with 0.15 units RNA polymerase for 10 min in a total volume of 0.1 ml under the conditions described above.


μ moles Ribonucleotide / mg DNA
0
1
2
3
0
10
20
30
40
50
60
70
80
90
Minutes
8 ng DNA
0.25 units RNA polymerase

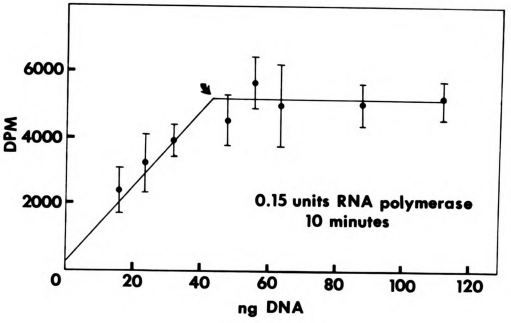

DPM
2000
4000
6000
0
20
40
60
80
100
120
ng DNA
0.15 units RNA polymerase
10 minutes

Figure 16

euchromatin, heterochromatin and pelleted heterochromatin (Figure 2). The DNA from each of these 3 fractions was purified as described in the Methods, and transcribed with E. coli RNA polymerase. The various parameters of in vitro transcription that are either directly measured, or can be calculated are listed in Table 12. The extent of RNA synthesis is determined from the incorporation of ^{3}H-UTP after 90 min of incubation. The percent of the DNA in the reaction mixture that was transcribed was calculated assuming that each DNA sequence was transcribed at maximum one time (88) and that there are 3.16 μmoles of nucleotides per mg DNA. The number of molecules of RNA polymerase present in the DNA titration experiments was calculated assuming an enzyme activity of 1200 U/mg (28) and an enzyme molecular weight of 475,000 daltons (28). This value was then divided by the amount of DNA at the end point of the titration, yielding the number of initiation sites per mg DNA. This was then divided into the total moles of ribonucleotide/mg DNA synthesized to estimate the number average RNA chain length. The rate of RNA chain elongation was calculated from the slope of the initial increase in average RNA chain size (Figure 17). Finally, the ability of the enzyme to reinitiate RNA synthesis was determined by omitting the $(NH_4)_2SO_4$ from the reaction. The amount of RNA synthesized after 60 min was divided by the amount synthesized when reinitiation was blocked, resulting in an estimate of the average number of reinitiations in 60 min.

To assess the effects of carcinogen ingestion on the template activities of isolated DNA, rats were fed 0.03% AAF for 4 days. This treatment substantially affected the purity of the isolated DNA

TABLE 12

Synthesis of RNA from Control DNA Samples Isolated from Chromatin Fractions[a]

	Slow Sedimenting Chromatin	Fast Sedimenting Chromatin	Pelleted Chromatin
Extent of RNA synthesis[b]: nmoles ribonucleotide/mg DNA	2392±147	2466±297	1750±107
% DNA transcribed	74.4%	77.9%	55.3%
Initiation sites[c] per 10^3 base pairs	4.92	4.06	3.78
per mg DNA	4.67×10^{15}	3.86×10^{15}	3.59×10^{15}
Average DNA chain length[d] (nucleotides)	308±19	385±46	293±18
Rate of RNA synthesis[d] (nucleotides/chain/min)	13.3	25.3	16.3
Enzyme Reinitiation/60 min[e]	6.3±1.5	4.8±0.9	7.8±1.0

[a]Chromatin was prepared and fractionated on a glycerol gradient. The chromatin fractions were stripped of protein and RNA as described in Methods. Each value is the mean ± SEM of 4 DNA samples each of which was assayed in duplicate.

[b]Maximal RNA synthesized on 8 ng DNA under conditions of blocked reinitiation.

[c]Determined from the end point of titration of 0.15 units RNA polymerase with DNA as illustrated in Figure 16. Calculation of the number of initiation sites is described in Results and assumes that there are 3.16 μmoles of nucleotides per mg DNA, and 1.58×10^{11} molecules enzyme/unit.

[d]Values calculated based on the extent and time course of synthesis and the average number of RNA chains formed.

[e]RNA synthesis with reinitiation [no $(NH_4)_2SO_4$] divided by values for synthesis without reinitiation [0.4 M $(NH_4)_2SO_4$].

Figure 17. Rate of RNA chain growth. The overall synthesis of RNA and the average number of RNA chains were determined as described in Figure 16 (upper and lower panels, respectively). The initial rate of RNA chain propagation is illustrated using a DNA template derived from the heterochromatin fraction of rats fed a basal diet (●), an AAF diet for 4 days (○), or an AAF diet for 4 days followed by 7 days on the basal diet (△). Each point represents the mean of DNA samples prepared from 4 rats.

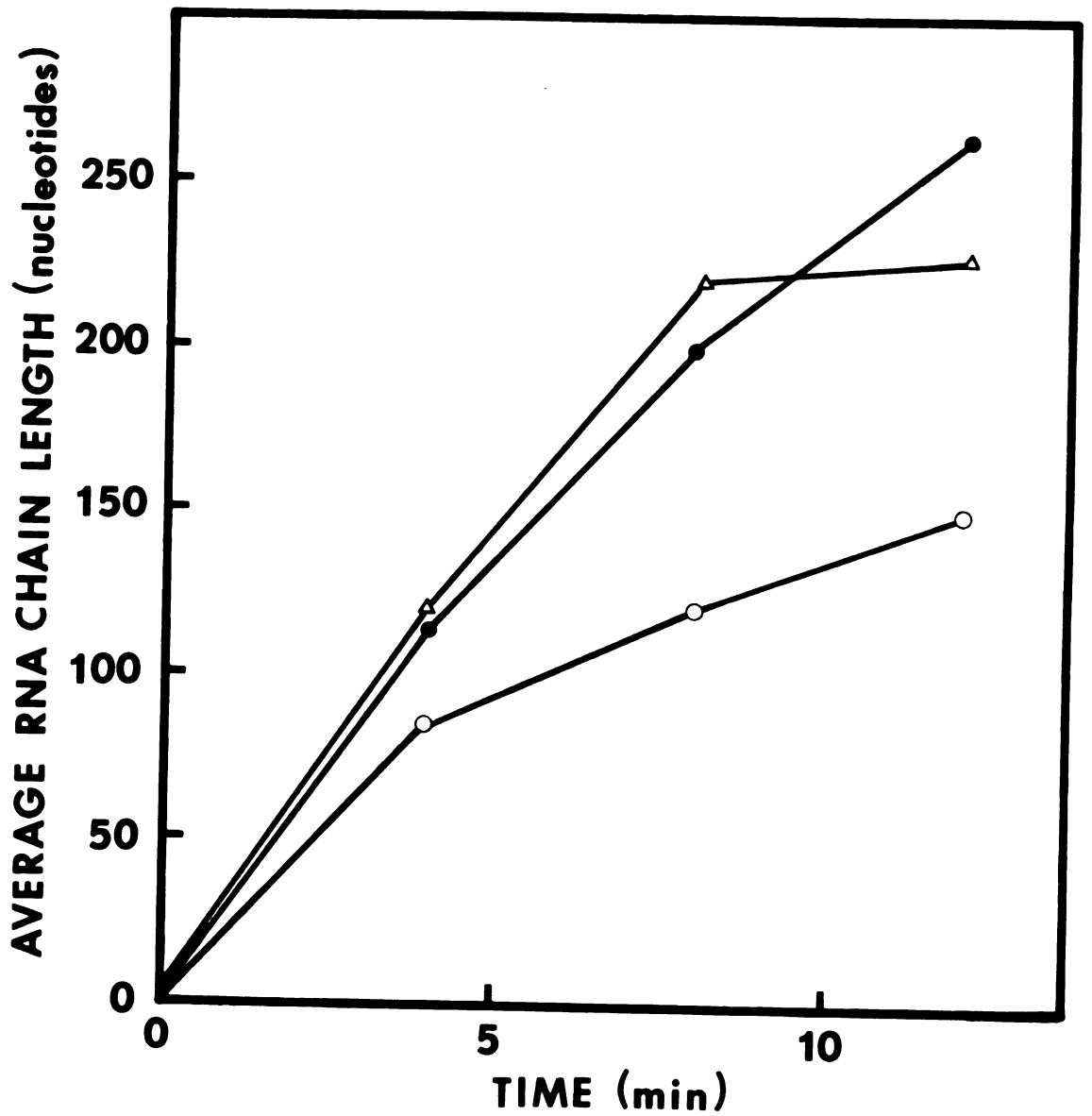

AVERAGE RNA CHAIN LENGTH (nucleotides)
250
200
150
100
50
0
0
5
10
TIME (min)

Figure 17

template; protein contamination increased to 23-44% (Table 13). When the rats were returned to a basal diet after 4 days of AAF ingestion, the degree of protein contamination of the DNA was reduced. RNA contamination of the DNA preparations remained consistently low in the control and treated groups.

As illustrated in Figure 18, AAF ingestion significantly altered in vitro transcription of the DNA derived from the eu- and heterochromatin fractions. After 4 days of AAF ingestion (single hatched bars), total RNA synthesis was reduced 50 and 41% using the DNA templates derived from the eu- and heterochromatin, respectively. This reduction is not the result of a decrease in the number of initiation sites, but rather is due to a decrease in the average size of the RNA chains formed. The calculated rate of RNA synthesis on the heterochromatin DNA was decreased (this decrease in rate, however, was not statistically significant). When the animals were returned to a basal diet for 7 days after ingestion of AAF for 4 days (cross hatched bars), both the extent and size of RNA synthesized, as well as the rate of synthesis returned to approximately control values. In contrast to the inhibition of RNA synthesis seen with eu- and heterochromatin DNA templates, there was no significant decrease in RNA size or extent on DNA of the pelleted chromatin.

