RNA SYNTHESIS AND DNA TEMPLATE ACTIVITY IN THE LIVERS OF RATS FED THE HEPATIC CARCINOGEN 2-ACETYLAMINOFLUORENE

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ABSTRACT

RNA SYNTHESIS AND DNA TEMPLATE ACTIVITY IN THE LIVERS OF RATS FED THE HEPATIC CARCINOGEN 2-ACETYLAMINOFLUORENE

By

Michael P. Adams

It is becoming increasingly evident that the mere presence or persistence of carcinogen-induced DNA base modifications does not automatically implicate these lesions as relevant to carcinogenesis. These lesions must be capable of producing some functional change in the DNA which will eventually result in initiation or promotion of carcinogenesis. One potential method of assessing the functional consequences of carcinogen-DNA interactions is to assess the ability of the DNA, in the target organ, to serve as a template for RNA synthesis. Significant decreases in hepatic DNA template activity have been reported after several weeks of feeding the hepatic carciongen 2-acetylaminofluorene (AAF), after a single injection of N-hydroxy-2-acetylaminofluorene (N-OH-AAF) and after reacting DNA in vitro with N-acetoxy-2-acetylaminofluorene. On the other hand, several investigators have been unable to demonstrate inhibition of chromatin or DNA template activity after N-OH-AAF administration. The studies described in this thesis were designed to examine the feasibility of using DNA template activity as a functional measure of carcinogen-induced DNA damage.

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RNA polymerase I activity and the capacity for RNA synthesis were assessed, under conditions optimal for RNA polymerase I activity, in hepatic nuclei isolated from rats fed 0.05% (w/w) AAF for 4, 7 or 14 days. RNA polymerase I activity progressively increased with time on the carcinogenic diet, while the capacity for RNA synthesis remained quite constant. These results suggest a progressive inhibition of DNA template activity during the early stages of AAF-induced hepatocarcinogenesis. The "permanence" of the increase in polymerase I activity was examined by switching carcinogen-fed animals to a control diet for either 2 or 5 days prior to making an assessment of the above parameters. The "permanence" varied, depending upon the duration of carcinogen exposure.

A more direct measure of DNA template activity was obtained by isolating hepatic DNA and transcribing the template in vitro with E. coli RNA polymerase. Template activity was assessed, in animals maintained on a schedule of carcinogen feeding similar to that described above, utilizing a method which separates the initiation step of RNA synthesis from the propagation step. Rate and extent of RNA synthesis, RNA chain length and the number of initiation sites were calculated from the resulting data. DNA was isolated from parenchymal cell (N-1) and non-parenchymal cell (N-2) nuclei. The rate of RNA synthesis on N-1 DNA was inhibited 24% and 48% after 4 and 14 days, respectively, of AAF ingestion but was unchanged after 7 days. The rate of RNA synthesis on N-2 DNA was inhibited (40%) only after 14 days of AAF feeding. No change in the number of initiation sites could be demonstrated on either template at the end of 4, 7 or 14 days of AAF feeding. When animals were returned

to the control diet for 2 days, statistically significant increases in the number of initiation sites were observed for N-1 and N-2 DNA after 4 days and for N-1 DNA after 14 days of AAF feeding.

The results of these studies suggest that an early effect of AAF ingestion is an impairment of hepatic DNA template activity. The major mechanism of action appears to be an inhibition of the elongation step of RNA synthesis, although some alterations in the number of initiation sites were observed.

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DEDICATION

This thesis is dedicated to my loving wife Susie, without whose compassion and understanding I would never have received this degree, and to my parents, John and Shirlee Adams for their never-ending love and guidance which will always be an inspiration for me to pursue higher goals.

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AAF	2-acetylaminofluorene
N-OH-AAF	N-hydroxy-2-acetylaminofluorene
N-Ac-AAF	N-acetoxy-2-acetylaminofluorene
ATP	adenosine 5' triphosphate
CTP	cytidine 5' triphosphate
DNA	deoxyribonucleic acid
GTP	guanosine 5'triphosphate
poly A	poly adenylic acid
poly (dA-dT)	a high molecular weight, doublt stranded copolymer composed of alternating dA and dT units
RNA	ribonucleic acid
TCA	trichloroacetic acid
UTP	uridine 5' triphosphate

INTRODUCTION

1.1 Chemical Carcinogenesis

In the past 50 years, a large number of chemicals have been identified as carcinogenic in animals. Structures of these compounds vary considerably, from highly complex natural products such as aflatoxin and cycasin to metals such as chromium, nickel and beryllium (1). Because these carcinogens exhibit considerable variability as to species and tissue specificity, there is currently no unequivocal method for extrapolating the production of experimental cancers in animals to environmentally produced human cancer. Chemicals have been implicated in human cancer largely on the basis of epidemiological studies. Such studies usually examine a small number of workers exposed to high concentrations of chemicals in an industrial situation or differences in the incidence of specific cancers between isolated geographical regions (1). As a result, several chemicals have been identified as probable causes of human cancer, including asbestos, cigarette smoke and the industrial compounds 4-aminobiphenyl, benzedrine and 2-napthylamine (2). Although no definitive data exist on the proportion of human cancer that develops as a result of environmental chemical exposure, epidemiological studies suggest that a large percentage, perhaps 80%-90%, of all human cancers have environmental factors in their etiology (2). The magnitude of the

problem demonstrates the need for a greater understanding of the underlying mechanism(s) of chemical carcinogenesis.

Carcinogenesis appears to consist of two major stages, initiation and promotion. Initiation of carcinogenesis is thought to consist of an essentially irreversible, heritable but unexpressed change in one or more informational macromolecules (3). Initiation may occur with "subcarcinogenic" doses, producing no apparent morphological change, such that the initiated state can be detected only by subsequent exposure to a promoting stimulus (4). The end result of initiation is the production of a potential cancer cell. If followed by an appropriate stimulus, the previously unexpressed change will become expressed and tumor progression will begin. Tumor promotors do not appear to be merely weak carcinogens. Application of promotor alone does not consistently result in tumor production and their action is dependent upon the order of application, i.e., promotors applied prior to initiation are generally not effective (3). In addition, a promotor may be effective when applied several months after the initiation event (5). Although not well understood, the process of promotion appears to involve a proliferatory stimulus resulting in tissue hyperplasia (3).

Although the model tissue for studying the two stages of carcinogenesis has been mouse skin, several studies have suggested that these processes also occur in the liver. Peraino et al. (6) demonstrated that feeding of phenobarbital or dichlorodiphenyltrichloroethane (DDT) to rats previously maintained on a 0.02% AAF diet for only 18 days, markedly enhanced hepatoma formation. Amobarbital, structurally very similar to phenobarbital, had no promoting action (6). Craddock (7)

significantly enhanced hepatoma frequency by performing partial hepatectomies on rats previously treated with the carcinogen dimethylnitrosoamine. Furthermore, Armuth and Berenblum (8) demonstrated these processes in mice by following a "sub-carcinogenic" dose of dimethylnitrosoamine with repeated injections of phorbol. While no hepatomas appeared in the group receiving phorbol alone, the group receiving carcinogen followed by phorbol developed a high incidence of tumors (8).

Of particular importance to the study of chemical carcinogenesis are the observations that; 1) the carcinogen appears to produce a very early, possibly irreversible change in an information macromolecule; 2) the carcinogen need not be present during the entire latent period of tumor production, and 3) "sub-carcinogenic" doses of a chemical carcinogen can produce high incidences of tumors if the appropriate promoting stimulus is applied.

1.2 Metabolism of AAF and Interaction with Cellular Macromolecules

While metabolism of most compounds results in their transformation to less chemically reactive species which can be readily excreted (9), metabolism of many chemical carcinogens results in small amounts of highly reactive species capable of interacting with cellular components (10). Formation of such highly reactive compounds may require several metabolic steps; i.e, transformation of a "non-carcinogen" to a proximate carcinogen and eventually to an ultimate carcinogen. A characteristic common to most and probably all ultimate carcinogens is the possession of strong electrophilic properties (10). These highly electrophilic reactants are capable of interacting non-enzymatically at a large number of nucleophilic sites, including those in glycogen, DNA,

RNA and proteins. The indiscriminate nature of this binding makes elucidation of the critical target(s) of chemical carcinogens a monumental task.

One of the most extensively studied carcinogens, in terms of its metabolic fate, is the hepatocarcinogen AAF. Early studies identified a metabolite of AAF, N-OH-AAF (11,12), which when administered to animals, exhibited greater carcinogenicity (13) and a higher degree of binding to nucleophiles (14,15) than the parent compound. The importance of the N-hydroxylation product in AAF-induced carcinogenesis has been demonstrated by several investigators. Irving et al. (16) demonstrated that the male Sprague-Dawley rat, a species highly susceptible to AAF-induced hepatocarcinogenesis, can readily N-hydroxylate AAF in the liver, as evidenced by the high percentage of this metabolite excreted in the bile. However, the female Fischer rat, a species refractory to AAF-induced hepatic tumors, excretes very little N-OH-AAF in the bile and thus appears to have an impaired capacity for N-hydroxylation in the liver (17). This correlation between inability to N-hydroxylate AAF and lack of significant carcinogenicity also exists for the rabbit (16) and guinea pig (18).

Substantial evidence has accumulated implicating esters of N-OH-AAF as ultimate reactive metabolites of AAF. Synthetic esters such as N-Ac-AAF bind avidly to proteins and nucleic acids in vitro (10,19) and several of the reaction products are identical with those obtained after in vivo administration of AAF or N-OH-AAF (10,19). In the liver, the ultimate carcinogenic form of AAF is believed to be the sulfate ester of N-OH-AAF. DeBaun et al. (15) demonstrated the importance of the sulfate

ester by showing a positive correlation between hepatic sulfotransferase activity and susceptibility to AAF-induced hepatomas in mice, hamsters guinea pigs, rabbits and male and female rats. This correlation was confirmed by Gutmann et al. (17) in two species of female rats. In addition, reducing the liver sulfate pool by treatment with p-hydroxyacetanilide (20) or decreasing liver sulfotransferase levels by hypophysectomy, gonadectomy or thyroidectomy (15) can inhibit carcinogenesis by AAF. However, it is probable that other metabolites of AAF can contribute to carcinogenicity by this agent since some tissues exhibiting AAF-induced tumors do not possess significant sulfotransferase activity (21).

Binding of chemical carcinogens to protein was first reported many years ago (22). Early studies demonstrated that certain proteins exhibiting binding to azo dyes soon after carcinogen administration are absent from the resulting hepatoma (23). Subsequent in vitro studies with N-Ac-AAF resulted in the identification of methionine, cysteine, tryptophan and tyrosine as target amino acids (19). De Baun et al. (15) identified two methionyl-AAF adducts in rats fed AAF or N-OH-AAF which appeared identical to those formed in vitro, thus supporting the existence of these reactions in vivo. In addition, this group also demonstrated that formation of methionyl-AAF derivatives in vivo correlated well with the hepatic carcinogenicity of N-OH-AAF in several species (15). Metabolites of AAF have been shown to bind to histones (24) and acidic nuclear proteins (24,25), both of which are thought to be involved in controlling gene expression. Interaction of a carcinogen with

a critical cellular protein could, theoretically, result in a permanent, heritable change in gene expression, possibly leading to carcinogenesis (26).

Several studies suggest, however, that proteins may not be critical targets of chemical carcinogens. The amount of protein-bound carcinogen does not always correlate well with carcinogenicity in certain rodents (27,28) and the ability to participate in protein binding is not unique to chemical carcinogens (29,30). In addition many protein-bound AAF adducts exhibit a relatively short half-life compared to some AAF-DNA moieties (24), although this may simply reflect the slow turnover of the latter macromolecule in many organs.

Another possible critical target of chemical carcinogens is RNA. N-Ac-AAF reacts well with RNA in vitro (31) and this interaction has been shown to produce defects in amino acid acceptance capacities, codon recognition and ribosomal binding of several transfer RNA molecules (32). Covalent binding of AAF metabolites to RNA has been reported to occur after in vivo administration of AAF or N-OH-AAF (33-35) and the major reaction product formed is believed to be N-(guanosin-8-yl)-2-AAF (31,36). The extent of binding of AAF metabolites to RNA is greater in male rat liver than female rat liver (34,37); the latter being relatively non-susceptible to hepatic carcinogenesis by this agent. In addition, two non-carcinogenic derivatives of N-OH-AAF, 1-hydroxy-2-acetylaminofluorene (35) and N-hydroxy-3-acetylaminofluorene (38), exhibit negligible binding to this macromolecule.

Several studies suggest that RNA may not be a critical target of chemical carcinogens. Irving et al. (35) demonstrated that detectable amounts of AAF metabolites are bound to RNA in tissues which are relatively non-susceptible to AAF-induced carcinogenesis, such as rabbit and guinea pig liver, after a single injection of AAF or N-OH-AAF. Matsushima et al. (33) prefed various inhibitors of AAF-induced hepatocarcinogenesis (m-acetatoluidine, indole, acetanilide, chloramphenicol) to rats and examined the binding of AAF metabolites to the various hepatocellular fractions after a single dose of N-OH-AAF. While prefeeding of these inhibitors generally decreased the initial binding of AAF metabolites to DNA, no such correlation could be demonstrated for RNA.

Numerous studies have presented data implicating DNA as a critical target of chemical carcinogens. The degree of covalent binding of a chemical carcinogen to DNA correlates well with its ability to induce tumors. This relationship has been shown to exist for a series of aromatic hydrocarbons (39), a series of alkylating agents (40) and certain metabolites of 4-nitroquinolin-1-oxide (41). Furthermore, prefeeding of several inhibitors of AAF-induced carcinogenesis, including indole, chloramphenicol, m-acetotoluidide and acetanilide markedly decreased the extent of AAF moieties bound to DNA after a single injection of N-OH-AAF (33).

Attempts have been made to identify specific structural alterations in DNA after carcinogen administration with the expectation that such knowledge would allow one to predict the functional consequences of such

an interaction on the molecular level. Experiments using double labelling techniques (^{3}H-acetyl group, ^{14}C-fluorene ring) indicate that 70% to 80% of the carcinogen molecules bound to DNA after a single injection of AAF (42) or N-OH-AAF (14) are deacetylated. This is in contrast to results with RNA which suggest that 70% of the bound AAF molecules retain the acetyl group (14,35,43). Eighty percent of the acetylated AAF moieties bound to DNA have been identified as N-(deoxyguanosin-8-yl)-2-AAF (31,42). Although not well characterized, the remaining 20% of the bound acetylated AAF moieties are believed to be 3-(guanin-N^2-yl)-2-AAF (19,42). Binding of AAF metabolites to adenine is not well understood and several investigators have failed to identify such base modifications (19,31,43). At least one study, however, has suggested the existence of modified adenine in DNA (44) and under certain circumstances, N-Ac-AAF can react with poly A *in vitro* (45,46). Failure to detect AAF modifications of adenine, cytidine or thymidine bases may be due to artifacts in the isolation procedure or to the limited sensitivity of the analytical methods used to identify these bases.

Numerous biochemical and biophysical studies on AAF-modified DNA and oligonucleotides have led to an "insertion-denaturation" ("base displacement") theory (46-49). According to this model, binding of an AAF metabolite to the C-8 of guanine causes a rotation of the base around the glycosidic bond from anti to syn (47,50). In such an arrangement, a modified guanine base is displaced from its normal position by the fluorene moiety and the latter becomes stacked coplanar with adjacent bases. This model explains the local denaturation of DNA

suggested in several studies (44,48,51,52) and predicts general destabilization of double helical conformation and disruption of normal hydrogen bonding between affected bases (52).

Several studies have demonstrated that certain AAF moieties bound to DNA can exhibit a prolonged biological half-life. Epstein et al. (53) demonstrated that hepatic DNA isolated from the hyperplastic nodules of AAF-fed rats exhibited an altered ultraviolet absorbance spectrum indicative of AAF-modified DNA, four weeks after discontinuation of the carcinogenic diet. These results were confirmed by Szafarz and Weisberger (54) who identified DNA-bound radioactivity in the livers of rats eight weeks after removal of a (9-^{14}C) N-OH-AAF diet. Persistant AAF-DNA adducts have also been identified in rat liver after a single injection of AAF (14) or N-OH-AAF (42). Irving and Veazy (14) determined the rate of disappearance of radioactivity from ribosomal RNA and DNA after a single injection of AAF and demonstrated that radioactivity remained bound to the former for only 2 weeks and with the latter throughout the entire eight week study. Kriek (42) isolated two reaction products after a single injection of N-OH-AAF, one having a half-life of seven days and the other remaining bound to DNA for eight weeks. The persistent moiety, accounting for 20% of the total bound AAF molecules, was later identified as 3-(guanin-N^2-yl)-2-AAF (19).

1.3 DNA Repair

Most cells possess a mechanism whereby certain types of DNA damage can be removed and the affected region repaired to its original structure. This phenomena, termed excision repair, has been extensively

studied in procaryotes (55) and mammalian cells in culture (56) and appears to involve at least five steps (58,57): 1) recognition of base damage; 2) production of a single strand break near the lesion by an endonuclease; 3) excision of the affected base(s); 4) re-insertion of a correct base sequence using the complementary strand as a template, and 5) annealing of the repaired region to the daughter strand by a ligase. The fidelity of this process has been assessed in mammalian cells and it appears to be an error-free process (60,59). Mutant cells deficient in excision repair have been isolated from patients with Xeroderma pigmentosum; a disease characterized by an increased susceptibility to ultraviolet light-induced skin cancer (61). The hypothesis that this increased susceptibility is due to a deficiency in a step of the excision repair process is attractive, but is as yet unsubstantiated. Certain variants possessing all the clinical symptoms of this disease appear to have no detectable defect in excision repair (62).

A second type of DNA repair which has been demonstrated in procaryotes and postulated to exist in eucaryotes is called post-replication repair (57). Although the process is poorly understood, it is thought to occur when a DNA polymerase bypasses a DNA lesion and continues synthesis distal to this site. A "gap" is created on the newly synthesized strand opposite the parental lesion and this "gap" is filled in at a later time by some unknown mechanism. This process does not actually repair the lesion but it does provide for cell survival by allowing the DNA polymerase to circumvent a potentially lethal block to DNA replication. The original lesion will be diluted out in subsequent

cell divisions. Painter (58) reviewed the available evidence for post-replication repair and concluded that this type of repair, as described by Lehman (63), does not exist in mammalian cells. In a recent article, Higgins et al. (64) proposed a model of replication repair which involves a bypass of the DNA lesion without the production of a "gap". In this model, the DNA synthesized off the undamaged strand acts as a template for replication of the damaged region in the homologous parental strand. Theoretically, this type of repair would be error free as opposed to the process of post-replication repair which, in procaryotes, is error prone (64).

Several studies have suggested that there are some reaction products formed between chemical carcinogens and DNA which are not readily repaired by the DNA excision repair process. Kriek (42) demonstrated the presence of two metabolites of AAF bound to DNA which exhibited markedly different biological half-lives. Whereas guanine modified by AAF at C-8 was removed with a half-life of 7 days, guanine modified by AAF at the 2-amino group remained in DNA eight weeks after a single injection. No persistent aminofluorene moieties could be identified. Utilizing molecular models, Kriek concluded that AAF substituted at the 2-amino group of guanine creates little distortion of the double helical conformation and thus may not be removed by DNA excision repair (19). Goth and Rajewsky (65) examined the initial extent of binding and rate of removal of three base modifications, O^6-ethylguanine (believed to cause mis-pairing during DNA replication [65]), N^3-ethylguanine and N^7-ethylguanine, produced by the alkylating agent N-ethyl-N-nitrosourea.

While the initial degree of alkylation to all three sites was essentially equal, the O^6 modification was removed considerably slower than the others. Furthermore, the rate of removal of O^6-ethylguanine was markedly slower in target tissue (brain) than in non-target tissue (liver) (65). These results suggest the importance of those reaction products not readily repaired by the excision repair process to carcinogenesis. However, in both of the studies cited above, the direct participation of the DNA excision repair process in preferential removal of a modified base was not demonstrated. The possibility exists that these modified bases were removed non-enzymatically due to instability or decomposition of the carcinogen-DNA adduct. The consequences of DNA lesions not readily repaired by the excision repair process have not been unequivocally determined, however, some appear to be potentially damaging (66) and may lead to cell death, mutation and possibly neoplasia.

1.4 Effects of AAF on RNA Synthesis

A potential method of assessing the functional consequences of carcinogen-DNA interactions is to measure the ability of the modified DNA in the target organ to serve as a template for RNA synthesis. Since hepatic RNA synthesis is occurring continuously at a relatively high rate, there is no need to perform a partial hepatectomy as is often the case when assessing DNA synthesis in the liver. Although several investigators have attempted to assess the effects of AAF on RNA synthesis and DNA template activity, the results obtained have been variable, depending upon the mode of carcinogen administration and the methods utilized to assess the experimental parameters.

Most investigators have reported a decrease in rat liver RNA synthesis several hours after a single injection of N-OH-AAF (67-72). However, there is disagreement as to whether this inhibition is due to an impairment in the capacity of DNA to serve as a template or to an inhibition of RNA polymerase activity. Initial studies by Grunberger et al. (69) indicated a decrease in hepatic nucleolar RNA synthesis with no change in the activity of RNA polymerase I, as measured on the synthetic template poly (dA-dT). They interpreted this data as indicative of an impaired DNA template activity. Herzog et al. (71), using an assay procedure similar to that used by Grunberger et al. (69), demonstrated a decreased RNA polymerase activity after AAF administration which they claimed could account for the observed inhibition in RNA synthesis. In addition, Herzog et al. could not demonstrate a decreased template activity on hepatic chromatin isolated from carcinogen-treated animals (71). In agreement with Herzog et al. were the results of Glazer et al. (67) and Zieve (68). Neither of these investigators could demonstrate an impaired template activity on DNA isolated from animals given a single injection of N-OH-AAF. In an attempt to resolve the apparent discrepancy, Yu and Grunberger (73) very recently confirmed their previous results (69) by demonstrating an impaired nucleolar template activity after a single dose of N-OH-AAF. However, their data also suggested the possibility of a direct alteration of RNA polymerase II by AAF metabolites, as was recently suggested by Glazer (74). Thus, AAF may have two mechanisms of RNA synthesis inhibition after acute administration; 1) alteration of

nucleolar template activity, and 2) selective inhibition of RNA polymerase II.

Troll et al. (75) were the first to isolate hepatic DNA from animals fed an AAF diet for several weeks and assess template activity using a bacterial RNA polymerase. They demonstrated that the ability of the DNA to serve as a template for RNA synthesis after AAF ingestion was substantially decreased. The degree of DNA template activity inhibition was somewhat variable, depending upon the duration of carcinogen feeding. Adams and Goodman (76) confirmed the results of Troll et al. (75), presenting data suggesting a decreased template activity in hepatic nuclei isolated from rats fed an AAF diet for 7 or 14 days.

Troll et al. (51) also demonstrated that DNA modified in vitro with N-Ac-AAF exhibits an impaired template activity. Extensive modification by N-Ac-AAF completely abolished the capacity of DNA to serve as a template for RNA synthesis. These results were confirmed by Zieve (77), Glazer et al. (67) and Millette and Fink (78), who also reported over 90% inhibition of DNA template activity after reaction of the macromolecule with high concentrations of N-Ac-AAF in vitro. In addition, Zieve reported template activity inhibition after reaction of DNA in vitro with N-OH-AAF, although very high concentrations were required to produce minimal changes (77).

Unfortunately, many studies assessing RNA synthesis on DNA isolated from carcinogen-treated animals have utilized relatively unsophisticated methods of measuring transcription. Some assay procedures (67,68,71, 77) measure a total amount of RNA synthesized after a fixed incubation period under conditions where results may be a complicated function of

the number of initiation sites available, the rate of chain propagation and RNA chain length. Such methods do not allow dissection of the mechanism of AAF-induced template activity inhibition. A few studies have attempted to distinguish among these mechanisms (74,78,77). The major effect of AAF upon transcription appears to be a decrease in RNA chain elongation (74,77,78), possibly due to premature chain termination (78). The number of initiation sites for RNA synthesis may also decrease with extensive DNA modification (77,78). It should be emphasized that these studies utilized DNA modified *in vitro* with N-Ac-AAF. No studies have been performed examining the mechanism of template activity inhibition of DNA modified *in vivo* with AAF.

The study of precancerous liver offers a particularly difficult problem in that the organ undergoes major histological changes following carcinogen feeding (79-82). In addition, the liver contains several cell types, some of which may change biochemically (83,84) as an animal progresses on a carcinogenic diet. Consequently, many studies examining effects of AAF on liver biochemistry measure parameters of a very diverse population of cells, most of which were probably not destined to become tumorogenic. In order to understand critical events leading to neoplasia, techniques should be developed to identify, isolate and study those cells which are direct precursors of the cancerous lesion (85). Experimental procedures have been developed to isolate nuclei from different hepatic cell types (86-88). A relatively simple procedure developed by Bushnell *et al*. (86) permits a relative separation of parenchymal cell nuclei (hepatocytes) from non-parenchymal cell nuclei (non-hepatocytes). Such an approach is potentially

very useful due to the fact that some carcinogens, e.g., AAF, produce primarily hepatocellular tumors which appear to arise from parenchymal cells (79,80) and other carcinogens, e.g., 3'-methyl-4-dimethylamino-azobenzene, produce tumors presumably arising from both cell types (79,80). Identification of selective effects upon the template activity of parenchymal cell DNA during the very early stages of AAF-induced hepatocarcinogenesis could have particular relevance to carcinogenesis by this agent.

1.5 Rationale

It has become axiomatic that chemicals induce cancer as a result of binding to one or more cellular macromolecules. Convincing evidence has been presented implicating DNA as a critical target of chemical carcinogens. Damage to DNA resulting in alteration in the capacity of the macromolecule to serve as a template for RNA synthesis could provide a mechanism for qualitative or quantitative changes in RNA production, eventually leading to synthesis of aberrant proteins. Abnormal production of certain critical proteins, such as those involved in nucleic acid biosynthesis or repression of genes controlling cell growth, could result in permanent, heritable alterations in gene expression. If the DNA damage producing the above sequence of events can be demonstrated very early in AAF-induced hepatocarcinogenesis, i.e., the time at which the initiation stage of carcinogenesis is thought to occur, such a phenomenon could have particular relevance as an initial molecular event leading to neoplasia.

1.6 Research Objectives and Long Range Goals

Research objectives and goals are summarized in Table #1.

TABLE 1

Research Objectives and Long Term Goals

I. Objectives

A. Does AAF feeding affect the ability of hepatic DNA to serve as a template for RNA synthesis?

B. If there is a change in template activity;

1. How is it altered as an animal progresses on the carcinogenic diet?

2. How permanent are the changes?

3. Is there a particular species of RNA or a particular hepatic nuclei population which is selectively inhibited?

4. What is the molecular mechanism of the template activity inhibition?

II. Long-term Goals

A. Can DNA template activity be used to assess the functional consequences of carcinogen-DNA interactions?

B. Are changes in DNA template activity relevant to carcinogenesis, i.e., is this an initial molecular event leading to neoplasia?

MATERIALS AND METHODS

2.1 Animals

Male Sprague-Dawley rats (Spartan Research Animals Inc., Haslett, MI), weighing 175 ± 25 g, were used in all experiments. Rats used for any given experiment were received on the same day and housed in a windowless room kept dark from 7 a.m. to 7 p.m. and light the other 12 hours of the day. Food and water were allowed ad libitum until the time of sacrifice, 9 a.m. ± 1 hour.

2.2 Carcinogenic Diet

A control basal diet (79) (carcinogenic basal diet) containing U.S. Pharmacopiea XIV salt mix and supplemented with p-aminobenzoic acid, 0.11 g/kg diet; inositol, 0.11 g/kg diet; and dry vitamin E acetate, 0.24 g (121 international units)/kg diet was purchased from Teklad Mills, Madison, WI. This control diet was supplemented with 0.05% (w/w) AAF purchased from Eastman-Kodak or Aldrich Chemical Co. Animals were fed the control diet for 3-5 days before beginning the carcinogenic diet, and were always started on the control and carcinogenic diets at 8 a.m. ± 1 hour.

2.3 Chemicals

Ribonuclease A (Sigma Type XI-A), protease (Sigma Type VI), actinomycin D and the sodium salts of cytidine triphosphate (Sigma Type III),

guanosine triphosphate (Sigma Type III), uridine triphosphate (Type I) and adenosine triphosphate were purchased from Sigma Chemical Co. Poly (dA-dT) and E. coli K-12 RNA polymerase were purchased from Miles Laboratories, calf thymus DNA from Worthington Biochemical Corp., and Multisol from Isolab Inc., Akron, Ohio. Uridine (5-^{3}H)5' triphosphate and adenosine (2,8-^{3}H)5' triphosphate were purchased from New England Nuclear.

2.4 Isolation of Hepatic Nuclei

Rat liver nuclei were selected as the initial system in this study because 1) the liver is the major target organ for AAF-induced carcinomas; 2) the procedure for nuclei isolation is relatively simple and well characterized; 3) the assay for RNA synthesis in hepatic nuclei has been extensively studied and parameters such as optimal ionic strength, optimal temperature of incubation and requirements for the polymerization reaction have been known for many years (89); and 4) the system has been used successfully by others attempting to assess carcinogen-induced changes in RNA synthesis (67,69,71,73,90).

Hepatic nuclei were isolated by the method of Yu and Feigelson (91). Animals were stunned by a blow to the head and their excised livers placed in a beaker of ice cold 0.34 M sucrose. Ten grams of liver were homogenized in 2.3 M sucrose; 3.3 M $CaCl_2$ utilizing a glass homogenizer fitted with a motor-driven teflon pestle. The homogenate was adjusted to a final volume of 100 ml, filtered through cheesecloth and centrifuged at 40,000 x g for 1 hour at 0°-5°C. The supernatant was discarded and the pellet resuspended in 10 ml of ice cold 0.34 M sucrose.

An aliquot of this nuclei suspension (0.2 ml) was stored at -90°C for assessment of DNA content on a later date. The remainder of the suspension was kept on ice until needed (15 ± 10 minutes).

2.5 Assay of the Capacity for RNA Synthesis in Hepatic Nuclei

A major disadvantage of measuring RNA synthesis in a nuclei preparation is that the total amount of RNA synthesized during a fixed incubation period is a complex function, depending upon the activity of endogenous RNA polymerases and the ability of the DNA to serve as a template for RNA synthesis, i.e, template activity. Chemical carcinogens could affect in vivo RNA synthesis by altering enzyme, template or both. Unfortunately, the ability of the DNA to serve as a template in the transcriptional process cannot be measured directly in hepatic nuclei. However, by concomitant assessment of the capacity for RNA synthesis and RNA polymerase activity, an indirect measure of changes in template activity can be obtained. The usefulness of this procedure has been demonstrated (76,69,73).

Mammalian cells contain multiple RNA polymerases (89). RNA polymerase I is nucleolar in origin and catalyzes the synthesis of ribosomal RNA while RNA polymerase II is nucleoplasmic in origin and synthesizes primarily messenger RNA (89). RNA polymerase III has been identified but its role in RNA synthesis is incompletely understood (89). To examine the possibility of AAF selectively inhibiting synthesis of a particular RNA species, a simple method was selected which separates RNA synthesis by the two major polymerases on the basis of ionic strength of the reaction mixture. RNA polymerase I is active when

assayed in a low salt media containing Mg^{++} and RNA polymerase II is active when assayed at high ionic strength in the presence of Mn^{++} (89).

The basic reaction mixture employed was modified from Grunberger et al. (69) and contained 100 mM Tris-HCl (8.2); 2.0 mM dithiothreitol; 0.4 mM each GTP, CTP, UTP; 0.4 mM ^{3}H-ATP (20 Ci/mole), and 50 µl nuclei (70 ± 20 µg DNA), in a final volume of 0.2 ml. Conditions optimal for RNA synthesis by the nucleolar RNA polymerase I included the basic reaction mixture described above, plus 6.0 mM $MgCl_2$ (89). Conditions optimal for RNA synthesis by the nucleoplasmic RNA polymerase II included the basic reaction mixture described above supplemented with 3.0 mM $MnCl_2$ and 0.2 M $(NH_4)_2SO_4$ (89). Reactions were initiated by the addition of nuclei and incubation was for 3 minutes at 30°C (92), during which time the rate was linear and directly proportional to the amount of nuclei added. Reactions were stopped by the addition of 2.0 ml ice-cold 5% TCA. Bovine serum albumin, 0.5 ml of a 0.5% solution, was added and the precipitate collected by centrifugation for 5 minutes at 1000 x g. The precipiate was washed twice with 2.0 ml portions of cold 5% TCA and once with ethanol; ether (4:1 v/v), prior to being dissolved in 0.5 ml of 88% formic acid (93). Multisol, 10 ml, was added and the radioactivity measured with a Packard Model 3380 liquid scintillation spectrometer.

2.6 Assay for RNA Polymerase Activity in Hepatic Nuclei

In the initial experiments, synthesis of poly A was selected as a potential method for assessing RNA polymerase activity, since the procedure is fast, simple and relatively inexpensive. Since AAF

metabolites do not appear to bind to thymine bases in DNA, thymine-rich regions should be relatively free of AAF modification and RNA synthesis in these regions should be somewhat independent of AAF-induced changes in template activity. Therefore, changes in poly A synthesis following AAF administration could potentially reflect changes in RNA polymerase activity relatively independently of changes in template activity.

Conditions utilized for assessment of poly A synthesis by polymerases I and II included the reaction mixtures described in section 2.5 for the respective polymerase; omitting GTP, CTP and UTP. All reactions were incubated 10 minutes, during which time the rate was linear and directly proportional to the amount of nuclei added.

RNA polymerase I activity was assayed using the basic reaction mixture described in Section 2.5 containing 20 µl of nuclei (28.6 µg of DNA), 8 µg poly (dA-dT), 4 µg actinomycin-D and omitting GTP and CTP. All reactions were run for 10 minutes at 30°C during which time the rate was linear and directly proportional to the amount of nuclei present. The reaction was terminated, precipitate washed and radioactivity measured as in Section 2.5.

2.7 Isolation of Hepatic Parenchymal and Non-parencymal Cell Nuclei

Hepatic parenchymal (class N-1, hepatocytes) and non-parenchymal (class N-2, "stromal") cell nuclei were prepared by the procedure of Bushnell *et al*. (86). The validity of the procedure has been reviewed (95) and the usefulness of the procedure has been demonstrated (70,84, 95).

2.8 Isolation of Hepatic DNA

The method of DNA isolation utilized was a modification (96) of the Marmur procedure (97). The Marmur procedure is widely used, well characterized and yields DNA reasonably free from nucleoprotein or RNA contamination. One disadvantage of the procedure is it involves the use of highly lipophilic agents such as chloroform and phenol. Since AAF is lipid soluble, it is possible that pieces of DNA containing multiple AAF modifications could become more lipid soluble and be lost in the organic phase during the isolation procedure. If this were to occur, there would be a loss of AAF modified DNA and data for template activity inhibition may represent minimum values. In other words, the true template inhibition may be greater than that actually measured in these experiments.

DNA was prepared from parenchymal (class N-1) and non-parenchymal (class N-2) cell nuclei. Nuclei were suspended in 10 mM Tris-HCl (pH 7.9); 0.1 M NaCl; 5.0 mM EDTA; 0.5 M $NaClO_4$; 1% sodium dodecyl sulfate and the solution was incubated for 40 minutes at 37°C with constant shaking. The suspension was deproteinized three times with chloroform; 3% isoamyl alcohol. To the resulting aqueous phase, an equal volume of ice-cold 95% ethanol was added and the nucleic acids were wound on a glass rod and transferred to a flask containing 10 mM Tris-HCl (pH 7.9); 5.0 mM EDTA. This suspension was treated with ribonuclease (50 μg enzyme/ml) for one hour and protease (3 mg enzyme/ml) for an additional two hours at 37°C. The solution was then deproteinized, once with an equal volume of chloroform; 3% isoamyl alcohol and once with an equal volume of freshly distilled phenol. The DNA was precipitated from the

aqueous phase by adding ice-cold 95% ethanol, wound on a glass rod, resuspended in 5.0 mM EDTA; 10 mM NaOH and sonicated for 50 seconds at low power in a Branson sonicator. The solution was adjusted to pH 7 and dialyzed overnight against 5.0 mM EDTA in a cold room (0°-5°C).

2.9 Measurement of the Rate and Extent of RNA Synthesis on Rat Liver DNA

The rate and extent of RNA synthesis was assessed by a method originally reported by Hyman and Davidson (98) and later fully characterized by Cedar (99) and Cedar and Felsenfeld (100). This method efficiently separates the initiation phase of RNA synthesis from the elongation phase. The high salt conditions of the assay appear to be as effective as the drug rifampicin in preventing reinitiation of RNA synthesis (100). Conditions of the assay are such that 1) sufficient time was allowed (15 minutes) for all possible initiation to occur (100), 2) RNA polymerase is present in excess such that all binding sites on the template should be saturated, 3) the high salt concentration during chain propagation should prevent a second polymerase molecule from initiating on a site already used for transcription (99), and 4) each polymerase molecule should be able to initiate only one chain (99). These conditions permit a quantitative estimation of RNA chain length and the number of initiation sites for RNA synthesis. The validity of the Hyman and Davidson method has been verified by others (101-105).

E. coli RNA polymerase was the enzyme selected to transcribe the various DNA templates isolated from control and AAF-treated animals. There are several advantages to using this enzyme for *in*

vitro template activity studies; 1) E. coli RNA polymerase initiates at one site per 1500 nucleotide pairs on DNA whereas the mammalian enzyme (RNA polymerase II) initiates at one site per 40,000 base pairs (99). Thus, the chances of detecting carcinogen-induced damage would be greater with the bacterial enzyme; 2) E. coli RNA polymerase appears to support initiation at every binding site on mammalian templates whereas calf thymus RNA polymerase II can support initiation at only a small number of binding sites (99,100); 3) the bacterial enzyme can be purchased from several manufacturers at a reasonably high purity and can be stored many months without loss of activity (Table 8). Several investigators have presented data suggesting that E. coli RNA polymerase does not initiate at the same sites as the mammalian enzyme (99,106). The bacterial polymerase, however, does synthesize hemoglobin messenger RNA on duck reticulocyte chromatin (96) but not from brain (107) or liver (96) chromatin. This suggests that E. coli RNA polymerase exhibits some degree of specificity as to initiation and termination sites on mammalian templates. Although there may be some advantages to using rat liver RNA polymerases, these initial studies were primarily concerned with the presence and recognition of template damage, as opposed to whether the damage is occurring in transcriptionally active regions of the genome.