As illustrated in Figure 19, there was an inverse correlation between the average RNA chain length and the extent of enzyme turnover (number re-initiations per 60 min), regardless of the source of the DNA template. Statistically significant differences in enzyme

TABLE 13

RNA and Protein Contamination of Purified DNA Samples[a]

		Slow Sedimenting Chromatin	Fast Sedimenting Chromatin	Pelleted Chromatin
Control	% RNA	1.0±0.3%	4.3±1.6%	0.67± .06%
	% Protein	6.8±1.8%	9.0±5.1%	12.6 ±0.6%
4 Days AAF	% RNA	2.2±1.1%	3.0±2.0%	2.7 ±2.0%
	% Protein	44±14%	31±5%	23±4%
4 Days AAF + 7 Days Basal	% RNA	4.7±2.5%	2.9±1.9%	3.3 ±1.5%
	% Protein	11.7±4.1%	12.9±5.5%	13.5 ±2.2%

[a]Chromatin was fractionated on a glycerol gradient into slow sedimenting, fast sedimenting and pelleted chromatin. The chromatin fractions were treated with RNase, pronase, made 3M with respect to NaCl, and centrifuged. The supernatant was applied to a Bio-Gel A-1.5m column. The material eluting just after the void volume was collected and dialyzed in 10 mM Tris (pH 7.9). The amount of RNA and protein relative to the DNA was determined.

Figure 18. Effect of AAF ingestion on DNA template activity. Several parameters of RNA transcription on DNA templates derived from slow sedimenting and fast sedimenting and pelleted chromatin fractions were determined. Rats were fed an AAF containing diet for 4 days (▨); or an AAF diet for 4 days followed by 7 days on the basal diet (▩). All values are expressed as a percent of those obtained from control rats fed the basal diet (Table 12). Each bar represents the mean of DNA samples prepared from 4 rats. (Signifies significantly different from control, t-test, $p<.05$.

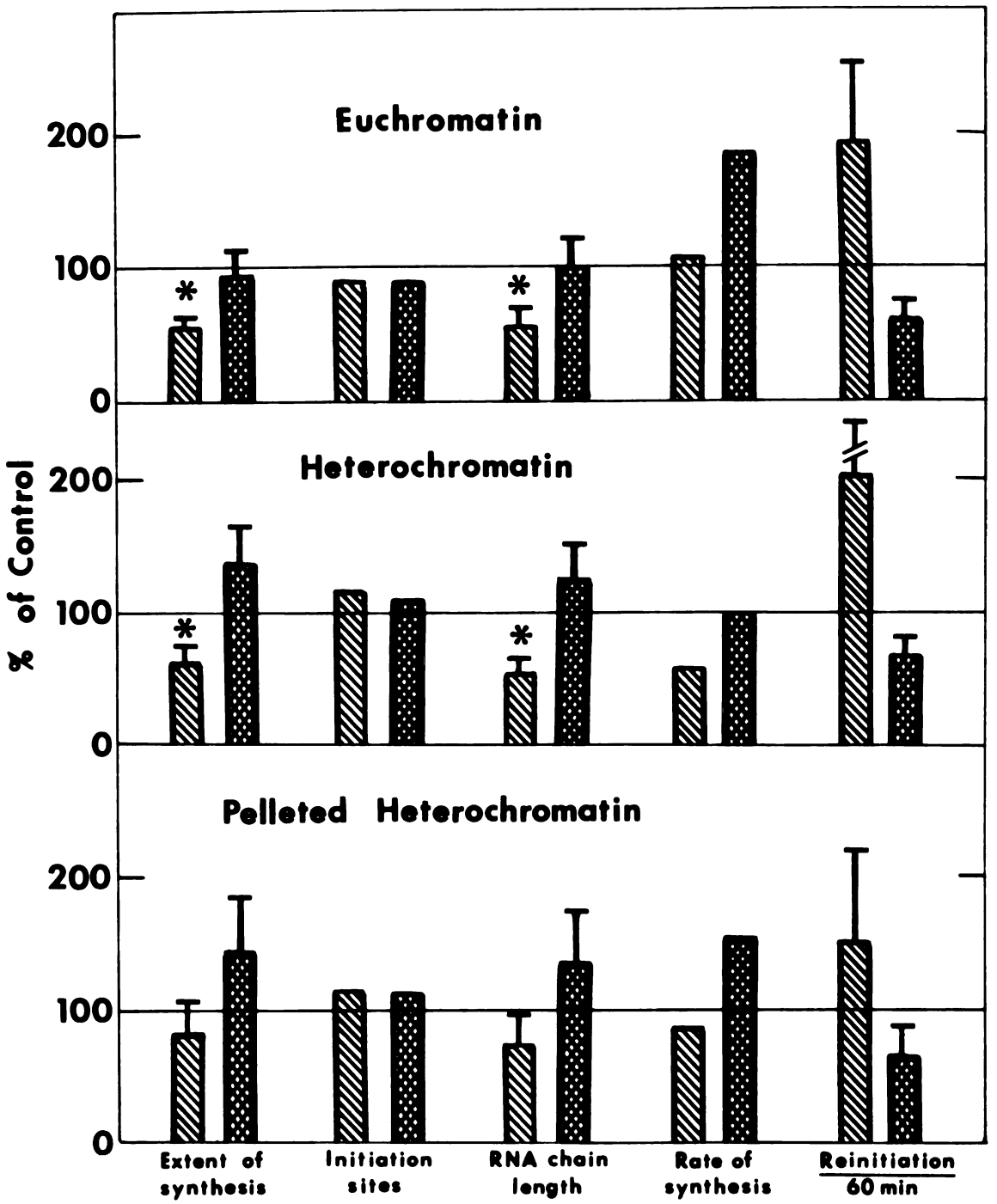

Euchromatin
Heterochromatin
Pelleted Heterochromatin
% of Control
200
100
0
*
Extent of synthesis
Initiation sites
RNA chain length
Rate of synthesis
Reinitiation
60 min

Figure 18

Figure 19. Correlation between RNA chain size and RNA polymerase turnover. Each point represents transcription of DNA from a single chromatin fraction, as described in Figure 16. Symbols are the same as for Figure 17. Correlation coefficient ($r = 0.91$) was statistically significant ($p<.01$).

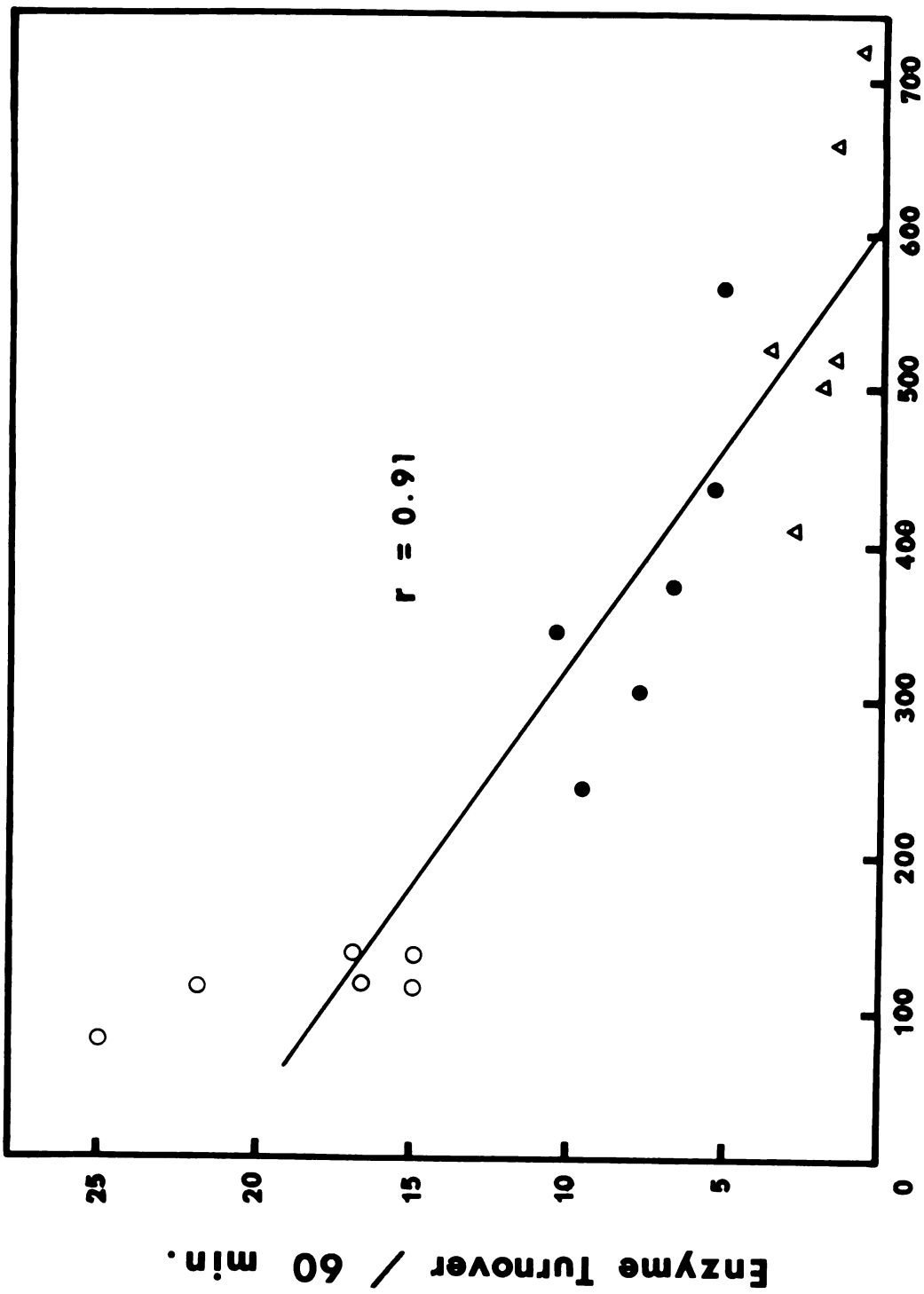

Enzyme Turnover / 60 min.
25
20
15
10
5
0
100
200
300
400
500
600
700
Chain Length (nucleotides)
r = 0.91

Figure 19

turnover were not observed, however, perhaps due to the large variability in measurement of this parameter.