E. coli RNA polymerase, 0.25 or 0.35 units[1], and 8 ng rat liver DNA were incubated for 15 minutes at 37°C in 10 mM Tris-HCl (pH 7.9); 1.0

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[1]One unit of enzyme is that amount catalyzing the incorporation of 1 mole of ^{3}H-UTP into acid insoluble material in the presence of calf thymus DNA in 10 minutes at 37°C.

mM $MnCl_2$; 0.08 mM each ATP and GTP; and 0.02 mM ^{3}H-UTP (500 Ci/mole) in a final volume of 100 µl. During this low-salt incubation period, the enzyme binds to DNA and initiates transcription but chain elongation is blocked due to the absence of CTP (100). Ammonium sulfate, 1.6 M, was added to prevent reinitiation and chain propagation was begun by the addition of $MgCl_2$ and CTP (final concentrations 5.0 mM and 0.063 mM, respectively)(100). Reactions determining the rate of RNA synthesis were incubated for 10 minutes. Those reactions determining the extent of RNA synthesis were allowed to proceed until the change in reaction rate approached zero (usually 140 minutes) as described in Section 2.10. Reactions were terminated, precipitate washed and radioactivity measured as described in Section 2.5, omitting the ethanol-ether wash.

2.10 Determination of the Number of Initiation Sites for RNA Synthesis

A fixed amount of E. coli RNA polymerase was titrated with increasing amounts of rat liver DNA to obtain an estimate of the number of initiation sites for RNA synthesis (100). The same procedure and basic reaction mixture described in Section 2.9 was employed using 0.05 units enzyme and 8, 16, 23, 48 or 64 ng DNA. Reactions were stopped after 10 minutes incubation in high salt. Since the conditions of the assay are such that no re-initiation is occurring (101,102), the incorporation of nucleotides after 10 minutes can be used as an indication of the number of initiated RNA chains. As the amount of template is increased, more chains are initiated until a plateau is reached. Since subsequent addition of DNA results in no increase in ^{3}H-UTP incorporation, the end point of the titration represents that amount of DNA which binds all

available RNA polymerase molecules. Thus, at the end point, the number of available RNA polymerase molecules is equal to the number of initiations, assuming that each initiation results in a propagating chain (100).

2.11 Calculations and Statistics

a. RNA polymerase activity, as measured in these experiments, was expressed as the amount of RNA synthesized on the exogenous template poly (dA-dT). Enzyme activity in hepatic nuclei was calculated from the following equation:

RNA Polymerase I Activity = A - B

where A = capacity for RNA synthesis with both actinomycin-D and poly (dA-dT) present in the reaction mixture and B = capacity for RNA synthesis with actinomycin-D present but poly (dA-dT) absent from the reaction mixture.

b. The extent of RNA synthesis is defined as the maximum number of nucleotides incorporated under the reaction conditions described in Section 2.9. It is equal to the incorporation of ^{3}H-UTP x 4 (assuming rat liver DNA contains 25% adenine bases) and was found by fitting a regression line to the data on the plateau region of the mean rate curve for a given treatment group. If the slope was not different from zero ($p<.05$), all the data on the plateau were combined to yield a mean ± S.E. representing the extent of RNA synthesis for a given treatment group. However, the control groups yielded statistically significant slopes. For these groups, analysis by a paired *t* test showed that incorporation of ^{3}H-UTP at 120 minutes was greater than that at 80

minutes but equal to that at 140 minutes. Thus, incorporation at 120 minutes was defined as the extent of RNA synthesis for these groups.

c. The number of molecules RNA polymerase in the reaction mixture can be calculated using the following formula:

$$\text{molecules RNA polymerase} = (U \times N)/(S \times M_w)$$

where U = that number of enzyme units added to the reaction; N = Avogadros number $6.02 \times (10)^{23}$; S = specific activity of RNA polymerase[1], 400 units/mg; M_w = molecular weight of E. coli RNA polymerase, 475,000 daltons (108).

d. The number of initiation sites for RNA synthesis/ng DNA was calculated from the data obtained by titrating the enzyme with template according to the following formula (100):

$$\text{Initiation sites/ng} = P/(\text{ng DNA})$$

where P = number of enzyme molecules added to the reaction mixture; (ng DNA) = amount of DNA at the end point of the titration. The end point of the titration was that point at which further addition of DNA resulted in no statistically significant increase in ^{3}H-UTP incorporation, as determined by a paired t analysis ($p<.05$) on each individual DNA preparation. The number of base pairs/molecule polymerase (Table 10) can be obtained by multiplying (ng DNA)/initiation x $(10)^{12}$ base pairs/ng DNA.

e. The average RNA chain length can be calculated by dividing the extent of RNA synthesis by the total number of initiation sites.

$$\text{Chain length} = \text{extent}/(\text{Initiations/ngDNA} \times 8)$$

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[1]The activity of E. coli RNA polymerase assayed by the method of Cedar and Felsenfeld (100) was one-third of that activity obtained when assayed by the Burgess method (109). Thus, the maximum specific activity of 1200 units/mg as described by Burgess was divided by 3 for the above calculations.

where extent = number of nucleotides incorporated (see Section 2.11 b); initiations/ng DNA = number of initiation sites (see Section 2.11 d.); 8 = ng DNA used to determine extent of RNA synthesis.

f. All other statistical analyses utilized the student's t-test ($p<.05$) for unpaired observations unless otherwise noted.

2.12 Determination of E. coli RNA Polymerase Activity Using Calf Thymus DNA

To examine the possibility of loss of activity occurring during storage of the enzyme, the activity of E. coli RNA polymerase was assayed every 8-12 weeks using calf thymus DNA as the template. No loss of activity could be demonstrated for any of the enzyme preparations used in these experiments. The assay for RNA polymerase was that described by Burgess (109), substituting ^{3}H-UTP for ^{14}C-ATP.

2.13 Miscellaneous Methods

DNA and protein were determined by the methods of Ceriotti (110) and Lowry et al. (111), respectively. For determination of RNA, each DNA sample was incubated at 37°C for one hour in 0.3 M KOH. Cold 5% TCA was added and the solution was centrifuged at 5,000 x g for 10 minutes. The absorbance of the supernatant was monitored at 260 nm in a Gilford UV spectrometer and RNA was calculated using 1 Absorbance unit = 32 µg RNA/ml (112).

RESULTS

3.1 Determination of the Capacity for RNA and Poly A Synthesis in Hepatic Nuclei

The effect of varying the amount of nuclei in the reaction mixture upon RNA and poly A synthesis is shown in Figures 1A and 1B, respectively. The data in these figures demonstrates that incorporation of ^{3}H-ATP into RNA and poly A is directly proportional to the amount of nuclei added to the reaction mixture. In addition, the four nucleotide triphosphates were present in excess in the reaction mixture (data not shown).

The optimum ionic concentrations for the assessment of RNA synthesis by RNA polymerase I were determined by measuring the rate of RNA synthesis while varying the concentration of $MgCl_2$ and $(NH_4)_2SO_4$ in the reaction mixture. The data in Figure 2A demonstrates that increasing the concentration of $MgCl_2$ in the reaction mixture from 2.8 mM to 14 mM has little effect upon the rate of RNA synthesis. Addition of up to 20 mM $(NH_4)_2SO_4$ also produced essentially no effect upon incorporation of ^{3}H-ATP into RNA, as shown in Figure 2B.

The optimal ionic concentrations for assessment of RNA polymerase II were also determined and the data is shown in Figure 3. Increasing the concentration of $MnCl_2$ in the reaction mixture from 1.2 mM to 3 mM increased the incorporation of ^{3}H-ATP after 6 minutes of reaction time. Increasing the concentration of $MnCl_2$ to 6 mM resulted in no

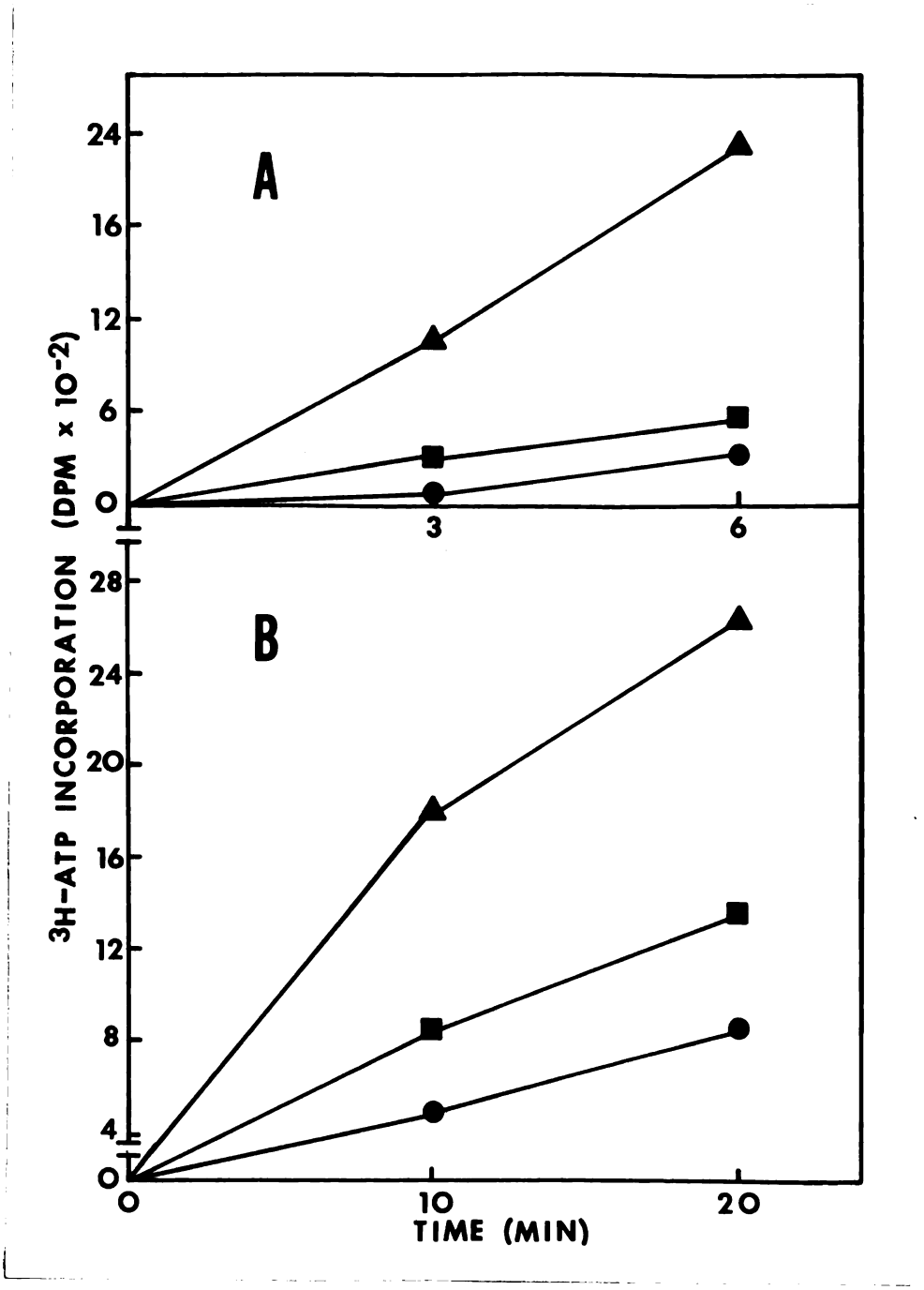

Figure 1: The Effect of Varying the Amount of Nuclei in the Reaction Mixture Upon Incorporation of ^{3}H-ATP into RNA and Poly A.

RNA (A) and poly A (B) synthesis were assessed under conditions optimal for RNA polymerase I as described in Section 2.5. Each point represents the mean of duplicate determinations using a single nuclei preparation. The amount of nuclei added to the reaction mixture was either 15 µl (●), 25 µl (■) or 50 µl (▲) where 50 µl equals approximately 70 µg DNA.

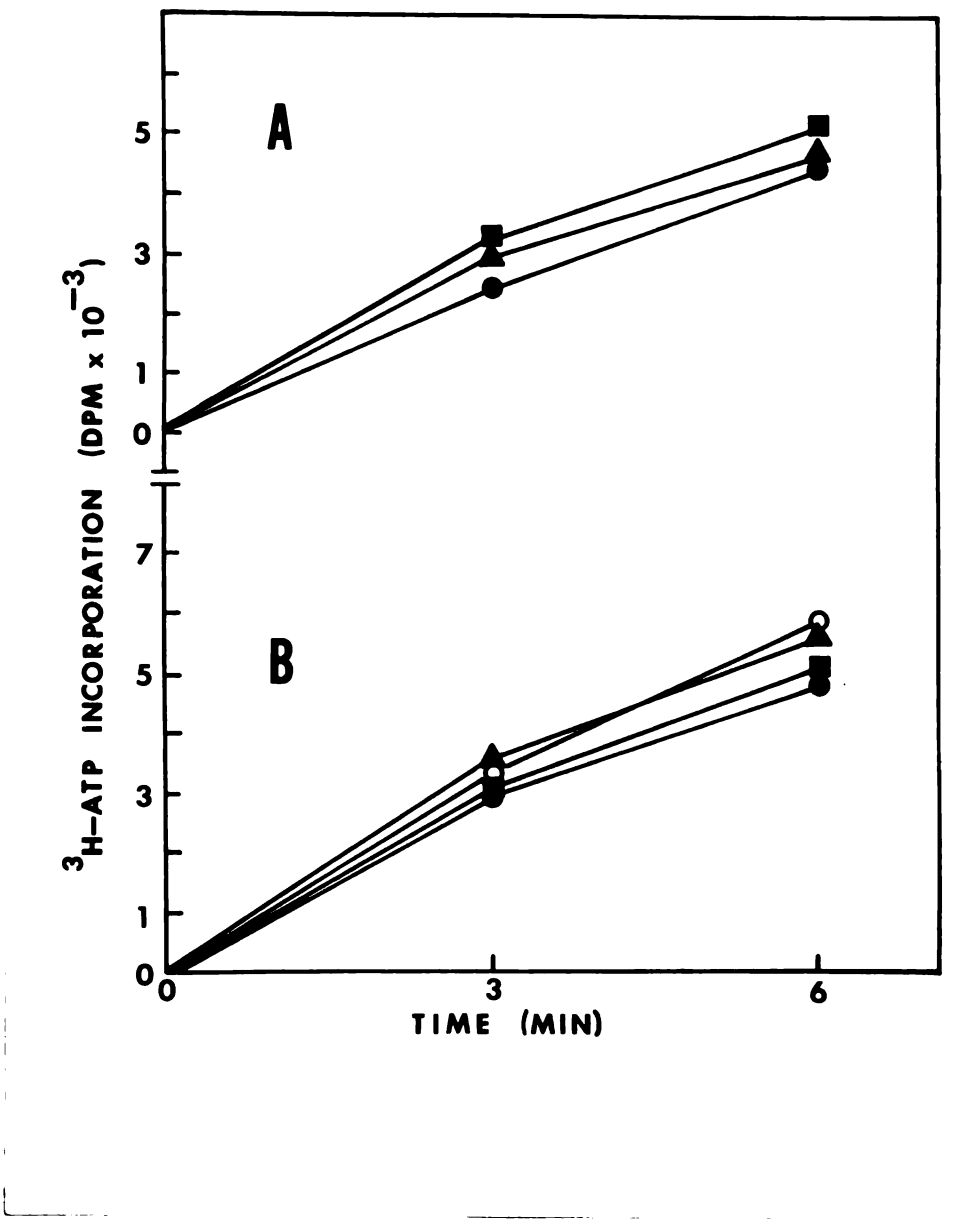

Figure 2: Determination of Optimal Ionic Conditions for Assay of RNA Synthesis by RNA Polymerase I in Hepatic Nuclei.

RNA synthesis by polymerase I was assayed as described in Section 2.5 except A) $MgCl_2$ concentration was varied keeping $(NH_4)_2SO_4$ concentration constant at 10 mM or B) $(NH_4)_2SO_4$ concentration was varied keeping $MgCl_2$ concentration constant at 7 mM. Each point represents the mean of duplicate determinations using a single nuclei preparation. Concentrations of $MgCl_2$ in A were 2.8 mM (●), 7 mM (■) or 14 mM (▲). Concentrations of $(NH_4)_2SO_4$ in B were 4 mM (●), 10 mM (■), 20 mM (▲) or no NH_4SO_4 (○).

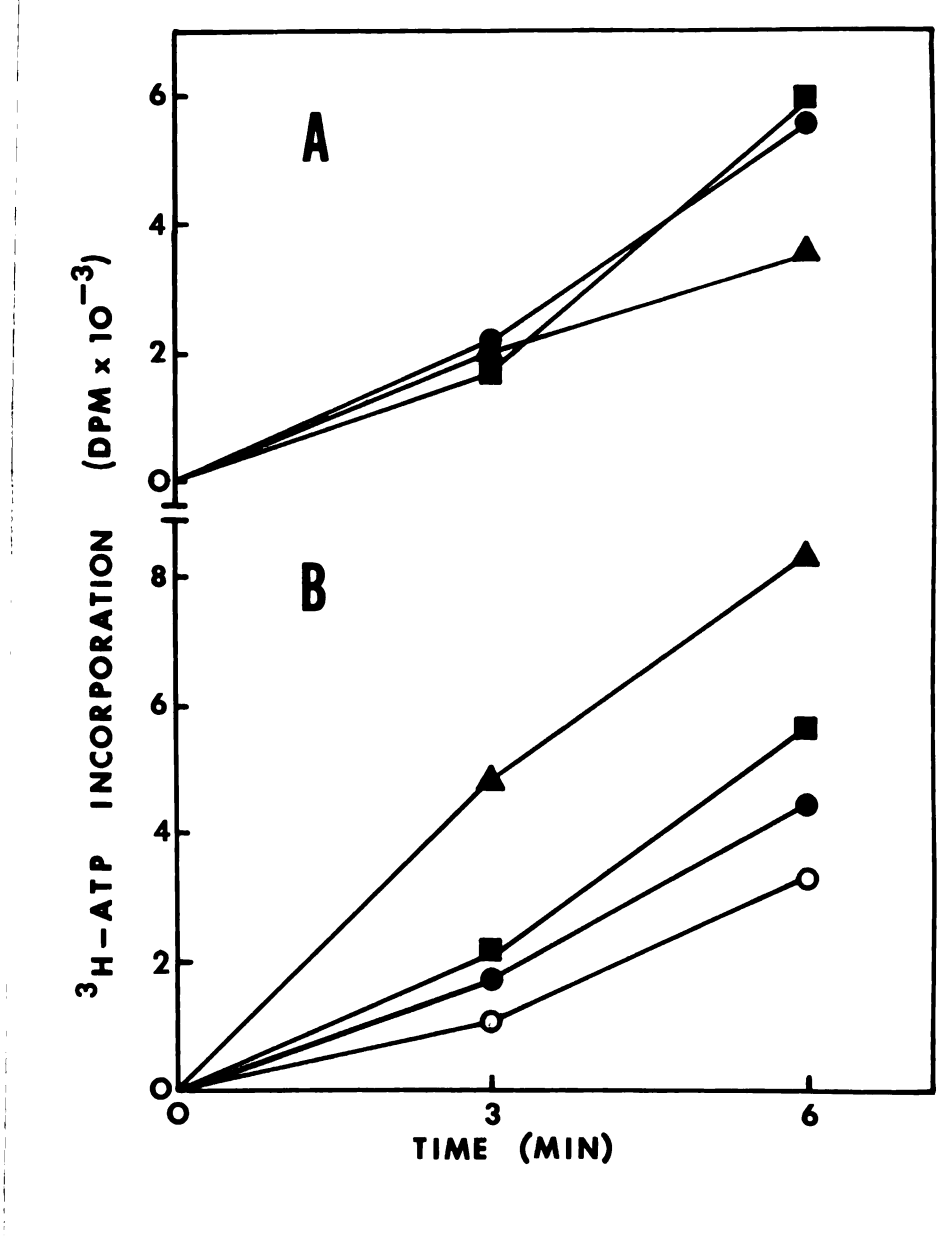

Figure 3: Determination of Optimal Ionic Conditions for Assay of RNA Synthesis by RNA Polymerase II in Hepatic Nuclei.

RNA synthesis by polymerase II was assayed as described in Section 2.5 except A) $MnCl_2$ concentration was varied keeping $(NH_4)_2SO_4$ concentration constant at 100 mM or B) $(NH_4)_2SO_4$ concentration was varied keeping $MnCl_2$ concentration constant at 3 mM. Each point represents the mean of duplicate determinations using a single nuclei preparation. Concentrations of $MnCl_2$ in A were 1.2 mM (▲), 3.0 mM (●) or 6.0 mM (■). Concentrations of $(NH_3)_2SO_4$ in B were 4.0 mM (●), 10 mM (■), 20 mM (▲) or no $(NH_4)_2SO_4$ (○).

further increase in ^{3}H-ATP incorporation. Addition of 40, 100 or 200 mM $(NH_4)_2SO_4$ resulted in a progressive increase in ^{3}H-ATP incorporation.

Figures 4A and 4B show the results of an experiment designed to examine the variability of the capacity for RNA synthesis by polymerases I and II. The data demonstrates that variability in the capacity for RNA synthesis between nuclei preparations obtained from 2 rats is low for both enzymes. Variability between two nuclei suspensions prepared from a single rat is also quite low.

RNA and poly A synthesis by polymerase I and polymerase II in nuclei isolated from carcinogen-treated animals is shown in Figure 5.

Feeding of the AAF diet for 4, 7 or 14 days resulted in little change in the capacity for RNA synthesis by RNA polymerase I (Figures 5A, 5B and 5C, respectively). However, removal of the carcinogenic diet for 2 days resulted in a 140% and 46% increase in the capacity for RNA synthesis by polymerase I in those animals ingesting the AAF diet for 7 or 14 days, respectively. Five days after returning to the control diet, RNA synthesis by polymerase I returned closer to control levels in nuclei isolated from animals ingesting the AAF diet for 7 days but remained elevated approximately 40% in those animals receiving the AAF diet 14 days.

RNA synthesis by polymerase II was inhibited 54% after 4 days, 10% after 7 days and essentially uninhibited after 14 days of AAF ingestion. The inhibition seen after 4 days of AAF feeding returned to control values 2 days after returning to the control diet.

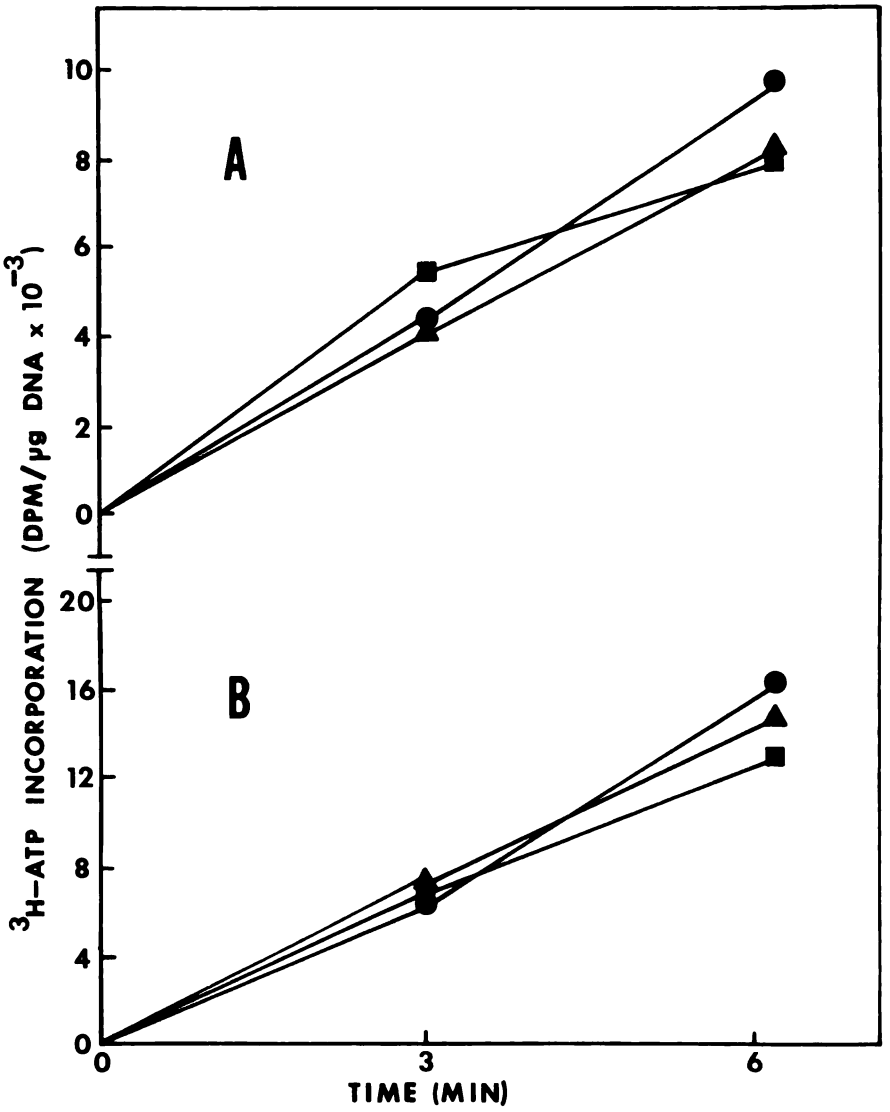

Figure 4: Variability of the Capacity for RNA Synthesis Between Nuclei Preparations.

Nuclei were prepared from two rats. The nuclei from rat 1 was divided into two parts. RNA synthesis by polymerase I (A) and polymerase II (B) was assayed as described in Section 2.5. Each point represents the mean of duplicate determinations. Rat 1, part 1, (●); Rat 1, part 2 (▲); Rat 2 (■).

Figure 5: Effect of AAF Ingestion on RNA Synthesis and Poly A Synthesis by RNA Polymerases I and II in Hepatic Nuclei.

RNA and poly A synthesis by RNA polymerase I and II were determined as in Section 2.5 in nuclei isolated from rats fed a 0.05% (w/w) AAF diet for 4 (A), 7 (B) or 14 (C) days. Groups of animals were killed at either the end of the period of carcinogen feeding (zero time) or at 2 and 5 days after being returned to the control diet. Each point represents the mean ± range of the data obtained from two animals. Control values (mean ± SE of the data obtained from 8 animals) were 5,000±333 DPM/min/mg DNA for RNA synthesis by polymerase I (●); 7000±667 DPM/min/mg DNA for RNA synthesis by polymerase II (○); 2700±600 DPM/min/mg DNA for poly A synthesis by polymerase I (▲); and 2100±200 DPM/min/mg DNA for poly A synthesis by polymerase II (△).

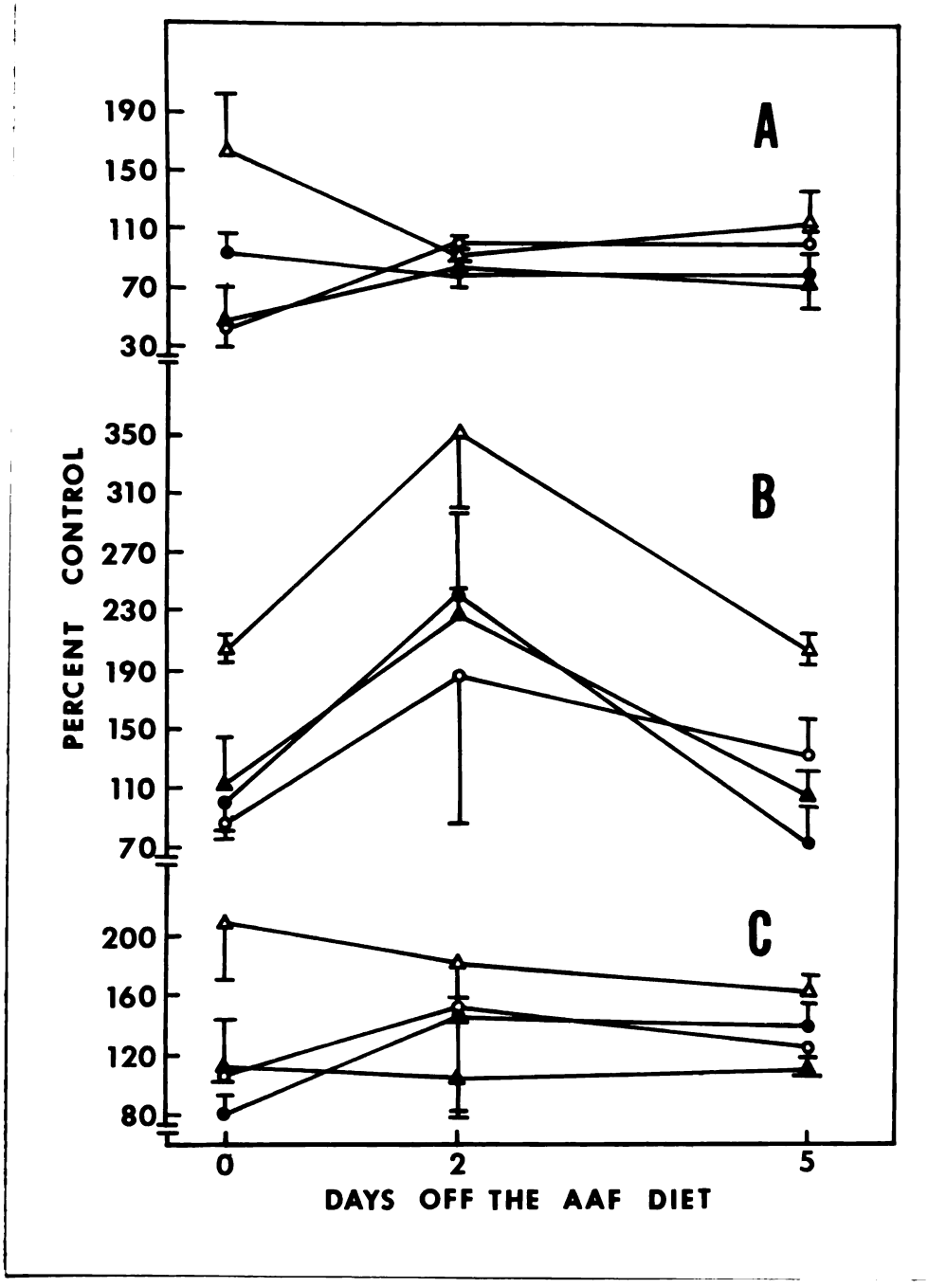
A
B
C
190
150
110
70
30
350
310
270
230
190
150
110
70
200
160
120
80
PERCENT CONTROL
0
2
5
DAYS OFF THE AAF DIET

Figure 5

However, removal of the carcinogenic diet for 2 days resulted in an 88% and 50% stimulation of RNA synthesis in those animals ingesting the AAF diet for 7 or 14 days, respectively. RNA synthesis by polymerase II remained elevated approximately 30% 5 days after returning to the control diet in those animals receiving the AAF diet 7 or 14 days.

Poly A synthesis by RNA polymerase II did not always mirror RNA synthesis by this enzyme (Figure 5). Poly A synthesis by polymerase II was increased 62%, 105% and 106% in nucleic isolated from animals ingesting the AAF diet 4, 7 or 14 days, respectively. Poly A synthesis by this enzyme remained elevated above control levels throughout the entire 7 and 14 days series.

Figure 6 shows the relationship between duration of AAF ingestion and RNA and poly A synthesis by RNA polymerases I and II. The data for 4, 7 and 14 days has been taken from Figure 5. The data in Figure 6 suggests that RNA and poly A synthesis by both polymerases reach a peak stimulation after 8 weeks of AAF feeding. An additional 4 weeks of carcinogen feeding results in all parameters except poly A synthesis by polymerase II returning close to control values.

3.2 Determination of the Capacity for RNA Synthesis and RNA Polymerase I Activity in Carcinogen-treated Animals

Figures 7A and 7B show the effect of actinomycin-D on the initial rate of RNA synthesis by RNA polymerases I and II, respectively. Controls included addition of 20 µl of glass distilled water or 95% ethanol, the latter being the solvent used to suspend the actinomycin D. The data in Figure 7A suggests that after 6 minutes of reaction time,

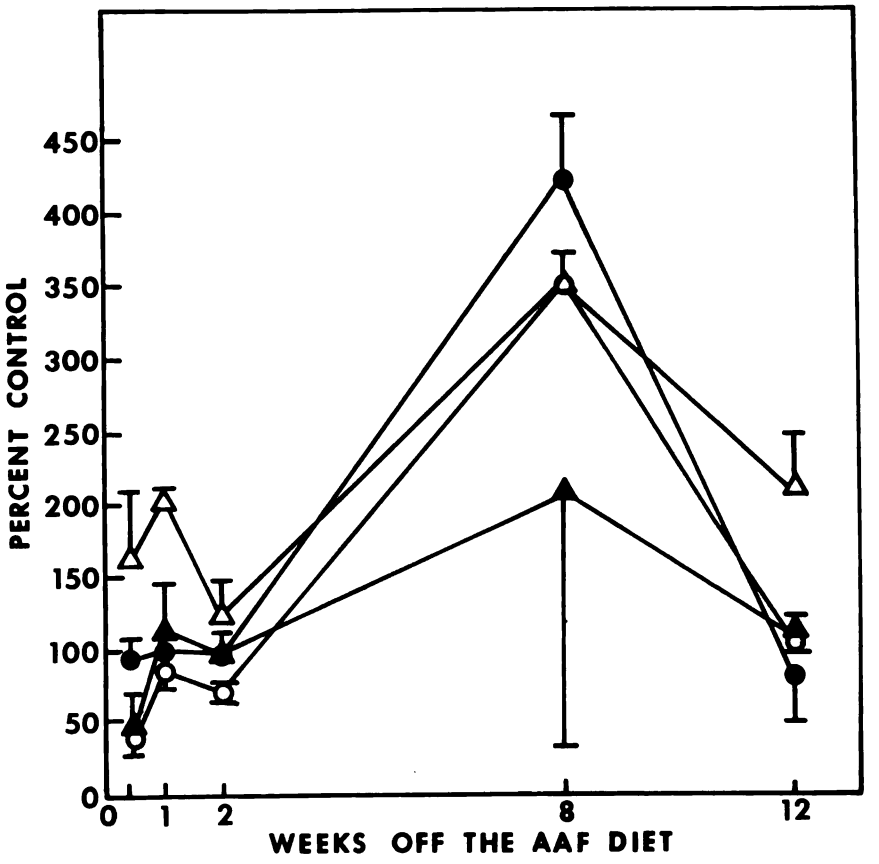

Figure 6: Effect of Duration of AAF Ingestion on RNA Synthesis and Poly A Synthesis by RNA Polymerases I and II in Hepatic Nuclei.

RNA and poly A synthesis by polymerases I and II were determined as in Section 2.5 in nuclei isolated from rats fed a 0.05% (w/w) AAF diet. Each point represents the mean ± range of the data obtained from two animals. Control values and symbols are as listed in the legend to Figure 5.

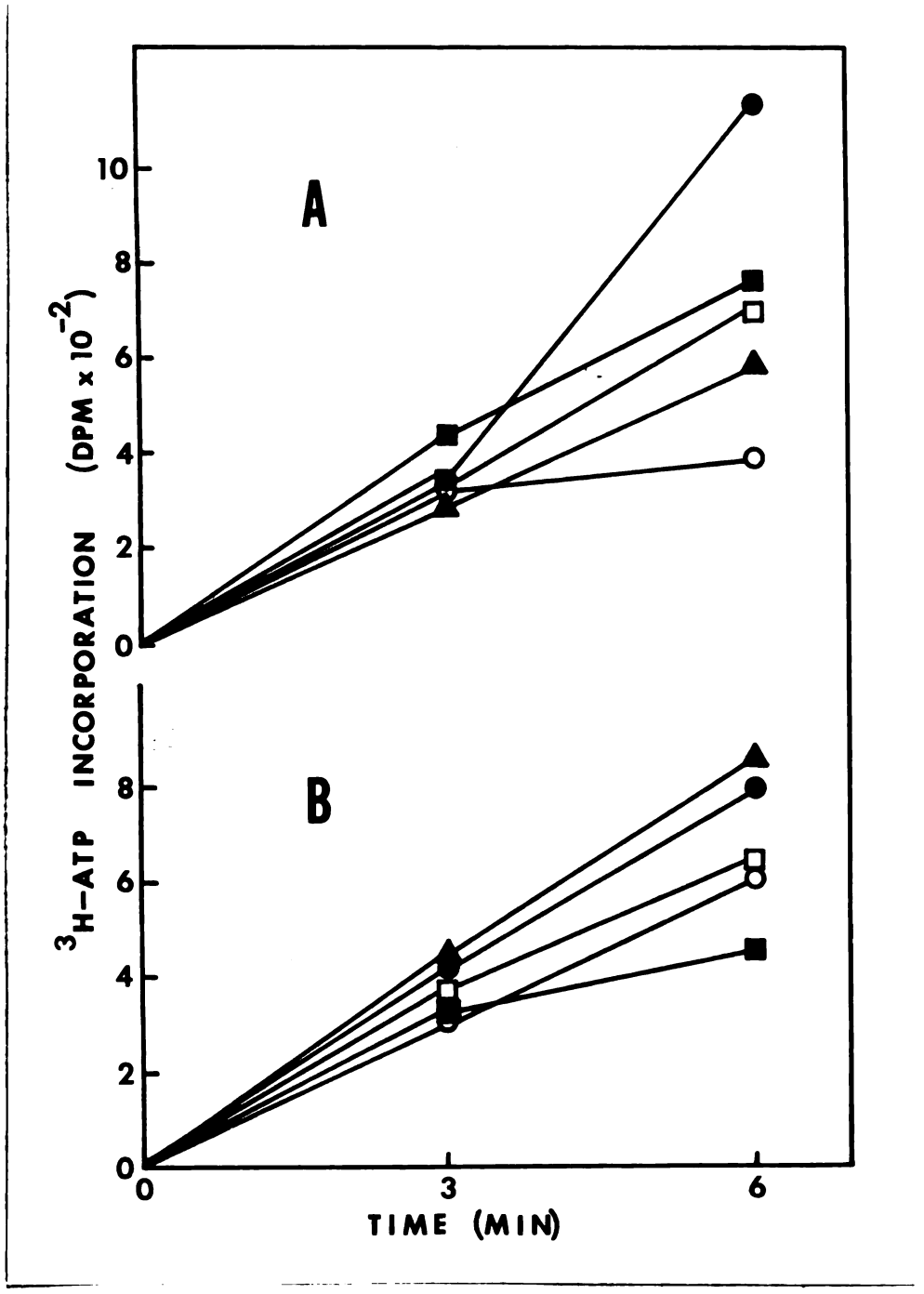

Figure 7: Effect of Actinomycin-D on RNA Synthesis by RNA Polymerases I and II in Hepatic Nuclei.

Assay conditions were optimal for either polymerase I (A) or polymerase II (B) as described in Section 2.5. The amount of actinomycin-D added to the reaction mixture was either 2 µg (▲); 4 µg (□); 8 µg (■); no actinomycin-D, 20 µl water (●); no actinomycin-D, 20 µl 95% ethanol (○). Actinomycin-D was suspended in 95% ethanol. Each point represents the mean of duplicate determinations using a single nuclei preparation.