The decrease in RNA synthesis after AAF ingestion occurred at the same time that protein contamination of the DNA template increased. An attempt was made to determine if there was a correlation between the amount of protein on the DNA and the amount of RNA synthesized. In Figure 20, percent protein was plotted against ^{3}H-UTP incorporated for both control and carcinogen treated animals. Within each group, there was not a significant correlation between percent protein and RNA synthesized, despite the fact that a wide range of protein contamination was examined. Similarly, there was no correlation ($r = 0.12$) between RNA contamination of the DNA template, and RNA synthesis, over the range of RNA contamination encountered (Figure 21). This suggests that an increase in tightly bound protein on the DNA template occurs at the same time that RNA synthesis decreases, but the protein contamination is not the cause of the altered transcription.

The rate of RNA chain elongation can be further analyzed by examining the kinetics of nucleoside triphosphate addition. Hyman and Davidson (88) have derived equations to describe the rate of nucleotide triphosphate incorporation into a growing RNA chain. Their analysis is summarized in Table 14. In these experiments, the concentration of one nucleotide triphosphate (S) is varied below saturating levels, while the concentration of the other 3 are held constant at saturating levels. The equation in Table 13 predicts that a Lineweaver-Burke plot of 1/v versus 1/s should be a straight line whose

Figure 20. Effect of protein contamination on RNA synthesis. Isolated DNA templates were prepared and transcribed with E. coli RNA polymerase as described in Methods. The relative protein contamination of the DNA is plotted on the abscissa, and the extent of RNA synthesized is plotted on the ordinate. Values were obtained for control rats fed a basal diet (●), or rats fed an AAF-containing diet for 4 days (○). In neither case was the correlation coefficient of the lines drawn significant ($p<.05$).

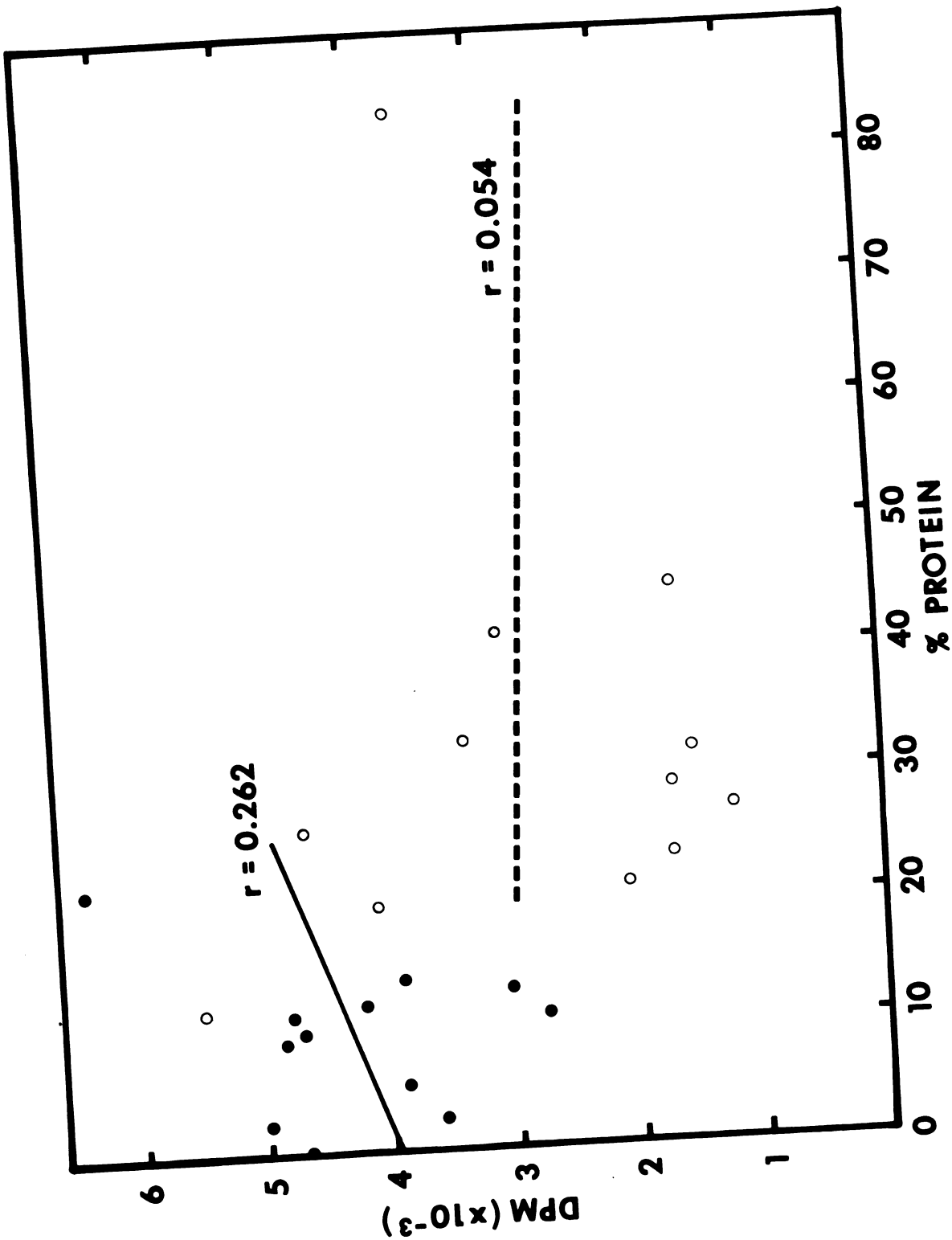


Figure 20

Figure 21. Effect of RNA contamination on RNA synthesis. Same as Figure 20, the correlation coefficient was not significant ($p<.05$).

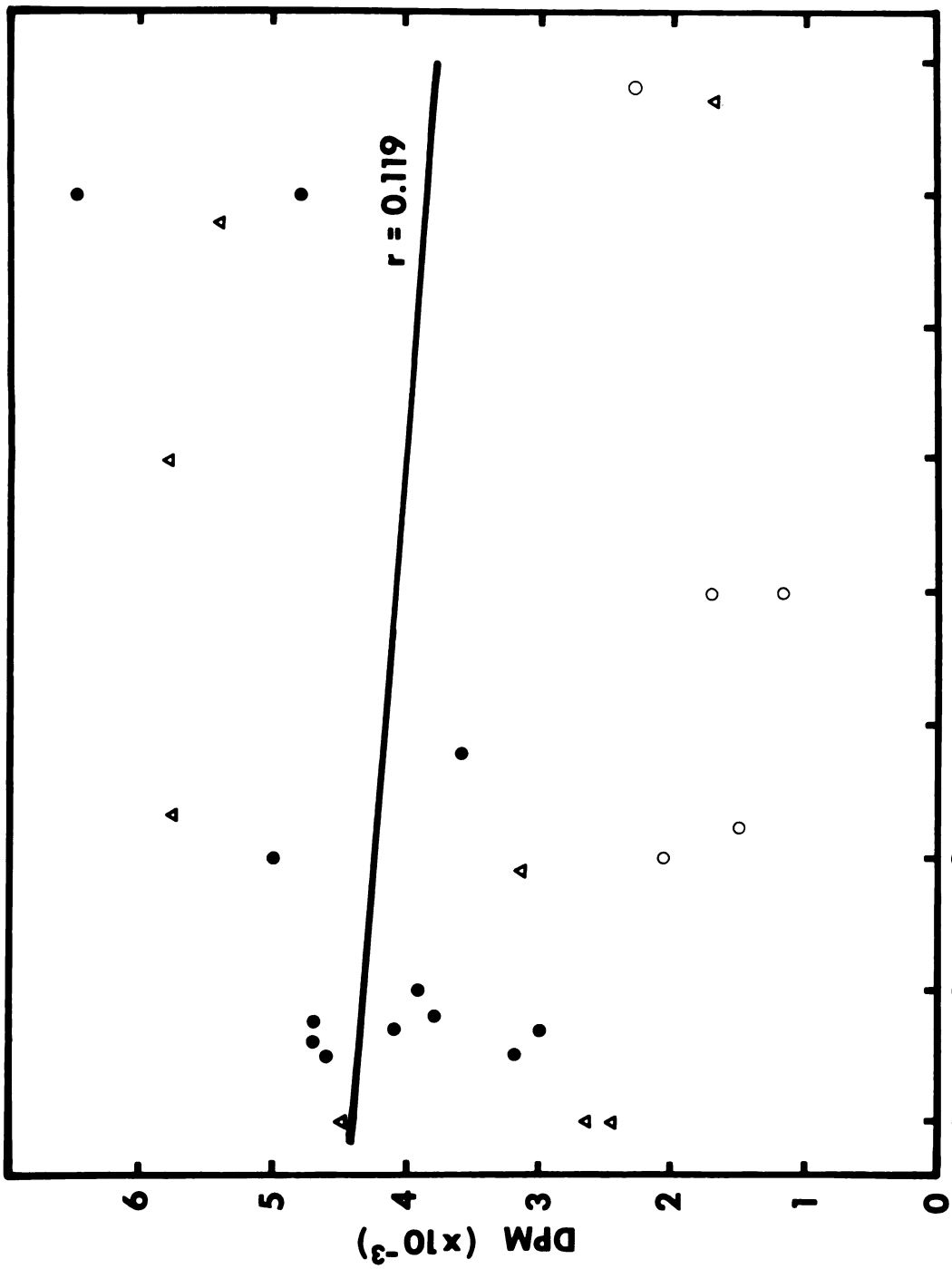


Figure 21

TABLE 14

$$E_\alpha + S_\alpha \underset{k_{2\alpha}}{\overset{k_{1\alpha}}{\rightleftharpoons}} ES_\alpha \xrightarrow{k_{3\alpha}} P$$

$$\frac{1}{v} = \sum_{\alpha=AGCU} \frac{f_\alpha K_\alpha}{k_{3\alpha}} \frac{1}{[S_\alpha]} + \frac{f_\alpha}{k_{3\alpha}}$$

$$\text{SLOPE} = \frac{f_\alpha K_\alpha}{k_{3\alpha}}$$

K_α = dissociation constant of ES complex.