95% alcohol alone can markedly inhibit RNA synthesis by polymerase I. Doubling the amount of actinomycin-D in the reaction mixture from 4 to 8 µg produced little effect upon RNA synthesis by polymerase I after 6 minutes of reaction time. As shown in Figure 7B, 95% ethanol also appears to have an inhibitory effect on RNA synthesis by polymerase II. The greatest inhibition of the rate of RNA synthesis by polymerase II was achieved by using 8 µg actinomycin-D in the reaction mixture.

Additional experiments on the effects of actinomycin-D and 95% ethanol on the initial rate of RNA synthesis by polymerases I and II produced the data shown in Figures 8A and 8B, respectively. In these experiments the stock solution of actinomycin D was concentrated to allow the addition of 2 µl of solvent (water-95% ethanol, 1:1 v/v) as opposed to the 20 µl of 95% ethanol added in the experiments shown in Figure 7. Also examined in these experiments was the effect of omitting CTP and GTP from the reaction mixture. The data for polymerase I, shown in Figure 8A, suggest 1) addition of 2 µl solvent (1 µl ethanol) can inhibit the rate of RNA synthesis after 6 minutes of reaction time, 2) addition of actinomycin-D can inhibit the rate of RNA synthesis greater than solvent alone and, 3) removal of two of the four nucleotide triphosphates can significantly lower RNA synthesis by polymerase I. In contrast to the results with polymerase I, the data for polymerase II (Figure 8B) suggest 1) addition of neither solvent alone nor actinomycin-D markedly alters the rate of RNA synthesis, and 2) removal of CTP and GTP did not decrease incorporation of ^{3}H-ATP.

Figure 9 shows the time course of the reaction for RNA synthesis in the presence and absence of poly (dA-dT) for polymerases I and II. All

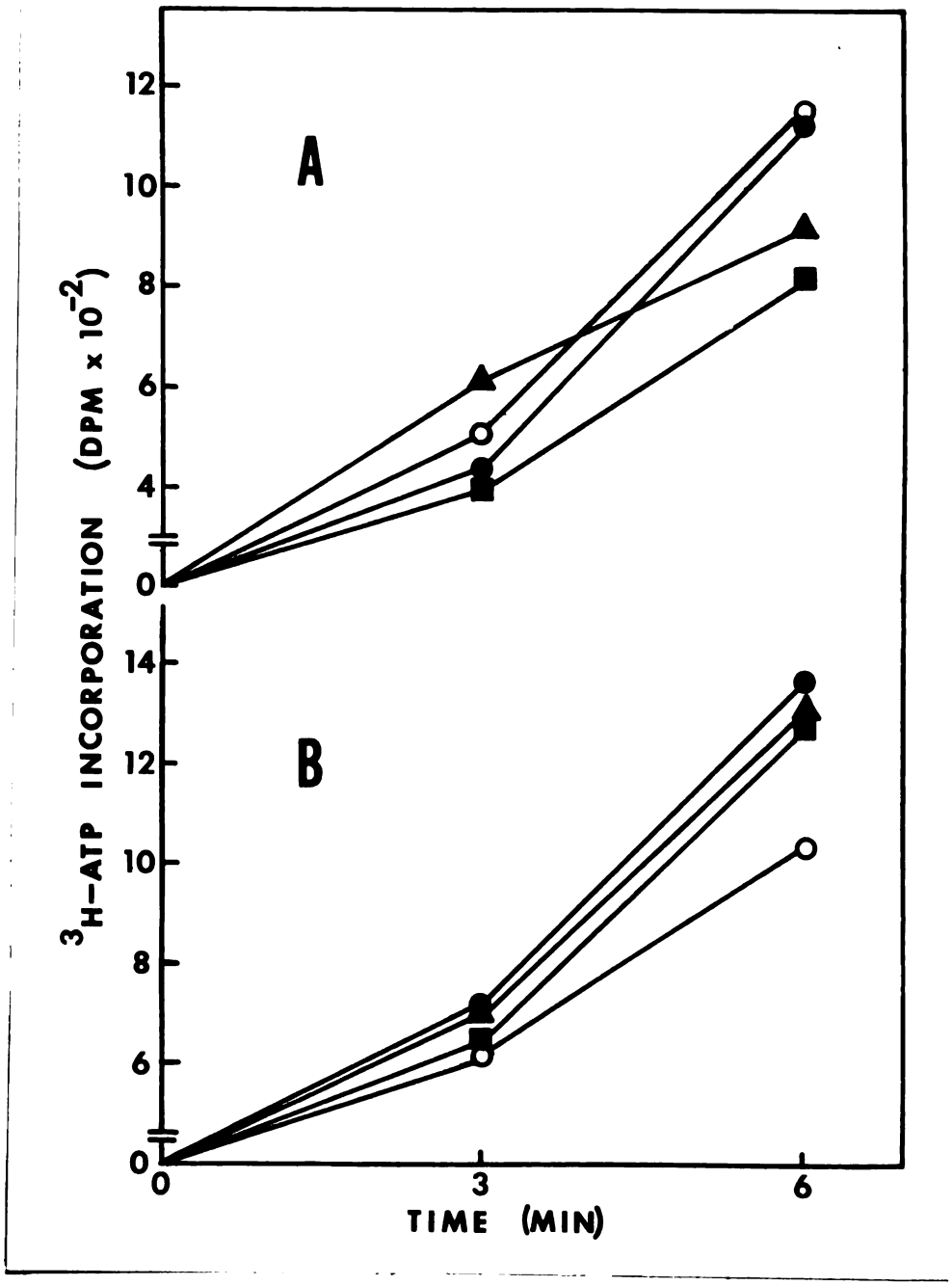

Figure 8: Effects of Ethanol and Actinomycin D on RNA Synthesis by RNA Polymerases I and II in Hepatic Nuclei.

Assay conditions were optimal for either RNA polymerase I (A) or polymerase II (B) as described in Section 2.5. Each point represents the mean of duplicate determinations using a single nuclei preparation. Actinomycin-D was suspended in water-95% ethanol (1:1 v/v). (○) 4 µg actinomycin-D; (■) 4 µg Act-D, omitting GTP and CTP from reaction mixture; (●) No actinomycin-D, omitting GTP and CTP from reaction mixture; (▲) No actinomycin-D, 2 µl water-95% ethanol (1:1 v/v), omitting GTP and CTP from reaction mixture.

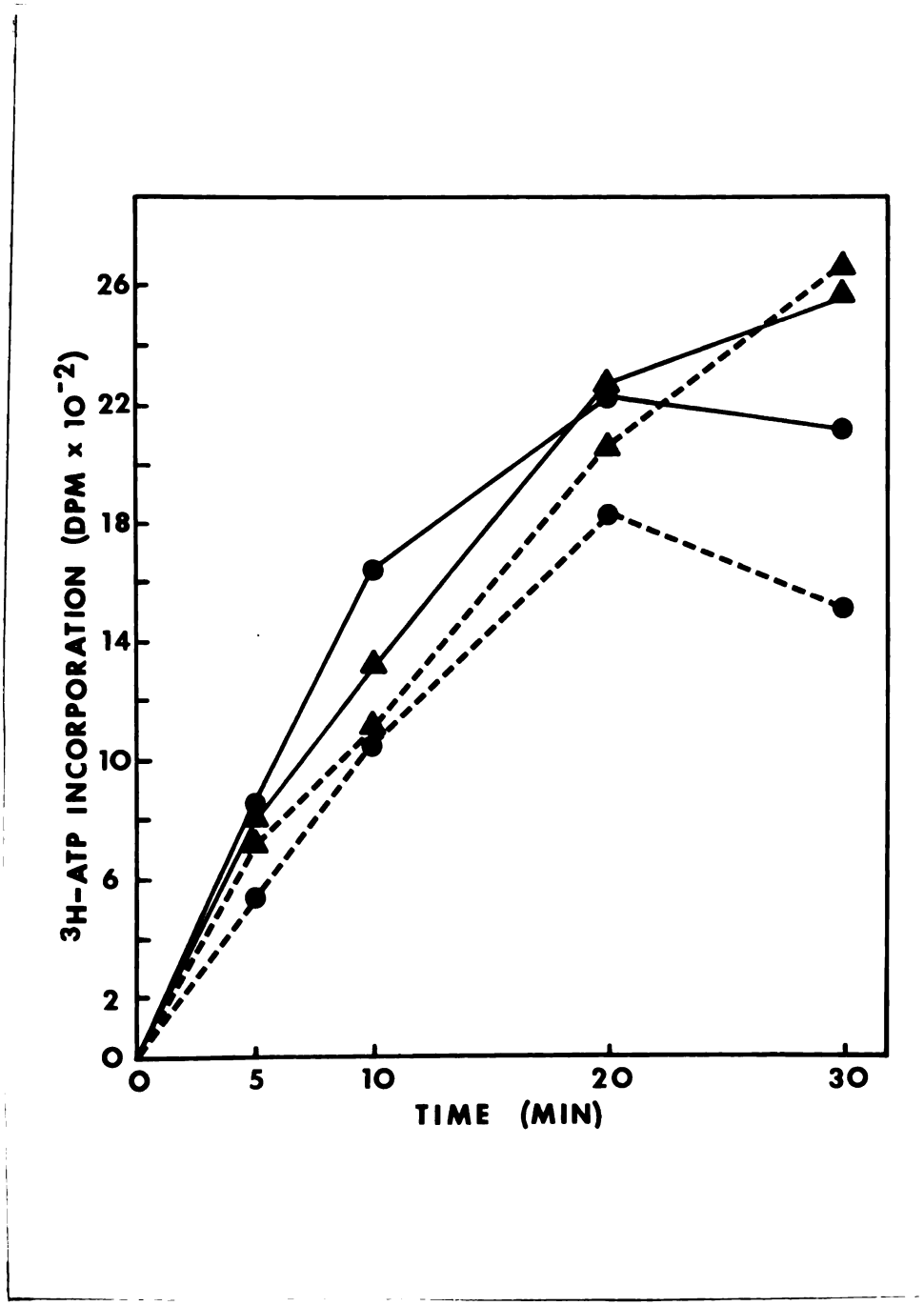

Figure 9: RNA Synthesis in the Presence and Absence of Poly (dA-dT).

Assay conditions for RNA synthesis by RNA polymerase I and polymerase II were as described in Section 2.5. Each point represents the mean of duplicate determinations using a single nuclei preparation. (●——●) Polymerase I, 8 µg poly (dA-dT); (▲——▲) polymerase II, 8 µg poly (dA-dT); (●- - -●)polymerase I, no poly (dA-dT); (▲- - -▲) polymerase II, no poly (dA-dT).

reactions were linear for at least 10 minutes. RNA synthesis by both enzymes was greater in the presence of the synthetic template than in its absence. RNA polymerase I activity (defined in Section 2.11) was greater than polymerase II activity at all time points measured.

Table #2 shows the effect of varying the concentration of nuclei in the reaction mixture upon the rate of RNA synthesis in the presence and absence of poly (dA-dT). The data suggest that the optimum amount of nuclei for assay of polymerase I is 25 µl (approximately 35 mg DNA). RNA polymerase II activity could not be detected with poly (dA-dT), despite the addition of up to 75 µl nuclei.

The above experiment was repeated for polymerase I only and the results are shown in Table #3. This experiment confirmed 20-25 µl nuclei as the optimum amount of nuclei to be added to the assay mixture.

The effect of AAF ingestion on RNA synthesis and RNA polymerase I activity is shown in Figure 10. No statistically significant changes in the capacity for RNA synthesis could be demonstrated in nuclei prepared from rats maintained on the AAF diet for 4 or 7 days. It was elevated approximately 40% in nuclei prepared from animals fed the AAF diet 14 days and returned to control level 5 days after returning to the control diet.

At the end of 4, 7 and 14 days of carcinogen feeding, RNA polymerase I activity was significantly ($p<.05$) increased to 145, 155 and 230% of control values, respectively. The stability of the carcinogen-induced changes in polymerase activity was variable, depending upon the duration of AAF feeding. Five days after being switched to the control diet, RNA polymerase I activity returned to control levels in those animals fed the AAF diet for 4 or 14 days. However, polymerase activity

TABLE 2

Effect of Varying the Amount of Nuclei in the Reaction Mixture Upon RNA Polymerase Activity

RNA Polymerase	Nuclei[1]	RNA Synthesis[5] Plus poly(dA-dT)[2]	RNA Synthesis[5] Minus poly(dA-dT)[3]	RNA Polymerase Activity[4,5]
I	5	7	8	--
	10	42	33	9
	20	86	38	48
	25	269	113	157
	50	267	181	86
	75	246	246	0
II	5	15	11	4
	10	6	11	--
	20	33	36	--
	25	97	180	--
	50	197	269	--
	75	305	305	--

[1]Nuclei; this suspension contained 1.9 mg DNA/ml.

[2]All tubes contained 8 µg poly(dA-dT) and 4 µg actinomycin D.

[3]All tubes contained 4 µg actinomycin D but no poly(dA-dT).

[4]Defined as in Section 2.11.

[5]DPM/min; All reactions were incubated 10 minutes.

TABLE 3

Effect of Varying the Amount of Nuclei in the Reaction Mixture Upon RNA Polymerase I Activity[1]

Nuclei	RNA Synthesis		RNA Polymerase Activity
	Plus poly(dA-dT)	Minus poly(dA-dT)	
10	39	28	11
15	72	64	8
20	76	48	28
25	93	71	22

[1]See footnotes to Table #1.

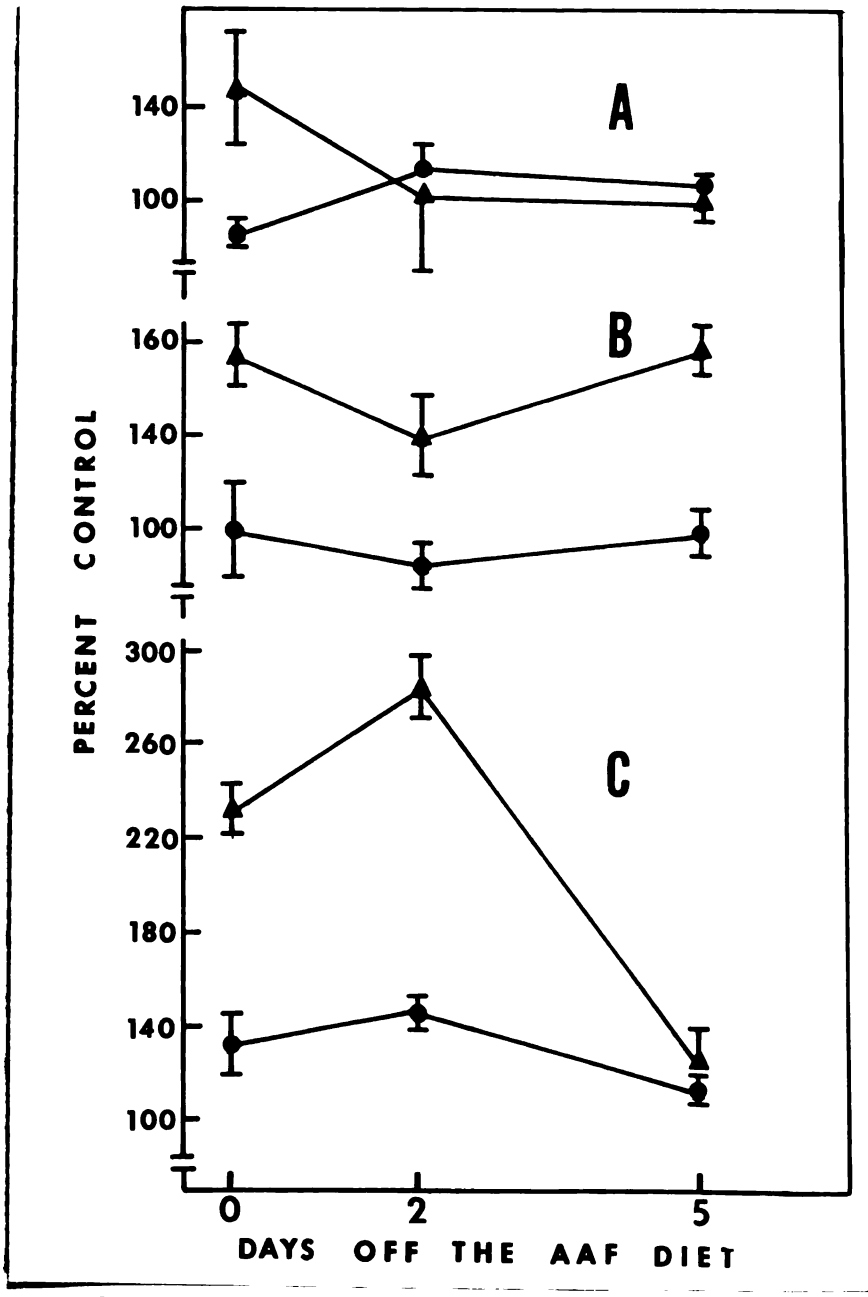

Figure 10: Effect of AAF Ingestion on RNA Synthesis and RNA Polymerase I Activity in Isolated Hepatic Nuclei.

RNA synthesis (●——●) and RNA polymerase activity (▲——▲) were determined, as described under Materials and Methods, in nuclei isolated from animals fed a diet containing 0.05% w/w AAF for 4 (A), 7 (B), or 14 (C) days. Groups of animals were killed at either the end of the period of carcinogen feeding (zero time on the chart) or at 2 and 5 days after being returned to the control diet. Each point represents the mean ± S.E. of the data obtained from 3 animals. The control values (mean ± S.E. of the data obtained from 12 animals) were 22,028±151 DPM/mg DNA/min and 488±67 DPM/mg DNA/min for RNA synthesis and RNA polymerase I activity, respectively.

remained significantly ($p<.05$) elevated (approximately 60%) in those animals fed the carcinogenic diet for 7 days.

3.3 Determination of the Rate and Extent of RNA Synthesis, RNA Chain Length and Number of Initiation Sites on Rat Liver DNA Isolated From Carcinogen-Treated Animals

Optimal conditions for incubation of DNA with ribonuclease and protease were determined and the results are shown in Table 4. Doubling the concentration of ribonuclease in the incubation media from 25 µg/ml to 50 µg/ml decreases the RNA contamination of the isolated DNA by approximately 50%. Doubling the protease concentration from 2 mg/ml to 4 mg/ml has no detectable effect on the percent protein contamination of isolated RNA. In addition, increasing protease incubation time from 2 hours to 4 hours has essentially no effect on percent protein contamination.

The effects of increasing E. coli RNA polymerase and DNA concentrations upon incorporation of ^{3}H-UTP into RNA is shown in Figure 11. Increasing the amount of RNA polymerase in the reaction mixture from 0.125 unit to 0.50 unit did not result in a marked increase in RNA synthesis using 10 ng of template, thus suggesting enzyme was present in excess at 0.125 unit. Increasing the amount of DNA from 10 ng to 20 ng stimulated RNA synthesis 2 to 3 fold, thus demonstrating the rate of reaction was directly proportional to DNA concentration.

The variability of template activity between DNA preparations is shown in Table 5. The data suggests that both rate and extent of RNA synthesis do not vary greatly between two DNA preparations isolated from different animals. In general, there are no marked changes in the rate or extent of RNA synthesis upon storage of the DNA preparation at 5-10°C

TABLE 4

Effect of Ribonuclease and Protease Concentrations on Percent Contamination of DNA

Enzyme	N[1]	Time[2]	Concentration[3]	% RNA[4]	% Protein[4]
Ribonuclease	2	1.0	25	19.0±5.5	------
	3	1.0	50	9.5±0.4	------
Protease	2	0	4000	--------	18.4±2.1
	1	1.0	4000	--------	23.6±
	2	2.0	2000	--------	9.6±0.7
	3	2.0	4000	--------	9.6±2.6
	2	2.0	6000	--------	13.8±0.8
	2	4.0	4000	--------	12.6±0.9

[1]Number of DNA preparations.

[2]Hours of incubation time.

[3]µg enzyme/ml.

[4]mean ± SE (n=3) or mean ± range (n=2).

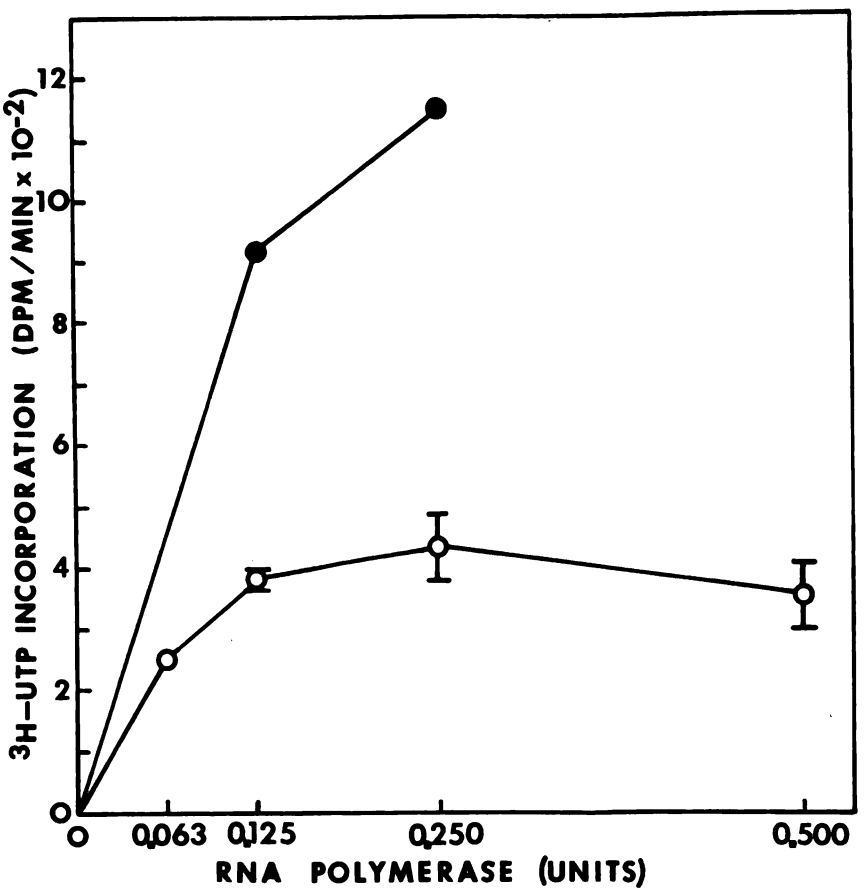

Figure 11: Effect of Increasing the Concentration of E. coli RNA Polymerase and DNA in the Reaction Mixture upon Incorporation of ^{3}H-UTP into RNA.

Assay for RNA synthesis was as described in Section 2.9. With the exception of the data at 0.063 unit, all (⊙) represent the mean ± S.E. of the data obtained from three DNA preparations. The data at 0.063 and all (●) represent the mean of duplicate determinations using a single DNA preparation. (⊙) = 10 ng DNA; (●) = 20 ng DNA.

TABLE 5

Variability of the Rate and Extent of RNA Synthesis Between DNA Preparations

Rat[1]	DNA	Nuclei[1]	Day[2]	Rate[3]	Extent[4]
1	Control[5]	N-1	1	77± 2	2.66±0.17
			3	63± 4	2.66±0.09
			5	78± 4	2.49±0.26
		N-2	1	82± 6	3.56±0.43
			3	78±16	3.01±0.26
			5	64±13	2.84±0.09
2	Control	N-1	1	105±14	2.06±0.26
			3	64± 8	3.27±0.54
			5	68± 3	3.18±0.09
		N-2	1	82±11	2.92±0.26
			3	64± 7	3.70±1.72
			5	74± 2	3.27±0.17
3	Calf Thymus	---	1	182±10	---------
			10	214±61	---------
4	AAF[6]	N-1	1	152± 7	---------
			5	161±13	---------
			15	140± 5	---------
		N-2	1	100± 1	---------
			5	196± 5	---------
			15	90± 1	---------

[1]N-1 = nuclei isolated from parenchymal cells; N-2 = nuclei isolated from non-parenchymal cells.

[2]Time (days) after sacrifice of animal. All DNA preparations were stored at 5-10°C between determinations.

[3]DPM/min; Each number represents the mean ± range of duplicate determinations.

[4]Nucleotides x $(10)^{12}$; extent in these experiments is defined as the total incorporation of nucleotides after 40 minutes of incubation in high salt.

[5]Prepared from rats maintained on a control diet.

[6]Prepared from rats maintained on a 0.05% (w/w) AAF diet for 6 weeks.

for up to 5 days. In all experiments, DNA preparations were assayed for template activity within five days after isolation of the template.

To examine the possibility of protein and RNA contamination affecting template activity, each DNA preparation was monitored for percent contamination by these macromolecules. The results are shown in Table 6. AAF treatment significantly ($p<.05$) affected the amount of protein covalently bound to N-1 DNA after 7 days on AAF-2 days off, 14 days on AAF-0 days off and 14 days on AAF-2 days off. No significant differences in percent protein could be detected for N-2 DNA. AAF treatment did not increase the amount of RNA bound to N-1 DNA but did increase the percent RNA remaining on N-2 DNA after 4 days on AAF-10 days off and 7 days on AAF-5 days off.

The relationship between the rate and extent of RNA synthesis and percent contamination of the DNA is shown in Figure 12. None of the regression lines have a slope significantly ($p<.05$) different from zero. This suggests that, within the limits of protein and RNA contamination encountered in these experiments, no significant correlation exists between these parameters and the rate of extent of RNA synthesis.

The relationship between the number of initiation sites and percent contamination of DNA is shown in Table 7. The differences seen are not statistically significant ($p<.05$), thus suggesting that there is no correlation between these parameters over the range of RNA and protein in these experiments.

The possibility of enzyme degradation occurring during storage was examined by assessing *E. coli* RNA polymerase activity on calf thymus DNA. Activity was checked upon arrival of the enzyme and after the vial

TABLE 6

Percent Protein and Percent RNA Contamination on DNA Isolated from Parenchymal (N-1) and Non-Parenchymal (N-2) Cell Nuclei

Treatment		Percent Protein		Percent RNA	
		N-1	N-2	N-1	N-2
Control		3.3±0.2[2]	6.0±0.4	1.8±0.1	4.7±0.4
4 Days on AAF[1]	- 0 days off	4.3±1.1[2]	4.7±1.3[2]	1.5±0.5[2]	2.2±0.6[2]
	- 2 days off	4.7±1.5[2]	7.8±1.7[3]	1.7±0.4[2]	5.0±0.6[2]
	- 5 days off	3.4±0.5[2]	6.6±1.7[4]	1.5±0.3[2]	4.5±0.6[4,6]
	- 10 days off	3.7±0.7[2]	6.1±0.6[4]	2.4±0.4[2]	7.1±0.5[4,6]
7 Days on AAF	- 0 days off	5.3±1.0[5]	6.7±1.8[5]	1.7±0.3[5]	3.4±0.6[5]
	- 2 days off	10.0±1.7[5,6]	8.3±1.8[5]	1.7±0.3[5]	4.1±0.3[4,6]
	- 5 days off	3.0±0.3[4]	5.3±0.4[4]	1.8±0.1[4]	6.4±0.1[4]
	- 10 days off	3.3±0.1[4]	6.6±1.2[4]	1.9±0.1[4]	4.9±0.6[4]
14 Days on AAF	- 0 days off	11.0±2.7[4,6]	10.2±5.2[2]	2.7±0.4[4]	3.4±0.2[2]
	- 2 days off	12.6±1.2[2,6]	5.6±1.5[4]	1.7±0.4[2]	2.8±0.6[2]
	- 5 days off	5.5±2.0[4]	2.4±0.3[4]	1.3±0.2[4]	2.6±0.3[4]
	- 10 days off	4.2±0.8[4]	5.5±1.2[4]	1.3±0.2[4]	4.6±0.9[4]

[1]Animals were fed a 0.05% (w/w) AAF diet for 4, 7 or 14 days. Groups of animals were killed at either the end of the period of carcinogen feeding (0 days off) or 2, 5 or 10 days after being returned to the control diet.

[2]Mean ± S.E., n=4.

[3]Mean ± S.E., n=3.

[4]Mean ± range, n=2.

[5]Mean ± S.E., n=6.

[6]Significantly different from controls, $p<.05$.

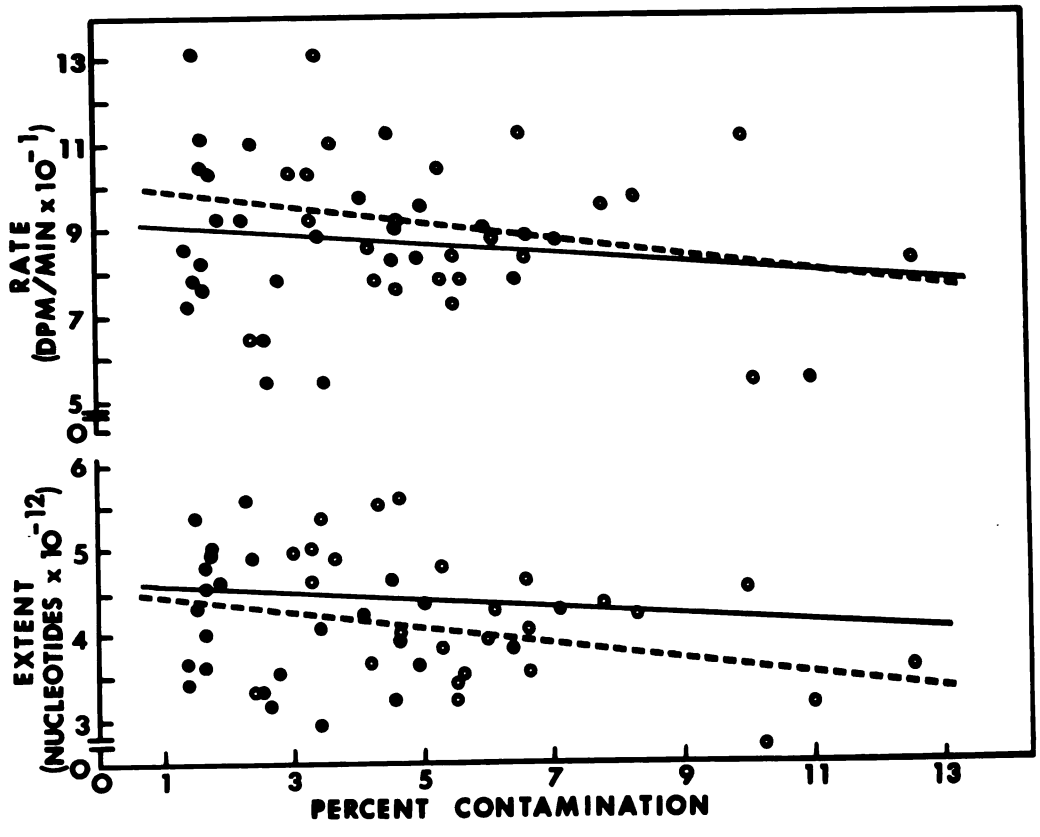

Figure 12: Relationship Between Percent Contamination of DNA and Rate and Extent of RNA Synthesis.

Rate and extent of RNA synthesis were measured as in Section 2.9. For the purposes of this study, all treatment groups were combined (n=26). Correlation coefficients are percent RNA (●—●) rate = 0.117, extent = 0.266; percent protein (O---O) rate = 0.295, extent = 0.313.

TABLE 7

Relationship Between Number of Initiation Sites and Percent Contamination of DNA

Initiations[1]	Percent Protein	Percent RNA
1000	8.4 ± 2.3[2]	2.8 ± 1.1[2]
2000	6.9 ± 0.6[3]	2.7 ± 0.1[3]

[1]Base pairs/molecule polymerase.

[2]Mean ± S.E., n=3.

[3]Mean ± S.E., n=11.

of polymerase was nearly empty. The results, shown in Table 8, demonstrate that enzyme activity was stable throughout the time of storage for each enzyme preparation.

The kinetics of incorporation of ^{3}H-UTP into RNA synthesized on DNA isolated from parenchymal (N-1) and non-parenchymal (N-2) cell nuclei fractions is shown for control animals in Figure 13. The reaction rate was linear for at least 10 minutes. Increasing the amount of enzyme in the reaction mixture to 0.35 units resulted in no increase in ^{3}H-UTP incorporation, indicating that RNA polymerase was present in excess. These conditions also existed when DNA from AAF-fed animals was used as a template in the reaction. Introduction of additional labelled and unlabelled nucleotide triphosphates (0.06 mM) after 140 minutes of reaction time resulted in no increase in ^{3}H-UTP incorporation, indicating the plateau was not due to depletion of substrate.

To examine the effects of an acute dose of the carcinogen, a single injection of 40 mg AAF/kg body weight was administered and the rate and extent of RNA synthesis on hepatic DNA assessed 24 hours later. As shown in Table 9, no change in the rate or extent of RNA synthesis on either N-1 or N-2 DNA could be demonstrated after this single large dose of AAF.

The effect of AAF ingestion on the rate of RNA synthesis is summarized in Figure 14. The rate of RNA synthesis on N-1 DNA is decreased approximately 25% (statistically significant, $p<.05$) after 4 days of AAF feeding and this inhibition remains after the animal has been returned to the control diet for 2 days. No inhibition is present after the animal has been returned to the control diet for 5 days. No

TABLE 8

Stability of E. Coli RNA Polymerase

Enzyme Preparation	Date of Assay	RNA Polymerase Activity[1]
A	12-10-75	0.85[2]
	2-27-76	0.82
B	2-27-76	0.88
	5-01-76	1.00
C	5-01-76	1.28
	8-30-76	1.20

[1] RNA polymerase activity was assayed as described in 2.12.

[2] nmols ^{3}H-UTP incorporated/10 min/unit enzyme; mean of a triplicate determination.

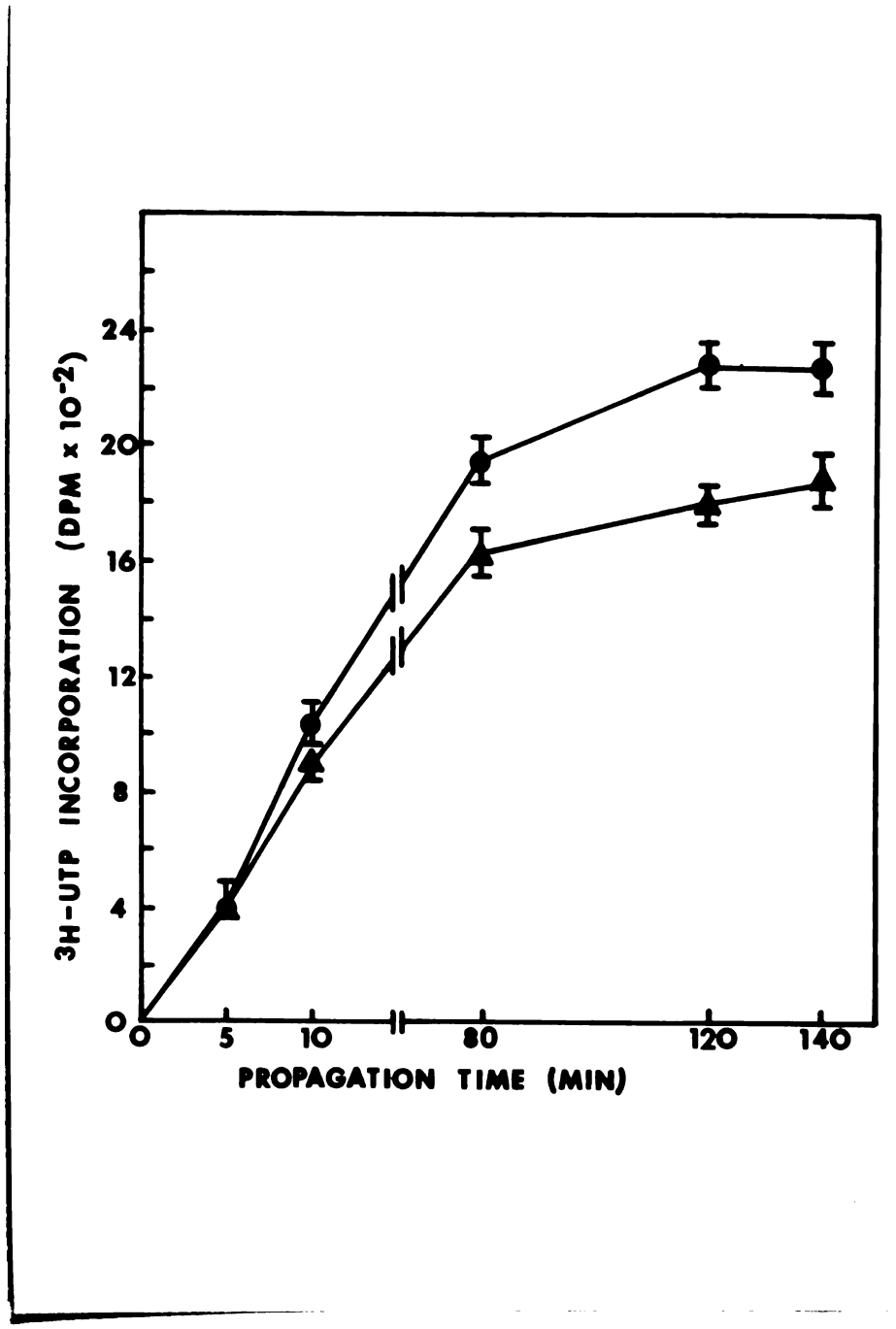

Figure 13: Rate and Extent of RNA Synthesis on Control DNA Isolated from Parenchymal (N-1) and Non-Parenchymal (N-2) Cell Nuclei.

DNA was isolated from hepatic parenchymal (●) and non-parenchymal (▲) cell nuclei and template activity was assessed as in Section 2.9. Incorporation of ^{3}H-UTP during the low salt incubation was less than 200 DPM and this value has been subtracted from the data. Each curve represents the mean ± S.E. of the data obtained from 14 DNA preparations.

TABLE 9

Effect of A Single Injection of AAF[1] on Rate and Extent of RNA Synthesis

Treatment	DNA[2]	Rate[3]	Extent[4]
Control[5]	N-1	100± 1	4.96±0.33
	N-2	101± 8	4.47±0.26
AAF	N-1	114± 1	4.92±0.21
	N-2	108±11	4.43±0.21

[1]40 mg AAF/kg body weight given i.p. in corn oil-dimethylsulfoxide (6:1 v/v), 24 hours prior to sacrifice.

[2]N-1 = DNA isolated from parenchymal cell nuclei.
N-2 = DNA isolated from non-parencymal cell nuclei.

[3]DPM/min; mean ± range of the data from two DNA preparations.

[4]Nucleotides x $(10)^{12}$; mean ± range of the data from two DNA preparations.

[5]Single i.p. injection of vehicle (corn oil-dimethylsulfoxide, 6:1, v/v) given 24 hours prior to sacrifice.