$k_{3\alpha}$ = rate constant of P formation.

E = enzyme (RNA polymerase)

S = substrate (ribonucleotide triphosphate)

P = product (RNA polymer)

α = ribonucleotide A, G, C or U

f = mole fraction of α in RNA chain

v = average rate of nucleotide incorporation

slope is inversely proportional to the rate of nucleotide addition (88,188).

In this manner, the rates of incorporation of the 4 nucleotide triphosphates were determined on DNA templates derived from the pelleted chromatin fractions. As illustrated in Figure 22, the relative rates of incorporation of GTP and CTP were not altered by carcinogen treatment. In contrast, the rates of incorporation of both ATP and UTP were substantially reduced when DNA templates from rats fed AAF for 4 days were used. When the rats were returned to the basal diet for 7 days, the apparent inhibition of ATP and UTP incorporation was reversed.

Figure 22. Kinetics of nucleotide triphosphate polymerization. RNA synthesis was initiated as described in Methods and elongation then measured for 10 min under varying concentrations of nucleotide triphosphate. In each panel, the concentration of the indicated nucleotide triphosphate was varied from 1.5 to 20 μM; the inverse concentration is shown on the abscissa. The remaining 3 nucleotide triphosphates were added at saturating levels (GTP, ATP or CTP = 80 μM; ^{3}H-UTP = 20 μM). Each tube contained 30 ng DNA and 0.3 units E. coli RNA polymerase. The initial rate of synthesis (pmole ribonucleotide/min) was determined for DNA derived from pelleted chromatin of rats fed a basal diet (●), an AAF diet for 4 days (○), or an AAF diet for 4 days followed by 7 days on the basal diet (△). Note the change in the scale of the ordinate in each panel. Each point represents the mean of values obtained from 4 rats.

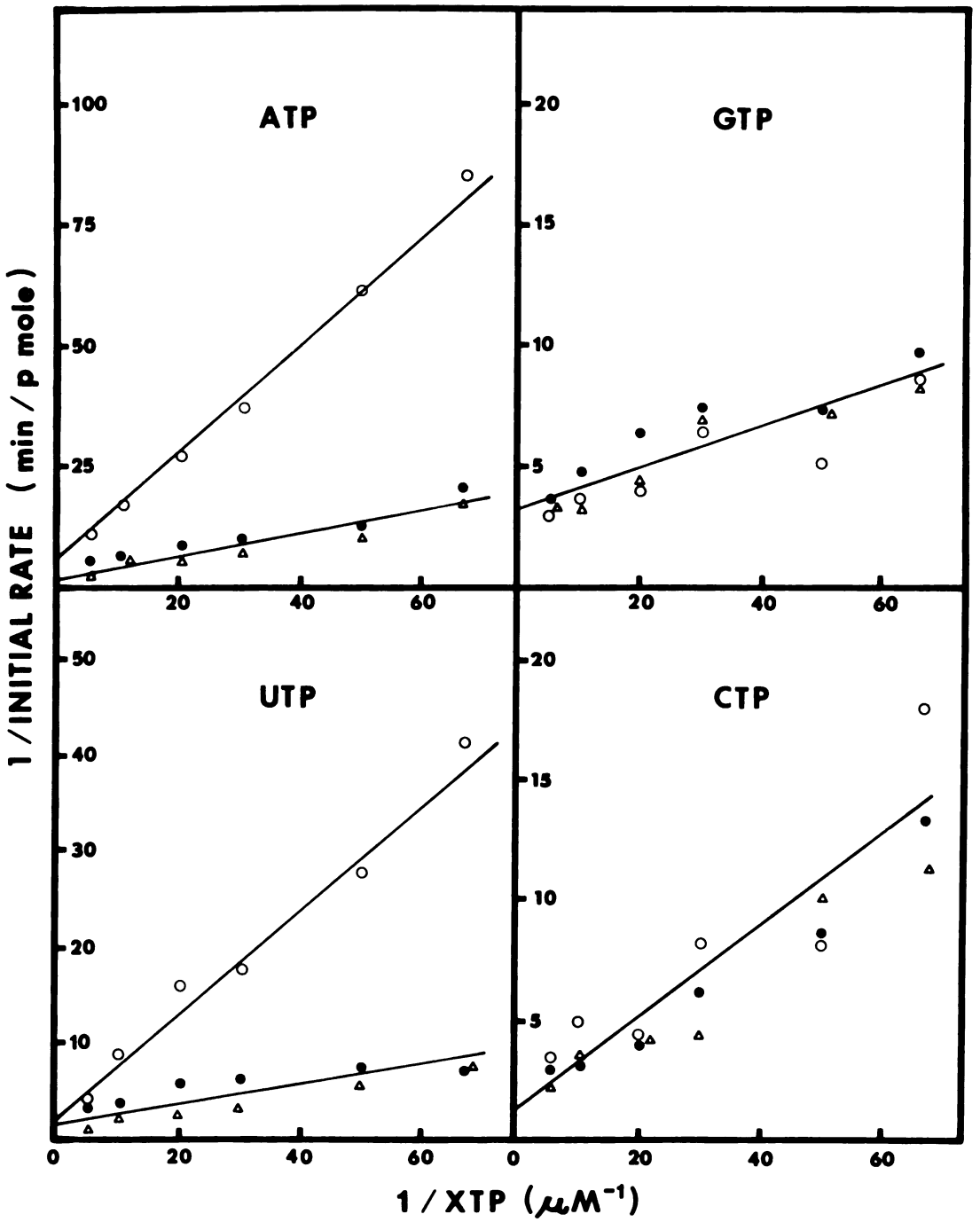

ATP
GTP
UTP
CTP
1/INITIAL RATE (min/p mole)
1/XTP (μM⁻¹)
100
75
50
25
20
15
10
5
40
30
0
20
40
60

Figure 22

DISCUSSION

It has been known for some time (54) that chromatin in interphase eukaryotic cells exists in two broadly classified conformations, euchromatin and heterochromatin. Only a small portion of the total nuclear DNA is contained in the extended, relatively uncondensed, euchromatin fibrils, while the bulk of the DNA is found in the more condensed heterochromatin. Furthermore, high resolution autoradiographic studies of mammalian nuclei demonstrated that RNA synthesis is confined to the extended euchromatin components of chromatin *in vivo* (199). It has been concluded that there is a definite difference in physical characteristics between the template active and template repressed chromatins *in vivo* (178).

The maintenance of native chromatin structure through the various isolation procedures employed is a subject of continuing debate. It is possible that the mere release of chromatin from its tight packing within the nucleus to a dilute solution introduces alterations of chromatin structure. It does appear, however, that isolated chromatin retains many of the physical characteristics of chromatin *in vivo* (19,39) as well as the tissue-specific restriction of transcription which results in organ-specific RNA *in vivo* (161).

It has been suggested that artifacts are introduced when chromatin is fragmented prior to separation into eu- and heterochromatin.

Since the euchromatin and heterochromatin regions of the genome are contiguous (53), any separation of the two depends on the shearing of the chromatin to fragments that are enriched in either eu- or heterochromatin regions. Many investigations have depended on random shearing techniques, including sonication, to fragment the chromatin. Although sonication does randomly shear a uniform macromolecule, it is not clear what its effect on chromatin is (178). There are conflicting reports as to the extent of perturbation of chromatin structure occurring after shearing or sonication. In one study (149), severe changes in the circular dichroism spectra were observed upon sonication of chromatin, while a second study (126) found only slight changes. Mechanical shearing has been reported (43) to induce movement of histones along the DNA strand; a more recent report estimates this rearrangement at only 5-10% after mild sonication or shearing (126). Upon reviewing the literature, however, it is clear that the chromatin fractionation patterns obtained after sonication were not reproducible when results from different laboratories were compared (see 14,42,156,179,202).

The chromatin used in the studies reported in this thesis was prepared from hepatic nuclei based on the method of Rodriguez and Becker (179), with modifications to minimize the number of washings used in an attempt to lessen mechanical disruption of the material. The chromatin complex obtained contained proportionately more protein and RNA compared to values often reported in the literature; they are in good agreement, however, with reports in which extensive chromatin washing was avoided (40). Upon sonication, the chromatin was fractionated into 3 fractions using either glycerol gradient

centrifugation or selective $MgCl_2$ precipitation. Thse fractions were characterized as template active or repressed based on either *in vitro* template activity (measured with *E. coli* RNA polymerase, Table 2) or *in vivo* template capacity (measured as nascent RNA, Figure 2, Tables 2 and 3). The nascent RNA measured in the glycerol gradient appears to be complexed with the chromatin DNA and proteins. Heating the chromatin at 70°C for 15 min dissociates the nascent RNA from the chromatin DNA (Figure 3). Since this mild denaturation would not be expected to have any effect on the RNA molecule *per se*, it is likely that under the control conditions, the nascent RNA is physically associated with the euchromatin rather than merely cosedimenting with it.

Chromatin is susceptible to aggregation by divalent cations in a concentration dependent manner (6). At a low concentration of cation the more condensed chromatin is selectively precipitated. When compared to the glycerol gradient procedure, fractionation at 1.75 mM $MgCl_2$ yields a smaller amount of chromatin that is more highly enriched in nascent RNA. It should be noted that Arnold and Young (6) have reported that treatment of chromatin with $MgCl_2$ might dissociate some of the nascent RNA. Therefore, relative comparisons of the purity of separation between the two procedures based on nascent RNA may be misleading.

In order to facilitate interpration of the numerous literature reports employing different chromatin fractionation procedures, a direct comparison of the glycerol gradient and $MgCl_2$ fractionation procedures was performed (Table 4). The bulk of the glycerol gradient slow sedimenting chromatin was not aggregated by 1.75 mM $MgCl_2$, while most of the glycerol gradient fast sedimenting chromatin was. The

majority of the glycerol gradient pelleted chromatin also precipitated when centrifuged in buffer without $MgCl_2$. This larger degree of overlap suggests that procedures such as glycerol and sucrose density gradients, differential aggregation by cations, and differential centrifugation are readily comparable in terms of physical characteristics of the chromatin fractions they yield when similar means of chromatin fragmentation (i.e., sonication) are used.

Despite the numerous reports utilizing chromatin fractionation procedures, there has been little examination of the chromatin fraction designated pelleted chromatin. This fraction, which represents approximately 15% of the chromatin DNA, is apparently more highly condensed than the remainder of the fast sedimenting heterochromatin. Interestingly, the pelleted chromatin from the glycerol gradient showed the greatest heterogeneity when assayed with $MgCl_2$. It is possible that the shearing forces occurring when the pellet was resuspended released portions of slow and fast sedimenting chromatin from the insoluble material. Murphy *et al.* (155) have examined the effect of various shearing times on the distribution of chromatin in glycerol gradients. Increasing the shear time from 1 and 5 min increased the template active fraction from 22 to 38% without altering the *in vitro* template activity of the euchromatin. They concluded that the increased shearing increases the likelihood of separating the interspersed template active segments from the heterochromatin. These findings have been extended to demonstrate that there is a stable portion of pelleted chromatin that apparently does not contain euchromatin or less condensed heterochromatin DNA segments.

The validity of the fractionation achieved by sonication and glycerol gradient centrifugation was questioned by the studies of Howk et al. (84). These investigators measured the degree of localization of DNA marker sequences between their putative eu- and heterochromatin fractions obtained from a mouse fibroblast cell culture line. They found an identical distribution between the eu- and heterochromatin of inactive DNA sequences (mouse mammary type B viral sequences and globin genes) and active DNA sequences (Moloney type C viral sequences). These investigators concluded that they were unable to separate active from inactive genes. Similar findings were reported by Krieg and Wells (103), who found only a slight enrichment of active genes (globin) and inactive genes (keratin) in the appropriate fractions of chicken erythroid chromatin.

In contrast, Higashi et al. (82) showed a ten-fold enhancement of ribosomal genes in the euchromatin of nucleoli which had been sonicated and fractionated on a sucrose gradient. Thus, it appears that under the proper conditions chromatin can be fractionated into eu- and heterochromatin by sonication and gradient centrifugation. Although further investigations might explain some of the discrepancies in the literature, the development of more reproducible chromatin fractionation procedures (e.g., DNAse digestion) has reduced the interest in random shear techniques. In terms of the studies reported in this thesis, it can be concluded that the extended and condensed fractions obtained with a brief sonication of chromatin retain many of the physical properties of in vivo eu- and heterochromatin (including "recognition" of transcription sites by RNA polymerase in vitro), but

cannot be unequivocally described as representing template active or repressed regions of the genome.

The development of endonuclease chromatin fractionation procedures has sparked new interest in the in vitro analysis of control of gene expression in vivo. In contrast to random shearing, endonuclease fractionation has an additional level of specificity: both DNAse I (8,203) and DNAse II (15,72) attack the template active regions of chromatin at a much higher rate than the nontranscribed regions, and the nontranscribed regions can then be removed from solution by precipitation with divalent cations. The DNAse II-$MgCl_2$ procedure separates template expressed from template repressed genes (Table 1). Both the transcribed and nontranscribed regions obtained are organized into repeating units of DNA and histone, and both have been shown to have the 100 Å, 200 base pair, nucleosomal structure (71). The template active regions are enriched in non-histone proteins and nascent-synthesized RNA (15,71). In one report (159) limited nuclease digestion was found to produce much less structural damage to chromatin than did shearing. Similarly, no detectable protein rearrangement occurs during either DNAse II digestion or subsequent chromatin fractionation (70). Since this method represents the "state of the art" of chromatin fractionation, it was important to analyze carcinogen binding after DNAse II digestion and to compare the results with those obtained after chromatin sonication. This allows for more definitive statements regarding carcinogen binding in terms of template active vs. template repressed regions of the genome.

The administration of ^{3}H-N-OH-AAF led to derivatives covalently bound to all three components of the chromatin complex: protein, RNA

and DNA. While the binding of aromatic amines to histone and non-histone chromatin proteins (95,138) and non-nuclear RNA species (91,104,106,132) has been reported, there have been no studies specifically describing carcinogen binding to chromatin RNA. That no carcinogen could be detected on the chromatin RNA (Figure 7) after 7 days renders it likely that loss of the carcinogen is due to the normal turnover of the RNA molecule, which has been estimated to have a half-life of 5 days (16). The initial binding of the carinogen is greater on RNA than it is on DNA (Figure 7 and 11). It has been postulated (107) that RNA is more susceptible to carcinogen binding due to its location in the cytoplasm; the demonstration of an equally high binding to nuclear chromatin RNA suggests that other differences between RNA and DNA (i.e., single vs. double stranded) might be the determining factor.

Rats were sacrificed two hours after a single injection of ^{3}H-N-OH-AAF to estimate the maximal binding of carcinogen to DNA (155,192). Carcinogen loss from DNA occurs over the next 7 days, however, approximately 15% of the initially bound carcinogen persists after 7-10 days (Figure 7). This is in good agreement with findings of other investigators, who reported a stable carcinogen binding to DNA of 5-13% of peak values for up to 8 weeks after a single injection (90,91,106). As illustrated in Figure 7, rats fed 0.03% AAF after a single injection of ^{3}H-N-OH-AAF had levels of DNA-bound carcinogen equal to those of the control rats. This suggests that carcinogen exposure at this level does not affect the gross amount of DNA repair occurring.

The susceptibility of DNA to carcinogen attack based on chromatin conformation was next examined. Two hours after injection of ^{3}H-N-OH-AAF, carcinogen binding to DNA on the extended euchromatin is greater than binding to the condensed heterochromatin DNA (Table 6). This finding was confirmed when the chromatin was fractionated by DNAse II-$MgCl_2$ (Table 9), indicating that there is a real difference in carcinogen binding between template active and template repressed portions of the genome. The relative distribution of carcinogen binding to DNA (euchromatin/heterochromatin) was 1.7 for sonicated-glycerol gradient chromatin, 6.7 for sonicated-$MgCl_2$ chromatin, and 16 for DNAse II-$MgCl_2$ fractionated chromatin. Differences in the relative amounts of DNA in the euchromatin fractions after sonication (25% for the glycerol gradient vs. 8% for the $MgCl_2$ procedure) may partially account for this difference in specific activity. Differences between sonicated and DNAse II fragmented chromatin probably reflects a real difference in the regions of DNA that are separated by these two procedures.

The greater initial binding of AAF residues to the DNA in euchromatin confirms similar findings for this carcinogen by Moyer et al. (155). Enhanced binding of carcinogens to DNA in euchromatin regions appears to be a near universal finding (94,139,140,155,171, 172). Since this phenomenon has been reported to occur with aromatic amines as well as polycyclic hydrocarbons and methylating agents, the structure of the chromatin rather than the chemical nature of the electrophile is probably the most important consideration in the relative amount of covalent binding occurring. This may relate to the apparent "extended" conformation of the euchromatin, which would have

potentially more DNA binding sites exposed than the more condensed heterochromatin.

The fact that the pelleted heterochromatin DNA also had a relatively high level of carcinogen bound (Table 6) suggests that the degree of chromatin condensation is not the sole determinant of carcinogen binding. An alternative possibility is that location within the nucleus may be an important consideration in susceptibility to carcinogen attack. For example, there is a "ring" of highly condensed heterochromatin in close apposition to the nuclear membrane (54). It is possible that the DNA in immediate contact with the nuclear envelope would be exposed to the highest concentration of ultimate carcinogens, particularly those that may be metabolically activated on the nuclear membrane (190,191). No information is available, however, as to the identity of either the pelleted heterochromatin or the DNAse II resistant chromatin.

Besides the differences in the internucleosomal binding of carcinogens discussed above, differences in intranucleosomal distribution have also been reported. Carcinogen binding is greatest in the "linker" regions of the nucleosome when compared to the "core" DNA of the nucleosome (94,139,140,171,172). Explanations for this observation are similar to those discussed above. Thus, the DNA in the linker region might be more accessible to carcinogen modification than the core DNA, which is closely associated with histones. It has been demonstrted (110) that the DNA in actively transcribed regions of the genome is organized as nucleosomes in a manner similar to the remainder of the DNA, indicating that differences in linker to core

ratios do not completely explain differences in carcinogen binding to eu- and heterochromatin regions.

The loss of the carcinogen from the euchromatin, heterochromatin, and pelleted heterochromatin DNA was not uniform over the 10 day period examined (Table 7); biologic half lives of approximately 2.0, 2.7 and 5.2 days, respectively, were observed. These values are comparable to those reported by Moyer et al. (155), who examined carcinogen loss from chromatin fractions over a 48 hour period. Similarly, Irving and Veazey (91) report a loss of AAF from total DNA with a half-life of 3.5 days, while Kriek observed a half-life of approximately 7 days (106).

The more rapid loss of carcinogen from the euchromatin DNA (Table 7) might result from a greater susceptibility of this damage to DNA repair enzymes. Increased repair may be due to either the physical nature of the chromatin conformation or the chemical nature of the carcinogen modification. It is unlikely that the observed loss of carcinogen residues was due to DNA turnover as a consequence of AAF-induced cell death. When male rats were maintained on a diet containing 0.03% (w/w) AAF there was no loss of prelabelled hepatic DNA during the first 5 weeks of carcinogen ingestion (3,207). This demonstrates an initial low degree of cytotoxicity in the target organ.