Figure 14: Effect of AAF Ingestion on the Rate of RNA Synthesis on Parenchymal (N-1) and Non-Parenchymal (N-2) Cell DNA.

Animals were fed a 0.05% (w/w) AAF diet for 4 (A), 7 (B) or 14 (C) days. Groups of animals were killed at either the end of the period of carcinogen feeding (zero time) or 2, 5 and 10 days after being returned to the control diet. Each point represents the mean ± S.E. of the data obtained from at least 4 DNA preparations, prepared from parenchymal (●) and non-parenchymal (▲) cell nuclei. Control values (mean ± S.E. of the data obtained from 14 DNA preparations) were 103±9 DPM/min and 90±6 DPM/min for parenchymal and non-parenchymal cell DNA, respectively.

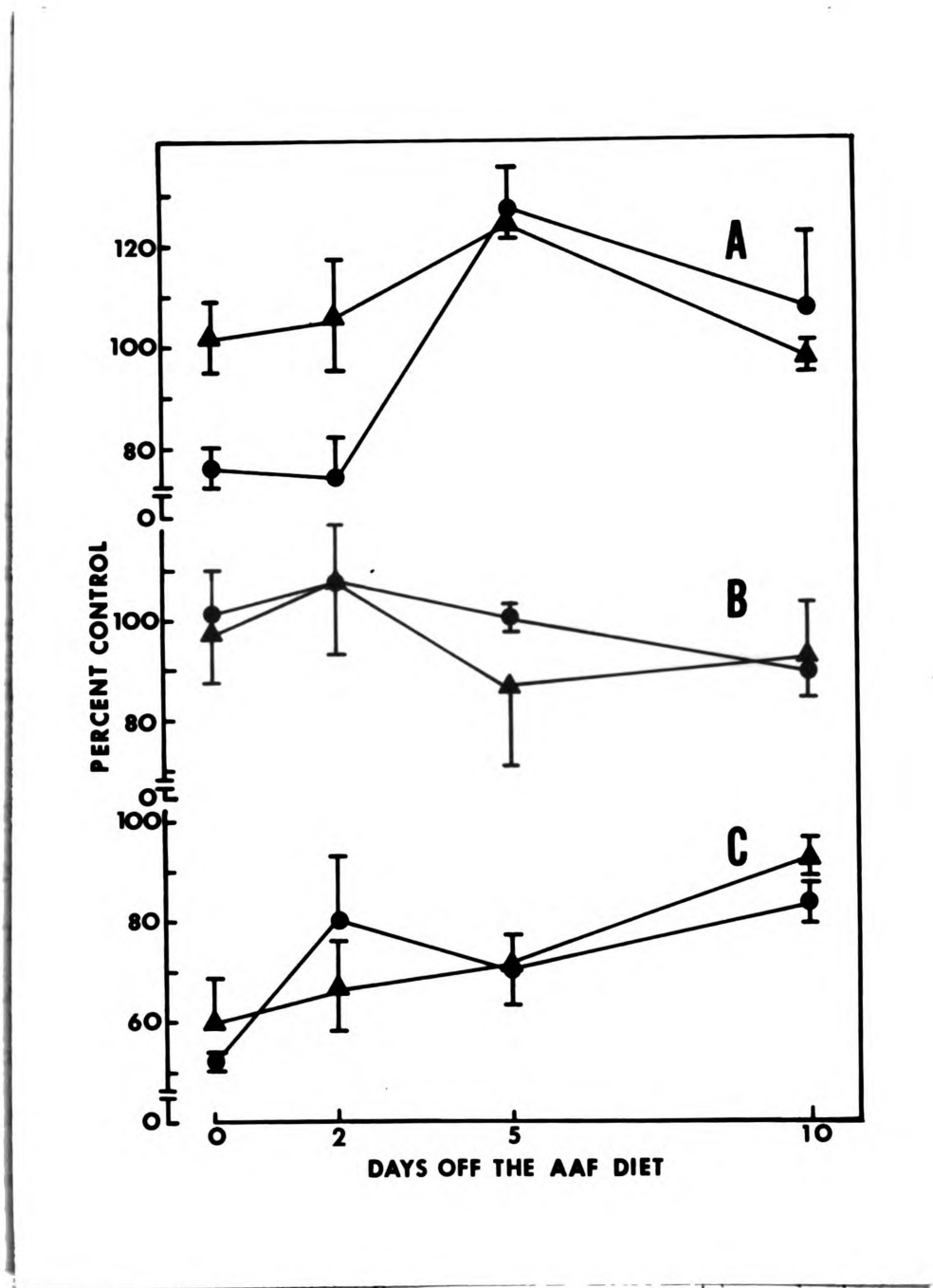
A
B
C
120
100
80
0
100
80
0
100
80
60
0
PERCENT CONTROL
0
2
5
10
DAYS OFF THE AAF DIET

Figure 14

statistically significant decrease in the rate of RNA synthesis on N-2 DNA could be demonstrated during the 4 day series.

No statistically significant decrease in the rate of RNA synthesis could be demonstrated after 7 days of AAF ingestion for either N-1 or N-2 DNA. Removal of the AAF diet for 2, 5 or 10 days had no significant ($p<.05$) effect on the rate of RNA synthesis on either template.

After 14 days of AAF ingestion, the rate of RNA synthesis was decreased 48% and 40% for N-1 and N-2 DNA, respectively (statistically significant, $p<.05$). The rate remained depressed for both templates despite the animals being returned to the control diet for 5 days. Ten days after removal of the carcinogenic diet, the rate of RNA synthesis on N-1 and N-2 DNA were 83% and 92%, respectively, of control values.

The results of titrating E. coli RNA polymerase with control rat liver DNA are shown in Figure 15. The titration curves for both N-1 and N-2 DNA plateau at 32 ng DNA for 0.05 unit enzyme. This corresponds to one RNA polymerase initiation site for every 2000 DNA base pairs.

The results of titrating E. coli RNA polymerase with DNA isolated from AAF-treated animals are shown in Figure 16. No change in the number of initiation sites could be demonstrated for either N-1 or N-2 DNA after 4, 7 or 14 days of AAF feeding. A statistically significant increase in the number of initiation sites was seen on N-1 and N-2 DNA after 4 days on AAF-2 days off and on N-2 DNA after 14 days on AAF-2 days off. The data is summarized in Table 10.

Data in these experiments was used to calculate a number average chain length of the RNA chains synthesized on the various templates. These are shown in Table 10. Feeding of AAF for 4 or 7 days did not

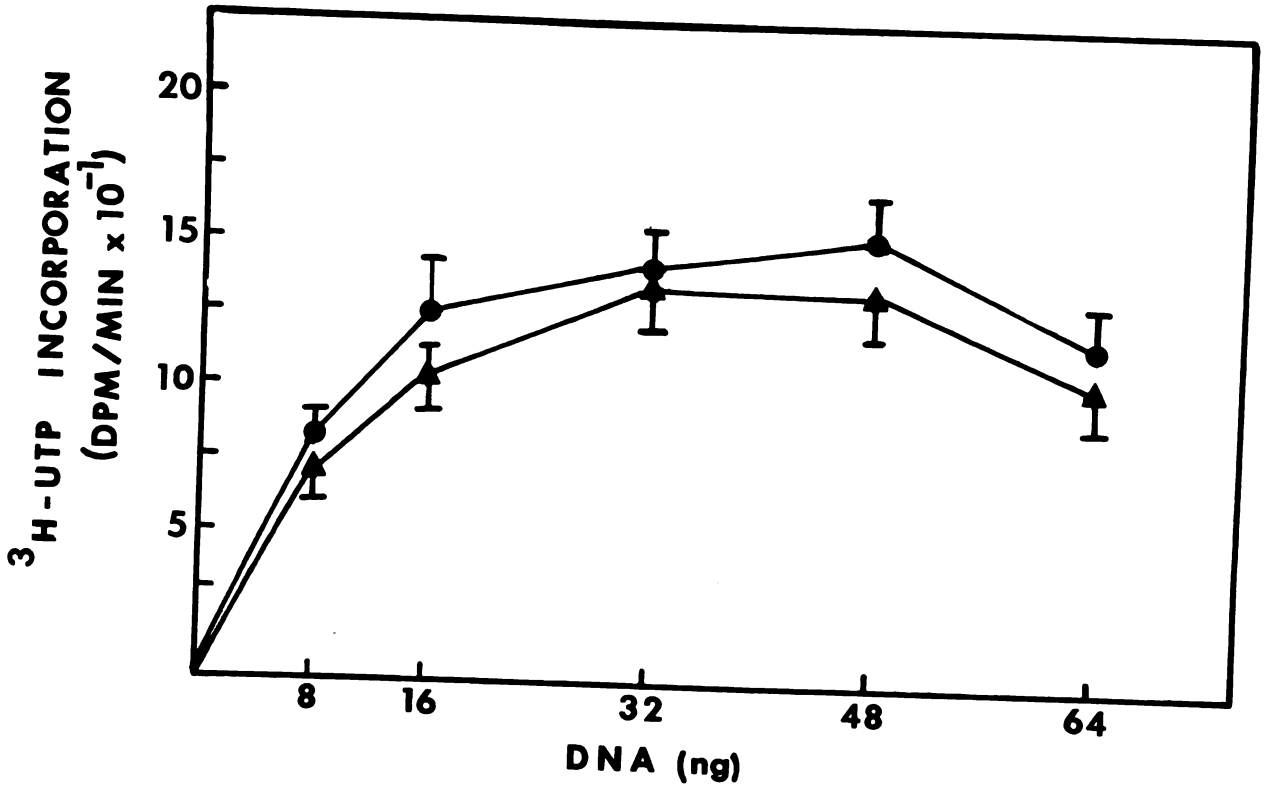

Figure 15: Determination of the Number of Initiation Sites on Control DNA Isolated from Parenchymal (N-1) and Non-Parenchymal (N-2) Cell Nuclei.

Incorporation of ^{3}H-UTP in low salt was less than 200 DPM and this value has been subtracted from the above data. Each curve represents the mean ± S.E. of the data obtained from 10 DNA preparations, prepared from hepatic parenchymal (●) and non-parenchymal (▲) cell nuclei.

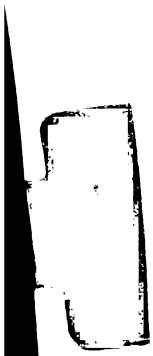

Figure 16: Determination of the Number of Initiation Sites on DNA Isolated from Parenchymal (N-1) and non-Parenchymal (N-2) Cell Nuclei from AAF-treated Animals.

Animals were fed a 0.05% (w/w) AAF diet for 4 (A), 7 (B) or 14 (C) days. Groups of animals were killed either at the end of carcinogen feeding or 2 days after being returned to the control diet. Each curve represents the mean ± SE of the data obtained from at least 4 DNA preparations. (●) Parenchymal cell DNA, 0 days off; (⊙) parenchymal cell DNA, 2 days off; (▲) non-parenchymal cell DNA, 0 days off; (△) non-parenchymal cell DNA, 2 days off. The arrow denotes the end point of the titration as defined in Section 2.10.

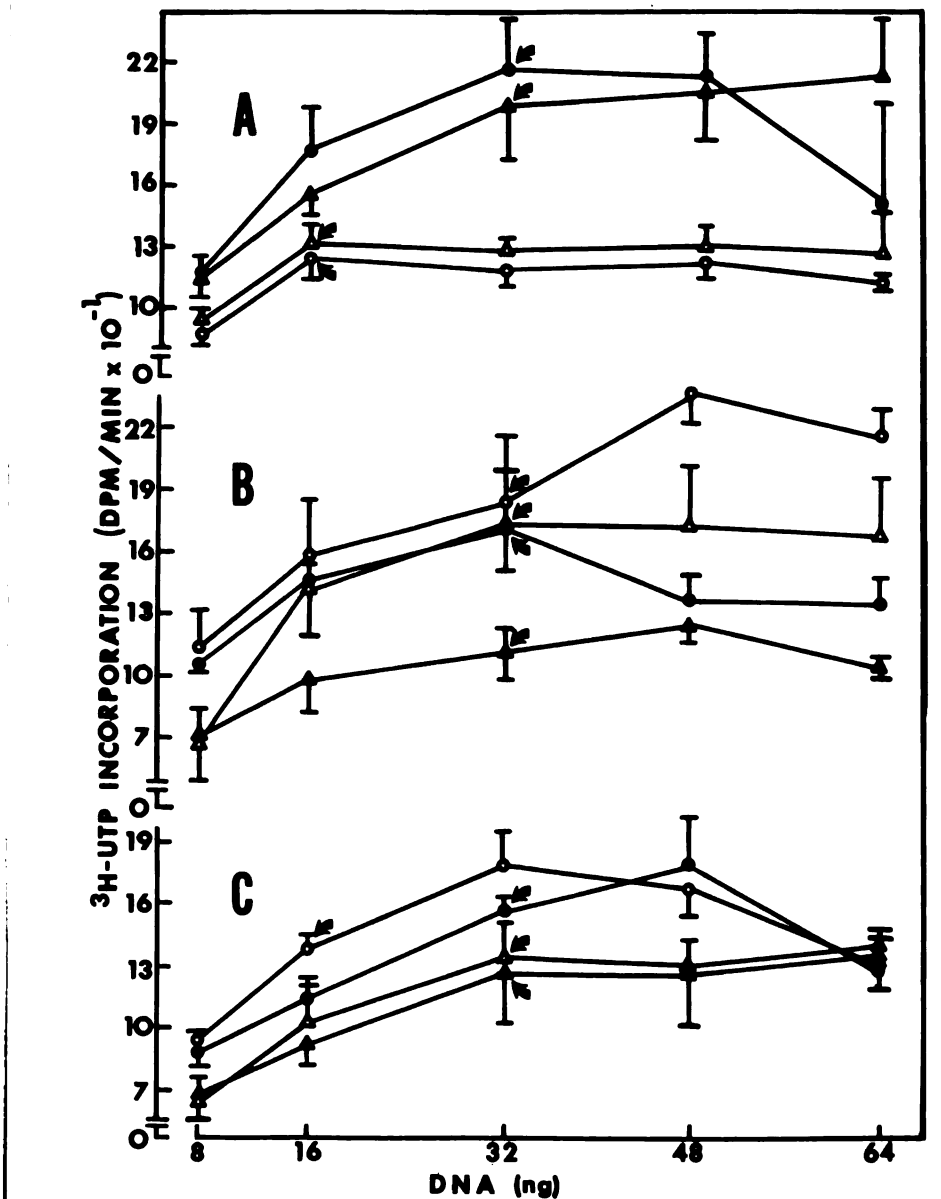
A
B
C
22
19
16
13
10
7
0
^{3}H-UTP INCORPORATION (DPM/MIN x 10^{-1})
8
16
32
48
64
DNA (ng)

Figure 16

TABLE 10

Effect of AAF Ingestion on the Extent of RNA Synthesis, Number of Initiation Sites and RNA Chain Length on DNA Isolated from Parenchymal (N-1) and Non-Parenchymal (N-2) Cell Nuclei

Treatment	Extent[1]		Initiation Sites[2]		RNA Chain Length[3]	
	N-1	N-2	N-1	N-2	N-1	N-2
Control[4]	5.00±0.16	3.94±0.13	2000	2000	1250	985
4 Days on AAF - 0 days off[5]	5.51±0.32	5.56±0.40	2000	2000	1378	1390
4 Days on AAF - 2 days off[5]	4.00±0.14	4.35±0.23	1000	1000	500	544
7 Days on AAF - 0 days off[6]	4.79±0.22	4.05±0.18	2000	2000	1198	1010
7 Days on AAF - 2 days off[6]	4.54±0.32	4.23±0.25	2000	2000	1135	1058
14 Days on AAF - 0 days off[5]	3.16±0.10	2.93±0.18	2000	2000	790	733
14 Days on AAF - 2 days off[5]	3.60±0.22	3.52±0.23	1000	2000	450	880

[1] Nucleotides x $(10)^{12}$.

[2] Base pairs/molecule polymerase.

[3] Nucleotides.

[4] Mean ± S.E., n=10.

[5] Mean ± S.E., n=4.

[6] Mean ± S.E., n=6.

result in a statistically significant decrease in the length of the RNA chain synthesized on either N-1 or N-2 DNA. A 37% and 26% inhibition of RNA chain length was seen after 14 days of AAF feeding for N-1 and N-2 DNA, respectively. Significant decreases in RNA chain length were also seen for N-1 and N-2 DNA after 4 days on AAF-2 days off and for N-1 DNA after 14 days on AAF-2 days off.

DISCUSSION

At least three conclusions may be drawn from the preliminary series of experiments shown in Figure 4. Firstly, changes in hepatic RNA synthesis and RNA polymerase activity appear to be an early consequence of AAF feeding. After only 4 days of AAF ingestion, a 52% inhibition of the capacity for RNA synthesis by polymerase II and a 60% stimulation of poly A synthesis by this enzyme could be demonstrated. Secondly, in several instances, the capacity for RNA synthesis by polymerase II was decreased relative to RNA polymerase II activity. This suggests that a decrease in template activity is an early consequence of AAF feeding. Thirdly, RNA and poly A synthesis by polymerase I did not always mirror RNA and poly A synthesis by polymerase II. This suggests that the assay for separation of RNA synthesis by the two polymerases is valid. In summary, these preliminary experiments demonstrated the need for further, more elaborate experiments to examine DNA template activity.

In actuality, poly A synthesis may not yield a true estimate of RNA polymerase activity. It is possible that initiation or elongation of an RNA chain could be blocked prior to the polymerase reaching a thymine-rich region. Therefore AAF, although not covalently bound to thymine, may affect transcription of these regions indirectly. In addition a poly A polymerase exists in mammalian cells (113) and the contribution of this enzyme in our assay was not determined. To avoid these variables,

an exogenous template, poly (dA-dT), was used to assess RNA polymerase activity. The template activity of endogenous DNA was abolished with the use of actinomycin-D and omission of CTP and GTP from the reaction mixture.

RNA polymerase I activity as assessed by poly A synthesis (Figure 4) did not always agree with enzyme activity measured on poly (dA-dT) (Figure 10). In several instances, synthesis on poly (dA-dT) was significantly increased above control values while poly A synthesis remained at or below control levels (4 days on AAF-0 days off; 7 days on AAF-0 days off; 7 days on AAF-2 days off; 14 days on AAF-0 days off; and 14 days on AAF-5 days off). This may be due to synthesis in thymine-rich regions being affected indirectly through an impaired template activity occurring in other regions of the genome, as discussed above. These data suggest that synthesis on poly (dA-dT) is a more sensitive measure of RNA polymerase I activity, probably because it is completely independent of AAF-induced changes on the endogenous template.

Attempts to validate the assay for RNA polymerase II activity utilizing poly (dA-dT) and actinomycin-D were unsuccessful (Figures 7B and 8B, Table 2). Although the exact reason(s) for failure to detect the nucleoplasmic enzyme activity is (are) not known, it may be due to differing ionic requirements between the endogenous chromatin template and the synthetic copolymer poly (dA-dT). The high ionic strength required to differentiate RNA synthesis by the two polymerases probably prevents initiation of RNA synthesis on poly (dA-dT). An ionic strength similar to that used for assessment of RNA polymerase II in these studies has been shown to markedly inhibit initiation of RNA synthesis

by E. coli RNA polymerase on phage DNA (114,115). Glazer et al. (67) also reported that poly (dA-dT) was a poor template for RNA polymerase II, although the ionic conditions in their assay were not specified. Since a high concentration of $(NH_4)_2SO_4$ is required for efficient separation of RNA synthesis by the two enzymes, assessment of polymerase II activity by this method may not be possible. Rather than pursue further experiments to validate this assay for RNA polymerase II, only polymerase I activity was measured in subsequent experiments.