When the animals were maintained on an AAF containing diet, loss of carcinogen from pelleted heterochromatin DNA was increased while loss from the euchromatin DNA was decreased, when compared to rats maintained on the control diet (Table 7). If carcinogen loss from DNA was due to DNA turnover, one would have expected an increased loss of

carcinogen from both fractions, due to the toxicity of continual AAF exposure. Thus, DNA repair is likely to play an important role in the removal of carcinogen that was observed. The effect of continued AAF ingestion on modifying rates of carcinogen loss might be explained by a gene modulating effect to alter the exposure of damaged DNA to repair enzymes.

Just as carcinogen binding appears to vary both internucleosmally and intranucleosomally, so it appears does DNA repair (76,171, 172, 186, 187,195). Within the nucleosome, repair occurs preferentially in the linker DNA; with time, a nucleosome rearrangement occurs which transforms core DNA into linker DNA, thus making it more accessible to repair enzymes (186,187,195). It would be of interest to determine the effects of continuous carcinogen exposure on this rearrangement repair process.

Westra et al. (205) have identified the persistently bound fluorene-DNA residue as 3-(deoxyguanosin-N^2-yl)-AAF, in contrast to the major guanine-AAF adduct, N-(deoxyguanosin-8-yl)-AAF, which is quantitatively removed by 4 weeks (106). In addition, there is a substantial amount of de-acetylated carcinogen which is bound to the C8 position of guanine (106) although it is not clear precisely how much of this adduct is formed in vivo (D. Grunberger, personal communication). Analysis of the distribution of these 2 adducts within the genome showed that there is a relatively larger N^2/C8 ratio in apparently repressed portions of the genome. This is seen in the pelleted heterochromatin DNA after sonication (Table 8) and the P2 heterochromatin DNA after DNAse II digestion (Table 9). Thus, a portion of the slower repair rate of the pelleted heterochromatin DNA

(Table 7) probably reflects the relative enhancement of the repair-resistant N^2 guanine lesion in this chromatin fraction.

Very little information is available as to the relative importance of the 2 guanine adducts in relation to carcinogenesis. The polycyclic hydrocarbon benzo(a)pyrene binds predominantly to the N^2 position of guanine (135). A recent study has suggested that there is a correlation between the extent of binding of isomers of benzo(a)-pyrene diol epoxide to the N^2 guanine in DNA and the mutagenic and tumorigenic potencies of these compounds (136). In another study (112), correlations between various carcinogen-DNA adducts and cell toxicity, mutagenicity, and carcinogenicity were examined in cell culture. There was a parallel between the repair of toxic, mutagenic, and transforming DNA damage. The removal of AAF molecules bound to the C8 of guanine followed kinetics similar to the repair of the biological lesions, while the N^2 adducts were not repaired to any appreciable extent by these cells. These investigators (112) concluded that the C8 guanine adduct causes most of the toxic and mutagenic damage exerted by AcAAF.

Besides the differences observed in the extent of carcinogen binding between the various chromatin fractions, it can be concluded that a substantial clustering of bound carcinogen along DNA also occurs. Examination of Table 9 reveals that more than 85% of the carcinogen is located on less than 25% of the total DNA (fractions P1 and S2). The possibility of a non-random distribution of AAF adducts was discussed on a theoretical basis by Kriek (107). He suggested that clustering could occur in DNA regions of high G·C base pair

content. Circular dichroism measurements indicate that these high G·C regions may be structurally different from other parts of the DNA molecule (125).

The observed clustering effect occurs not only in terms of the total carcinogen bound, but is also reflected as a separate localization of the two different guanine adducts (Figure 15). A review of the physical aspects of carcinogen binding to the C8 and N^2 positions of guanine might provide an explanation of this finding. The structure of DNA in the region of a C8-guanine AAF adduct has been predicted based on the "base displacement" model of Levine et al. (116) and the "insertion-denaturation model" of Fuchs et al. (59). Both groups of investigators have provided evidence that the covalent binding of carcinogen causes a localized region of denaturation of the DNA double helix due to the rotation of the modified guanine base around the glycosidic bond from an anti to a syn conformation (56, 5974,116,157). Evidence has been reported suggesting that the binding of AAF to the C8 position is favored in nucleic acids having characteristics of single strandedness:

1) binding to the C8 position occurs to a greater extent on RNA as opposed to DNA (Figure 11);
2) binding is greater on denatured DNA (single stranded) when compared to native, double stranded DNA (116);
3) a high ionic environment, which stabilizes DNA secondary structure, decreases the rate and extent of AAF binding (116);

4) binding of carcinogen to tRNA occurs primarily in guanine residues to the single stranded loop positions as opposed to guanine residues in the double stranded regions (61).

In contrast, the binding of AAF to the N^2 position appears to be favored in nucleic acids having characteristics of double strandedness:

1) N^2-guanine adducts can only be detected on double stranded DNA but not on single stranded RNA (Figure 11, ref. 106);
2) analysis of binding based on the density of chromatin indicated that formation of N^2 adducts was greatest on a highly condensed pelleted chromatin (Figure 8). DNA in this region presumably has a highly coiled, stabilized secondary structure.

Based on these physical aspects of carcinogen-DNA interactions, the following model is proposed to explain the clustering effects described above:

1) AAF binds to the C8 position of guanine preferentially in regions of chromatin where the reaction is sterically favored. These regions are mainly euchromatic in nature. Thermal denaturation studies (14,69,134) indicate that DNA in euchromatin has a lower melting temperature. Therefore, this chromatin would be more susceptible to the natural "breathing" of DNA base pairs, which is sterically required for binding of AAF to the C8 position of guanine (57). Furthermore, RNA synthesis, which by definition occurs exclusively in this region, also produces a temporary disruption of normal DNA double-stranded base pairing.

2) Upon binding of the carcinogen to the C8 position, a local denaturation of the DNA helix occurs (see discussion of base displacement model, above). This local denaturation further enhances binding of carcinogen to the C8 position of guanine (see points 1-4 under "characteristics of single strandedness", above) in that local region.

3) The local denatured region prevents binding of carcinogen to the N^2 position of guanine in that region of the genome (see points 1-2 under "characteristics of double strandedness", above).

4) AAF binds to the N^2 position of guanine mainly in regions of chromatin where C8 binding is sterically unfavorable. This occurs mainly in heterochromatic regions where a more stabilized secondary structure inhibits DNA strand opening. This could explain why the N^2 adducts are clustered in regions of chromatin having low overall susceptibility to carcinogen modification.

5) Binding of AAF to the N^2 position of guanine causes little or no distortion of the DNA double helix (209), hence subsequent carcinogen binding (to the C8 position of guanine) is not enhanced in this region.

A similar hypothesis to explain the effect of carcinogen binding to C8 guanine was proposed by Harvan et al. (78) based on their studies of adduct formation between AcAAF and synthetic polydeoxyribonucleotides in vitro. In these studies, binding of the carcinogen to the C8 position was seven-fold greater on an alternating poly(dG-dC)

polymer than it was on a poly dG·poly dC homopolymer. These investigators conclude that "in the case of the homopolymer, the poly dG strand would be sterically hindered from further attack near the site of initial AAF attack. On the other hand, AAF attack on the alternating polymer would generate two single-stranded, guanine-containing polymers. These polymers would be less sterically hindered, due to cytosine spacers between the guanine bases. Furthermore, AAF-purine stacking interactions are known to be stronger than AAF-pyrimidine interactions. Thus, the addition of an AAF moiety to a poly dG region should tend to preserve stacking interactions, contribute to stability, and reduce the extent of further attack; while in the alternating polymer stacking interactions should have little effect." These studies indicate that the increased reactivity of AAF-modified DNA for further binding to the C8 position is controlled by steric factors and the ease of forming single-stranded regions, but not by the absolute G·C content of the DNA itself.

If this model to explain the clustering of carcinogen-guanine adducts is correct, several predictions can be made. First, previously bound carcinogen residues would be expected to alter the site (i.e., C8 vs. N^2) of subsequent carcinogen binding. Thus, the modified DNA would have an altered conformation due to the presence of local regions of denaturation. According to the model above, the altered conformation should tend to favor binding to the C8 position rather than the N^2 position of guanine. This prediction is supported by the data in Table 11. Continuous carcinogen ingestion resulted in an enhanced binding of carcinogen to the C8 position relative to the N^2 position.

The second prediction is that the binding of carcinogen to the C8 position of guanine should demonstrate cooperativity. Binding of the first carcinogen residue would depend on the natural "breathing" of the DNA double helix. Once binding occurred, the DNA would be "locked" in an open conformation making it easier for the next carcinogen residue to bind to the DNA. Evidence for a cooperative effect have been reported by Poirier et al. (167) for low levels of AAF binding to the C8 position of guanine detected by radioimmunoassay. Their findings are illustrated in Figure 23. The extent of modification in Figure 23 is in the range (i.e., adducts/10^6 base pairs) of that observed in vivo (see Table 6). In contrast, at higher levels of DNA modification (i.e., in the range of adducts/10^3 base pairs), a linear relationship between carcinogen concentration and amount bound has been reported (137). This may represent the linear portion of the curve in Figure 23. At even higher carcinogen concentrations, when saturation of binding sites occurs, the curve will taper off resulting in an overall sigmoid relationship between carcinogen concentration and amount bound.

Other interpretations besides a "cooperative base displacement" model could explain the findings described above. The clustering effect might result from relatively high concentrations of activated carcinogen in localized areas of the nucleus. Alternatively, the clustering could result from inherent structural conformation differences in selected chromatin regions, without the need to invoke the "cooperative" aspects of the base displacement model. The effect of continued carcinogen ingestion on the site of subsequent binding could be explained by differential effects on metabolic activation pathways.

Figure 23. Extent of formation of dG-8-AAF, *in vitro*, in relation to the concentration of N-AcAAF. Data of Poirier *et al*. (167) and unpublished observations of M. Poirier. N-AcAAF was added to primary BALB/c epidermal cell cultures. After 1 hour, DNA was isolated, enzymatically hydrolyzed and assayed for dG-C8-AAF by radioimmunoassay.

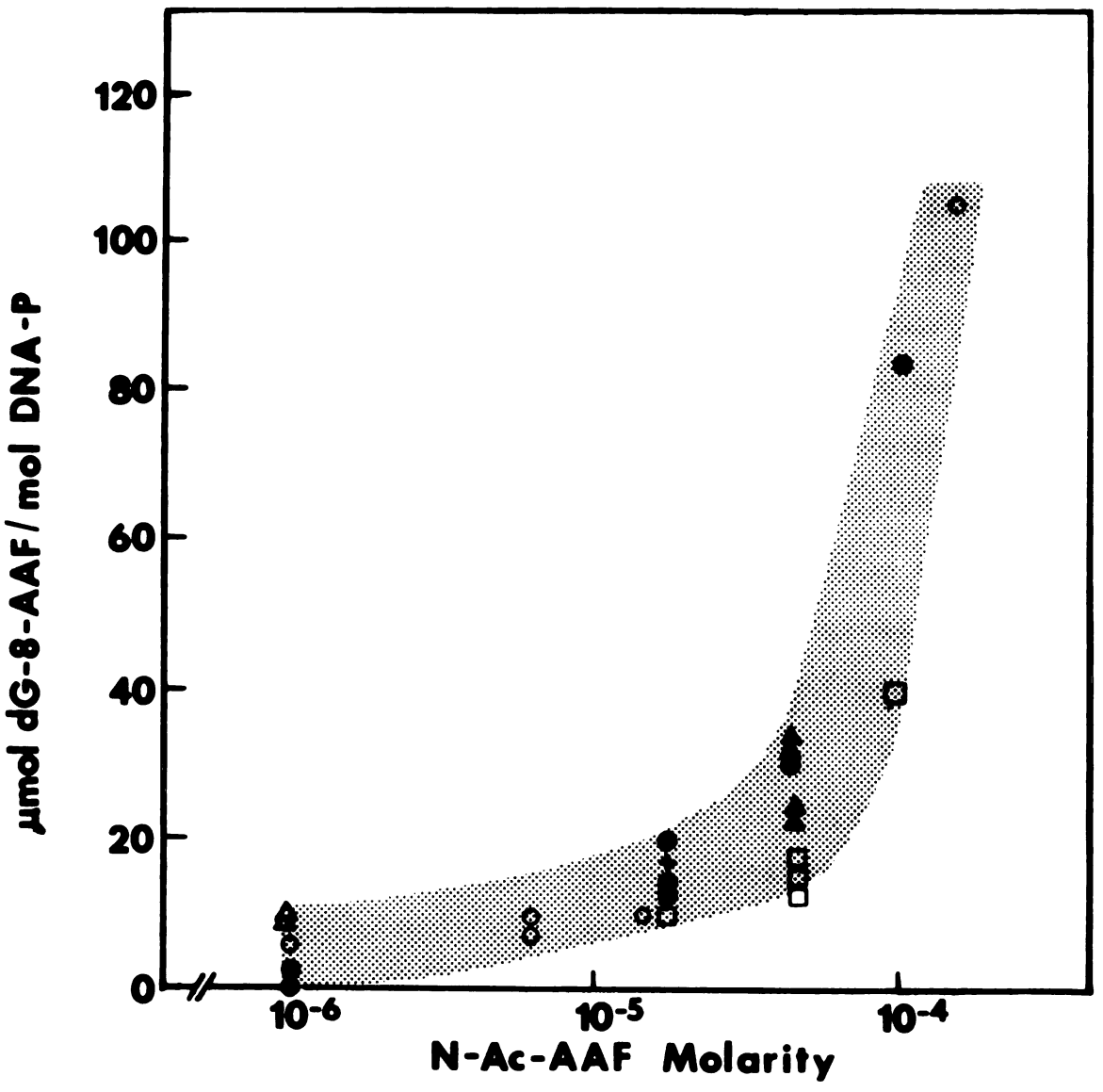

120
100
80
60
40
20
0
µmol dG-8-AAF/mol DNA-P
10-6
10-5
10-4
N-Ac-AAF Molarity

Figure 23

Different carcinogen-DNA adducts have been speculated to result from separate metabolic activation pathways (91), although no direct experimental evidence is available for this. The deacetylated adducts might result from reaction of N-glucuronide-AAF with DNA, while acetylated adducts could be due to reaction of reactive AAF esters with DNA (91). Finally, the apparent cooperative curve illustrated in Figure 23 could potentially be an artifact of radioimmunoassay or some property unique to cell culture systems.

Analysis of metabolic activation capacities with time on an AAF diet indicate there is a progressive decrease in formation of macromolecule-binding reactive species over a two week period (Table 10). Other investigators have shown that there is less carcinogen bound to DNA after 10 weeks of continuous carcinogen ingestion than after 2 weeks of ingestion (192). Comparison of the relative inhibition of AAF and N-OH-AAF binding indicates that part of the decrease is due to an inhibition of the N-hydroxylation of AAF. Most of the reduction, however, is due to an inhibition of the second metabolic activation step.

Briefly summarized, the carcinogen-DNA binding studies indicate that binding of carcinogen is greater on DNA in euchromatin regions when compared to heterochromatin regions. The nature of the carcinogen binding to DNA, expressed as the N^2/C8 ratio, is inversely proportional to the overall extent of carcinogen attack. With continued carcinogen exposure, the amount being bound decreases. There also appears to be a tendency for clustering of adducts in a relatively small proportion of the total DNA; this effect would be

expected to persist with continued carcinogen exposure. DNA repair occurs during this time as well, and differences were observed in repair rates between the various chromatin fractions. Once AAF exposure ends, complete repair of C8-guanine-carcinogen adducts can occur, while the N^2-guanine adducts should persist (106). The cooperative base displacement model also predicts that the persistent N^2 adducts may be localized within certain regions of the genome.

Some interesting implications of the cooperative base displacement model and resultant clustering of DNA damage can be considered. This phenomenon might explain how a repairable DNA lesion can nevertheless persist with time and continued carcinogen exposure. It is presumed that DNA damage is biologically significant only when the DNA is called upon to be transcribed or replicated. Although the C8 adduct can be repaired, the DNA region in which the adducts are clustered would also be highly susceptible to further damage. Therefore, the relative balance of binding and repair would determine how long damage in a specific region of DNA would persist. It would also be of interest to examine these DNA regions in relation to mechanisms of DNA repair and enzymatic recognition of damaged DNA. Repair of AAF-induced DNA damage occurs by the long patch excision repair mechanism, however, this repair mechanism appears to differ from repair of UV damage, which also is a long patch type repair (2,4). Finally, the effect of carcinogen clusters should be examined in terms of functional damage and inhibition of DNA template capacities.

The inhibition of transcription of AAF-damaged DNA templates has been previously reported (1,73,96,150,195,208,212). DNA isolated from

chromatin fractions obtained by sonication and glycerol gradient centrifugation was used to assess functional carcinogen damage. After 4 days of AAF ingestion, a significant reduction in transcriptional capacity occurred on the DNA isolated from the slow and fast sedimenting chromatin fractions. No changes in RNA polymerase initiation sites were seen on the DNA derived from the AAF exposed rats. This finding is in agreement with other reports (150,212). Therefore, the inhibition of transcription was due to a premature termination of RNA synthesis resulting in a reduced average RNA chain size. The re-initiation studies demonstrated that the enzyme is released upon premature RNA chain termination, and can reinitiate another round of RNA synthesis. Thus, the effect of AAF can be distinguished from that of benzo(a)pyrene, which besides causing a reduction in average RNA chain length, also prevents recycling of the RNA polymerase (115).

Millette and Fink (150) have suggested that the premature termination of transcription results from release of the RNA polymerase at or near the site of the binding of AAF to DNA. Presumably the E. coli RNA polymerase cannot continue polymerization distal to the site of the carcinogen induced modification (150). It is not known whether the enzyme can re-initiate RNA synthesis at a site distal to the DNA damage but within the same transcript.

Transcription of mammalian DNA by E. coli RNA polymerase is faithful in terms of the nucleotide sequence polymerized (30). However, the enzyme is less accurate compared to eucaryotic RNA polymerases in that it initiates transcription at a much larger number of sites than does the homologous RNA polymerase (27) and also probably

transcribes both strands of DNA and spacer as well as gene regions (174). It should be emphasized that these studies were not designed to faithfully reproduce the RNA transcript products occurring *in vivo*, but rather to use the bacterial RNA polymerase as a probe for discerning carcinogen-induced DNA damage. Any DNA damage that is directly relevant to the neoplastic process is likely to be only a small fraction of the total carcinogen modification of DNA that occurs. Assuming that carcinogen binding is equally likely to occur on both strands of DNA, the use of *E. coli* polymerase can yield a valid estimate of total carcinogen-induced modification of the DNA template, although not necessarily providing a measure of the extent of functional alterations that might occur in the intact cell.

The degree of inhibition of template capacity that was observed can be used to estimate the extent of carcinogen binding to DNA. An average transcript size of 350 nucleotides (Table 12) and an average reduction of 40% in RNA synthesis after 4 days of 0.03% AAF ingestion (Figure 18) suggests one modified base per 438 nucleotides or 23 per 10^4 nucleotides (this assumes a random average distribution of AAF within the DNA transcript and that all bound AAF molecules result in chain termination (150). Szafarz and Weisburger (192) report binding of approximately 1.25 molecules of carcinogen per 10^4 nucleotides after feeding 0.016% N-OH-AAF for 2 weeks. The higher estimate of binding compared to that of Szafarz and Weisburger may be due to several factors. Both the amount of carcinogen fed and the length of time of ingestion may affect the extent of carcinogen bound, since metabolic activation and extent of binding decrease with continued AAF

ingestion (ref. 192 and Table 10). Covalent binding of AAF to a single DNA nucleotide may actually result in a perturbation of DNA structure extending up to 50 base pairs (206), potentially extending the chain terminating effect of a single carcinogen molecule over adjacent DNA transcripts. Thus, the degree of inhibition of RNA synthesis that was observed is reasonable based on these previous studies.

When the initial rate of RNA synthesis was examined, a small reduction in the rate of chain elongation was observed on the DNA template derived from the heterochromatin (Figure 17). It is not clear whether this effect is due to a carcinogen-induced reduction in the actual movement of the enzyme along the template, or may merely reflect the decrease in the average RNA chain size formed. A more direct method to measure the rate of RNA chain elongation was devised by Hyman and Davidson (88). In this procedure, the rate of RNA synthesis is measured using varying subsaturating concentrations of one nucleotide triphosphate, while the concentration of the other 3 nucleotide triphosphatase are held constant at a saturating level. Intuitively, the polymerization of the nucleotide triphosphate present at subsaturating levels will be the rate-limiting step, and total RNA synthesis will be proportional to the concentration of the nucleotide triphosphate. Anything which further reduces this polymerization (e.g., inhibited base pair match-up due to carcinogen damage) will further also reduce total RNA synthesis. This effect will be magnified as this particular nucleotide triphosphate concentration decreases relative to the Km for incorporation of the nucleotide.

The kinetics of this reaction has been formally derived by Hyman and Davidson (88), and is summarized in Table 14. The reciprocal of the rate of RNA synthesis is a linear function of the reciprocal of the substrate concentrations; the slope of this line is inversely related to the rate of addition of the individual nucleotide. These investigators (88) have used this analysis to demonstrate that actinomycin D interacts with G-C base pairs.

The DNA isolated from the pelleted chromatin showed no significant reduction in template capacity as judged by total RNA synthesis, initiation sites, or average RNA chain length. It has been previously shown, however, that carcinogen residues can be found on the DNA from this chromatin fraction after injection of ^{3}H-OH-AAF (Table 6). Therefore, the nucleotide addition analysis described above was utilized as a means of assessing the fidelity of transcription of RNA from this template. A large reduction in the rate of incorporation of A and U was observed. The fact that the alteration of adenosine and uridine polymerization occurred on a DNA template on which no apparent premature chain termination was measured indicates that at least 2 different types of carcinogen modification occurs in vivo. The selective effect on A and U, but not G and C suggest that this damage may be due to adenine modification rather than a minor guanine product.

Studies with adenine-containing synthetic polynucleotides indicate that under the proper conditions, covalent binding of AcAAF to adenine can occur (78,97,109,116,130,141). In one of these studies, more than 95% of the bases in a poly (A) homopolymer were modified

(109). Nevertheless, these reports have all concluded that reaction of A-AAF with adenine in synthetic polynucleotides is less than that of guanine. On the other hand, Kapuler and Michelson (97) have calculated that reaction of DNA in vitro with AcAAF results in approximately equal amounts of carcinogen bound to guanine and adenine. Westra et al. (205), however, reported that the amount of carcinogen bound to A was less than 4% of that bound to G after in vitro reaction of DNA with AcAAF. Other investigators have been unable to detect any carcinogen-modified adenine residues after reaction with AcAAF (56). In addition to the findings regarding potential covalent binding of AAF to adenine, there have been reports of non-covalent, tight binding of carcinogen to native DNA and polyadenylic acids (78,97,116). It is not clear whether the substantial functional alterations observed results from a relatively low extent of adenine modification, or perhaps reflects conditions under which a more extensive modification can be detected than had been previously believed to occur.

Studies by Corbett et al. (35) have suggested that a binding of AAF to adenine may be significant in terms of a mutational event. AcAAF-induced mutations in T4 phage resulted 25% of the time from A/T to G/C base transitions. The major type of mutation observed was a frameshift mutation; no G/C to A/T transitions were observed in this study. Furthermore, Hyman and Davidson (88) reported that although actinomycin D binds to G, an inhibition of both C and G incorporation occurs. Thus, our findings are consistent with the hypothesis that AAF can modify adenine and interfere with its normal base pairing properties.

All of the alterations in template capacity occurring after 4 days of AAF ingestion appear to be rapidly repaired. This apparent return to control DNA template capacities does not preclude the possibility that some damage persists in a small number of cells or that there may be an error-prone repair mechanism in operation. Although AAF ingestion for the brief time examined probably would not result in tumor formation in and of itself (3), Peraino et al. (164) have shown that 0.02% AAF ingestion by weanling rats for a nontumorigenic length of time (18 days) followed by exposure to phenobarbital results in a significant enhancement of hepatocarcinogenesis. This implicates initial DNA damage as important to the overall carcinogenic process.

At the present time, the biological effects of the minor guanine-AAF adduct [3-(deoxyguanosin-N^2-yl)-N-AAF] (205) on DNA function have not been determined. It was somewhat surprising to find that no apparent alteration of C incorporation occurs after 4 days of AAF ingestion. However, it is possible that the N^2 modification of guanine by AAF might result in either premature chain termination or base mispairing which may not have been detectable using the double reciprocal analysis of the rate of nucleotide addition. Despite the fact that the pelleted heterochromatin DNA contains a relatively high amount of the persistent N^2 adduct after a single injection (Table 6), there were no detectable persistant alterations in DNA template capacity on the DNA from this chromatin fraction. Due to the impracticality of feeding a diet containing radiolabelled carcinogen, the extent of DNA modification occurring after carcinogen ingestion

cannot be quantified. Thus, it is possible that the degree of DNA damage occurring is below the sensitivity of the transcriptional analyses used. Furthermore, as discussed above, the appropriate tests to determine the biological effect of N^2-guanine modification may not have been employed.

SUMMARY AND CONCLUSIONS

An analysis of the interaction of a chemical carcinogen with target tissue DNA was performed. The DNA was fractionated utilizing procedures which differentiate the template active from the template repressed DNA. These studies demonstrated that different regions in the genome are susceptible to alternate carcinogen modification based on the conformation of the chromatin *in vivo*. Differences in the rate of removal of carcinogen residues from various regions of the genome could then reflect both the predominant nature of the modification (i.e., C8 vs. N^2 position of guanine), and the general accessibility of the DNA to repair enzymes perhaps based on the degree of chromatin coiling. Template active euchromatin DNA is more susceptible to carcinogen binding, while a greater proportion of the carcinogen adducts on heterochromatin DNA are likely to persist with time. This suggests that factors which alter the location of target genes (eu- vs. heterochromatin) could modify the initiation phase of carcinogenesis, including both binding and repair.

An attempt was also made to assess the functional consequences of carcinogen binding to DNA. It was shown that AAF ingestion for as little as 4 days can result in substantial alterations in DNA transcriptional capacity. The primary effect appears to be a reduction in RNA chain size. This damage occurs on both expressed and repressed

portions of the genome, and is rapidly repaired when the rats are taken off the carcinogen-containing diet. In contrast, DNA derived from a highly condensed, apparently repressed chromatin fraction exhibited no change in the average RNA chain length synthesized. Analysis of the rate of nucleotide incorporation, however, demonstrated a large reduction in the rate of incorporation of A and U. This suggests that previous studies may have underestimated the potential functional significance of covalent carcinogen modification of adenine.

The binding of carcinogen to DNA in vivo is a decidedly non-random event. Thus, 85% of the bound carcinogen is localized on less than 25% of the total DNA. A "cooperative base displacement" model has been proposed to at least partially explain this phenomenon. Experimental evidence and predictions based on physical studies of carcinogen-DNA interactions support this model.

The non-random nature of AAF-DNA interactions is particularly intriguing from a biological point of view. In several studies, the frequency of malignant transformation in vitro was found to be larger than the frequency of mutation in vitro (85,197). These investigators speculate that either the DNA "target" for transformation is larger than the target for a specific mutation, or alternatively the target gene for transformation is located in a "hot spot" within the genome, and is more susceptible to damage from chemical carcinogens (85,197). Based on the findings reported in this thesis, further study of the latter proposal would be of interest.

A recent study (170) has examined the interaction of AcAAF with the SV40 genome. The nucleotide sequence of this viral DNA has been

totally elucidated, and the DNA can be enzymatically fragmented into 5 defined segments. Carcinogen binding to one fragment was found to be twice that of the binding to the other 4 fragments. This is the first study demonstrating that carcinogen binding to DNA can be analyzed in terms of specific genes. Shoyab (184) has examined the binding of a polycyclic hydrocarbon to reiterated and unique DNA sequences in skin cells in culture. He reports a dose-dependent preferential binding to the reiterated DNA. Furthermore, there is an inverse relationship between the total amount of carcinogen bound, and the frequency of that binding occurring on reiterated DNA. Juxtaposed with Figure 15 of this thesis, one can speculate that specific regions of the genome are susceptible to certain types of purine modification based on the particular carcinogen used. The clustering of specific types of carcinogen-induced DNA damage provides potential sites for abnormal gene expression in selected regions of the genome. Situations in which the potential for aberrant gene expression is increased may enhance the probability of initiating malignant transformation, or might otherwise influence the progression of the carcinogenic process. Thus, the possibility exists that DNA interactions important in carcinogenesis do not occur at random, but are governed by steric characteristics of the carcinogen and the DNA.

Any attempt to draw conclusions as to the biochemical events occurring in the ultimate neoplastic cell based on parameters measured in the whole liver is fraught with difficulty. Various cellular subpopulations exist even in the normal liver (26); during the pre-cancerous period the number of these subdivisions increase and the

differences between them are accentuated (68,165). The possibility must be kept in mind that the biochemical events relevant to the carcinogenic process might be at variance to those occurring in the liver as a whole. Measurement of parameters in the whole liver might then result in an overestimation, underestimation, or completely opposite effects from those that are critical in carcinogenesis.

Increasingly sophisticated techniques are needed to analyze carcinogen binding to DNA. More specific fragmentation of DNA (i.e., using restriction endonucleases and specific cDNA probes) may shed further light on those specific genes that are important in carcinogenesis. It would also be helpful if preneoplastic cellular subpopulations could be specifically tested. The cooperative base displacement model which has been proposed, but by no means proven, is a testable hypothesis that may have important implications in understanding DNA-carcinogen interactions.

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