A single injection of N-OH-AAF, at a dose of 30 mg/kg (68,70) or 40 mg/kg (72) has been reported to inhibit hepatic RNA synthesis in vivo. Acute administration of AAF (40 mg/kg), however, does not appear to affect this process (72) unless extremely high doses (160 mg/kg) are administered (116). Feeding of AAF for 4, 7 or 14 days does not inhibit the capacity for RNA synthesis in hepatic nuclei (Figure 10). In fact, a 40% stimulation in this process was seen after 14 days on the AAF diet. These results are in fair agreement with those obtained in the preliminary experiments seen in Figure 4.

While the capacity for RNA synthesis remained quite constant, RNA polymerase I activity progressively increased with duration of carcinogen exposure (Figure 10). These results are indicative of a progressive inhibition of the capacity of DNA, in the target organ, to serve as a template for RNA synthesis. These results are in agreement with Troll et al. (75) who also reported impairment of template activity after several weeks of AAF feeding.

A possible alternative explanation is that RNA polymerase was present in excess in the nuclei preparations, such that the enzyme was not rate limiting. However, several studies have reported a direct correlation between the amount of RNA synthesis and the level of RNA polymerase activity in hepatic nuclei. For example, cortisone treatment (91) or partial hepatectomy (117,118) results in increases in RNA synthesis and this stimulation appears to be due to an increase in RNA polymerase activity. Each of these studies utilized poly (dA-dT) and actinomycin-D to assess RNA polymerase activity. To validate their measurements, Organtini et al. (118) and Yu (117) solubilized the polymerases and confirmed the increase in enzyme activity. In addition, Schwartz and Roeder (119) calculated the number of RNA polymerase molecules in several cell types and concluded that in normal cells, RNA polymerase I is not saturating to ribosomal RNA cistrons. In rapidly growing mouse myeloma cells (MOPC 315), the quantity of the nucleolar RNA polymerase approaches saturation levels (119). These studies suggest that RNA polymerase is rate limiting.

Recent studies (83,94,120) have verified a previous report (121) suggesting that RNA polymerase exists in two functional states in rat liver nuclei: "engaged" and "free". The "engaged" population of RNA polymerase is bound to DNA and is probably the form responsible for endogenous RNA synthesis (120). The "free" population of RNA polymerase is not bound to the template and is believed to be responsible for RNA synthesis on poly (dA-dT) (120). Thus, in the present studies, RNA synthesis on poly (dA-dT) may have been initiated by a different

population of RNA polymerase than RNA synthesis on the endogenous template. If alterations in enzyme activity following AAF feeding are not equal for both populations of RNA polymerase, the results obtained in these experiments may be complex and any inferences as to changes in DNA template activity may be quite tenuous. Assuming that the suggested template activity inhibition is real, it is impossible to distinguish whether the impairment was due to AAF lesions on the DNA blocking RNA synthesis or to physiological repression of transcriptionally active regions of the genome. In addition, if AAF lesions were indeed responsible for the observed inhibition, did they block initiation or elongation of RNA synthesis? To answer these questions, hepatic DNA was isolated from animals maintained on a schedule of AAF ingestion similar to that used in the above experiments (Figure 10) and template activity was assessed directly utilizing a purified bacterial RNA polymerase.

The values obtained in these studies for the rate of RNA synthesis and number of initiation sites on control DNA compare fairly well to literature values. Our estimate of the rate of RNA synthesis, 46 pmols/min/μg, is close to that obtained by Millette and Fink (78) on T7 DNA, 53 pmols/min/μg, and that of Chiu et al. (103) using rat liver DNA, 34 pmols/min/μg. Our estimate of the number of initiation sites on rat liver DNA, 2000 base pairs/molecule polymerase, is slightly greater than that obtained by Cedar and Felsenfeld on calf thymus DNA (100), 1000-1400 base pairs/molecule polymerase, but agrees very well with the value obtained by Chiu et al. using rat liver DNA (103), 1850 base pairs/molecule polymerase. Measurement of initiation sites on calf thymus DNA

(γ-^{32}P)GTP and (γ-^{32}P)ATP yields 1100 base pairs/molecule polymerase (100).

The data shown in Figure 14 confirm the results of Troll et al. (75) who demonstrated a significant decrease in the template activity of rat liver DNA after several weeks of AAF ingestion. These results also confirm those of Grunberger et al. (69) and Yu and Grunberger (73) who demonstrated decreases in template activity following a single injection of N-OH-AAF. However, several studies have not demonstrated an impairment of template activity after N-OH-AAF administration. Possible reasons for the apparent discrepancies are differences in 1) the mode of carcinogen administration, 2) the nature of the template used, and 3) the strain of animals.

All of the studies reporting no change in template activity after N-OH-AAF administration have measured this parameter after a single injection of the carcinogen (67,68,71). Many years ago, Turner and Reid (122) demonstrated that changes in hepatic RNA synthesis after an acute injection of a carcinogen are different from those occurring after short periods of carcinogen feeding. While a single injection of ethionine depressed RNA synthesis, feeding of this carcinogen for 2 to 20 days resulted in a marked stimulation of RNA synthesis in hepatic nuclei (122). Szafarz and Weisburger (54) compared the extent of carcinogen binding to DNA and its subsequent removal from this macromolecule after a single injection of N-OH-AAF and after 2 weeks of N-OH-AAF feeding. Their results indicate that a single acute administration of N-OH-AAF produces a lesser degree of binding which is more transient relative to those AAF moieties bound after 2 weeks of carcinogen feeding. Thus, the

AAF metabolites bound to DNA after a single injection of N-OH-AAF may differ both quantitatively and qualitatively from that bound after continuous feeding of AAF. The present results and those cited above, indicate that hepatic RNA synthesis can vary depending upon the mode of AAF administration. However, two studies have demonstrated an inhibition of DNA template activity after a single injection of N-OH-AAF (69,73), thus other experimental variables must be important in assessment of template activity.

Another difference between the present studies and those of others (71,90) is the nature of the template utilized. Herzog et al., in recent studies, measured template activity on liver chromatin rather than DNA (71,90). If carcinogen molecules were bound to regions of the DNA covered by chromosomal proteins, those modified bases would probably be repressed in vitro thus decreasing the chances of detecting changes in template activity. It may be possible that AAF lesions repressed in the in vitro chromatin preparation could become derepressed in vivo and inhibit DNA template activity. Therefore, the measurement of chromatin template activity in vitro may not give an accurate measure of the true ability of AAF to inhibit transcription in vivo.

Another factor probably influencing the results of template activity experiments is the sex or strain of rat used. An early study by Farber (79) indicated that while histological changes can be observed in the livers of male Wistar rats 14 days after beginning a 0.04% AAF diet, no such changes could be demonstrated in female rats of this straing after 49 days of AAF ingestion. Female Holtzman (13) and Sprague-Dawley

(17) rats also do not exhibit the high incidence of hepatic carcinomas seen in male rats of these strains after carcinogen administration. In addition, Reuber (123) recently demonstrated that five strains of male rats exhibit markedly differing susceptibilities to hepatic carcinogenesis by N-2-fluorenyldiacetamide. The differences in the susceptibility of different strains to hepatocarcinogenesis by AAF should be considered when evaluating template activity studies, particularly those studies which utilize strains considered relatively non-susceptible to AAF-induced hepatomas (71).

Several other factors could be responsible for the seemingly inconsistent results reported in the literature. Glazer et al. (67) studied the effects of N-OH-AAF on hepatic template activity using partially hepatectomized rats. Since partial hepatectomy itself can alter gene expression (124) and increase levels of hepatic RNA polymerase (117), results on DNA template activity obtained from animals given this operation can not be strictly compared to our data or those from Grunberger and co-workers (69,73). In addition, Glazer et al. (67,72) and others (69,71) utilized a method of nuclei isolation which loses the "free" RNA polymerase population. Changes in enzyme activity are not necessarily equal for both populations of RNA polymerase. Treatment of nuclei with an estradiol-receptor complex can increase both "free" and "engaged" RNA polymerase (125) whereas treatment of animals in vivo with N-OH-AAF appears to increase the "free" population of RNA polymerase III and decrease the "engaged" form (73). Those studies measuring only the "engaged" population may have to be re-evaluated in light of this information.

While the experiments utilizing hepatic nuclei (Figure 10) suggested that feeding of AAF for 4, 7 or 14 days results in a progressive decrease in DNA template activity, the method could not distinguish whether this inhibition was due to AAF-DNA interactions or to physiological repression of the genome. The present studies confirm that template activity is decreased after 4 and 14 days of AAF ingestion and strongly suggest that at least some of the inhibition demonstrated in the previous study was due to carcinogen-DNA interactions. However, the suggestion of template activity inhibition seen after 7 days of AAF feeding (Figure 10) was not confirmed in the present studies. This could indicate that 1) after 7 days of AAF ingestion, RNA polymerase was present in excess and was thus not rate limiting in the nuclei suspensions, or 2) significant repression of the DNA was present. The presence of chromosomal proteins can reduce the availability of the template for AAF modification (126), thus a relative increase in the amount of DNA repressed at 7 days could decrease the number of AAF molecules bound to DNA. This would explain the fact that a decrease in template activity was observed in the nuclei preparation (due to repression) and not in the present study (due to fewer AAF-DNA modifications). The reasons why a significant increase in repression would occur at this particular time of AAF feeding remains to be elucidated.

The fact that template activity is inhibited after 4 and 14 days of AAF feeding and not after 7 days is rather puzzling. The data suggest that either the amount of AAF available for binding to DNA has decreased significantly between 4 and 7 days of carcinogen feeding or that those DNA modifications present at 7 days are qualitatively different from

those at 4 days, such that they no longer adversely affect transcription by E. coli RNA polymerase. In support of the former possibility, several studies have demonstrated that pre-feeding of AAF or N-OH-AAF can alter subsequent handling of the carcinogen (12,127-129). In particular, excretion of N-OH-AAF (as a glucuronide) appears to increase with carcinogen feeding. Feeding of AAF has been shown to affect the activity of several enzymes (130-134), some after only 3 days of ingestion (132-133). Of significant interest is the fact that the enzyme, sulfotransferase, responsible for the metabolic conversion of N-OH-AAF to a highly reactive sulfate ester, is inhibited after 7 days of AAF feeding (130). Such an inhibition could result in fewer active metabolites of AAF being available for interaction with DNA. However, sulfotransferase levels were also decreased after 3 (133) and 14 (130) days of AAF feeding; the latter being the time at which a large decrease in template activity was observed in the present studies. This suggests that the availability of AAF in precancerous liver is affected by levels of other enzymes involved in activation and detoxification of the carcinogen, such as those catalyzing hydroxylation (17,135), acetylation (138-139), glucuronidation (127,129,135) or isomerization (138).

Alternatively, there may be qualitative differences in the predominant type of DNA modification present at 7 days relative to 4 days. AAF is capable of producing several types of DNA modifications (see Section 1.2), at least two of which differ in their biological half lives (42). It is possible that each base modification may have a unique biological half-life and perhaps a characteristic effect on RNA synthesis. Significant advances in methodology are required before it

is possible to identify and determine the functional importance of these different DNA modifications during the early stages of hepatocarcinogenesis.

In view of the fact that AAF produces primarily hepatocellular tumors arising from parenchymal cells (79,80), a method of nuclei isolation was selected which allows separation of parenchymal (N-1) and non-parenchymal (N-2) cell nuclei (86). Previous studies have documented the distribution (84) and synthesis (95) of DNA in these two classes of liver nuclei during azo dye-induced hepatocarcinogenesis. Glazer (70) examined the effect of a single injection of N-OH-AAF upon the incorporation of ^{3}H-orotic acid into N-1 and N-2 nuclei in sham and partially hepatectomized rats and concluded that RNA synthesis was inhibited to the same degree in both classes of nuclei. However, the following data, obtained in the present experiments, suggests a preferential inhibition of template activity on DNA isolated from N-1 nuclei: 1) the rate of RNA synthesis on N-1 DNA is selectively inhibited after 4 days of AAF ingestion and this inhibition remains despite the animal returning to a control diet for 2 days (Figure 13); 2) the number of initiation sites on N-1 DNA was increased relative to N-2 DNA after 14 days on AAF-2 days off (Table 10). Changes in the number of initiation sites are consistent with high degrees of AAF modification (78); 3) the chain length of RNA synthesized on N-1 DNA was decreased to a greater extent and exhibited a slower recovery to control values relative to RNA synthesized on N-2 DNA after 14 days of AAF feeding (Table 5). This selective inhibition of DNA template activity in cells which presumably are precursors of the AAF-induced carcinoma in the target organ suggests

that this parameter may be a contributing factor in hepatocarcinogenesis by this agent.

There are three general mechanisms by which AAF moieties could inhibit RNA synthesis: by preventing initiation, slowing polymerization or terminating an RNA chain prematurely. An attempt was made in these studies to elucidate the mechanism(s) of AAF-induced template activity inhibition.

The rate of RNA synthesis after 10 minutes of incubation (Figure 14) is a function of the number of initiated RNA chains and the speed of the polymerization process. Thus, a decrease in the initial rate of RNA synthesis with no change in the number of initiation sites is indicative of an impairment of the process of elongation. After 4 days of AAF feeding, a 25% decrease in the rate of RNA synthesis was observed on N-1 DNA with no change in the number of initiation sites. Since chain length was not affected by this treatment, the major effect at this time appears to be a slowing of the polymerization process. An analogous situation is present for both N-1 and N-2 DNA after 14 days of AAF ingestion, however, this treatment produces significant decreases in RNA chain length. Thus, after 14 days of AAF treatment, at least part of the impairment in elongation appears to be due to premature termination. Early termination has been reported to be a primary factor in AAF-induced template inhibition (78). Premature termination of RNA chains synthesized on DNA modified in vitro by N-Ac-AAF has also been reported by Glazer (74),

Several treatments in this study produced increases in the number of initiation sites for RNA synthesis (Table 10). Millette and Fink

(78) have reported that initiation is increased only when the amount of carcinogen bound to DNA is quite high. However, highly modified DNA should also exhibit a severely depressed rate of RNA synthesis (78) and this was not seen after 4 days on AAF-2 days off for either N-1 or N-2 DNA. These experiments were designed to measure only large changes in the number of initiation sites and variability between experiments was sometimes quite extensive. Additional experiments are needed to confirm the validity of the observed increases.

Whether or not these findings are relevant to hepatic carcinogenesis by AAF remains to be demonstrated. However, several studies have found a correlation between inhibition of the capacity for RNA synthesis and carcinogenesis. Zieve (77) examined the carcinogenicity of a series of arylhydroxamic acids and related compounds and demonstrated that neither non-carcinogens nor carcinogens with target organs other than liver could elicit a significant decrease in hepatic RNA synthesis after in vivo administration. Eker (139) demonstrated that the carcinogen urethane markedly decreased RNA synthesis in its target organ (lung) but had no such effect in a non-target organ (liver). Two non-carcinogenic derivatives of urethane had no effect on lung RNA synthesis (139). Support for the role of template activity in carcinogenesis by AAF was obtained in the present studies. In several instances, template activity on N-1 DNA appeared to be selectively decreased relative to N-2 DNA. Since parenchymal cells have been implicated as precursors of AAF-induced neoplasms (79,80), template activity inhibition in these cells could represent an initial molecular event leading to neoplasia.

Modification of the DNA template could potentially result in qualitative and/or quantitative changes in RNA production. Qualitative changes could occur when an RNA polymerase molecule inserts a non-complementary base or series of bases thus altering the primary structure of the RNA molecule. This could produce conformational changes possibly resulting in defective transfer RNA codon recognition or amino acid acceptance capacities. If the misincorporation occurred in a messenger RNA molecule, and this molecule was translated, an aberrant protein molecule could result. Production of aberrant proteins could potentially result in non-functional repressors or perhaps aberrant RNA or DNA polymerases. Although the methods utilized in this thesis do not allow detection of qualitative RNA changes or their functional consequences, these may be important parameters for further study in precancerous liver.

The results of the present studies, indicating a decrease in the rate of elongation (perhaps due to premature termination) of RNA synthesis, suggest that AAF may create quantitative changes in RNA synthesis.[1] Quantitative changes in RNA synthesis could result in decreased production of certain specific RNA molecules. If AAF binds nonrandomly to certain regions of the genome, i.e., preferentially binds to transcriptionally active regions, then the opportunity for bound AAF moieties to affect cellular levels of certain critical RNA molecules (and thus critical proteins) may be significant. The

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[1] If premature termination did occur and the RNA molecule was transcribed, the resulting protein molecule may be aberrant. Thus, decreases in elongation may also be a mechanism for qualitative changes in RNA (and protein) production.

decreased production or absence of certain chromosomal repressors could potentially result in derepression of the genome. Thus, by this mechanism, inhibition of DNA template activity could account for the alterations in gene expression seen in some tumors (ectopic hormones, fetal isozymes).

SUMMARY AND CONCLUSIONS

Two different methods have been utilized to demonstrate that ingestion of the hepatic carcinogen AAF results in an inhibition of the capacity for DNA, in the target organ, to serve as a template for RNA synthesis. This inhibition appears very early, perhaps as soon as 4 days, following the start of carcinogen feeding. The mechanism of RNA synthesis inhibition appears to be an impairment in the process of elongation. Although not conclusively demonstrated, this impairment of elongation appears to involve a slowing of polymerization after 4 days of AAF ingestion and a premature termination of RNA synthesis after 14 days on the AAF diet. Thus, it appears that DNA bases modified by AAF metabolites in vivo serve as a barrier to transcription either by merely slowing the RNA polymerase or by completely halting synthesis. AAF modification may also increase the number of initiation sites for RNA synthesis, although this data needs verification.

Chemical carcinogenesis appears to be an extremely complex process, occurring in several stages and possibly involving multiple mechanisms of induction. It is now becoming evident that assessment of the extent of binding of a chemical carcinogen to DNA does not conclusively demonstrate the relevance of the interaction to the carcinogenic process. A very important factor to be considered is the nature of the DNA base damage. Since several carcinogens exhibit binding to more than one DNA base and/or several atoms on a single base, it is possible that certain DNA base modifications are more damaging than others. For example, the

nervous system-specific carcinogenesis by ethylnitrosourea has been correlated to a particular DNA modification; ethylation at the 06 of guanine (65). In addition to the initial type of DNA base damage present, the persistence of certain types of modifications may be critical to carcinogenesis. It is possible that certain DNA lesions are more damaging due to slower removal from this macromolecule, either because of nonrecognition by the excision repair process or due to unusually high stability of the carcinogen-DNA adduct. This was illustrated by Goth and Rajewksy (65) who demonstrated that, although the initial degree of alkylation by ethylnitrosourea at the 06 of guanine was greater in nontarget tissue than target tissue, the rate of removal of this modification from target tissue DNA was considerably slower.

The mere presence or persistence of a DNA base modification, however, does not automatically implicate the lesion as relevant to carcinogenesis. The lesion must be capable of producing some functional change in the DNA, perhaps defects in DNA or RNA synthesis, which will eventually result in initiation and/or promotion of carcinogenesis. Thus, the ultimate test of whether a particular DNA modification is relevant to carcinogenesis may depend upon the ability of the lesion to alter normal DNA function. Because inhibition of the capacity for RNA synthesis appears to be selective for carcinogens in their target organ (77,139), assessment of template activity may be an important parameter to examine in those studies aimed at elucidating the functional consequences of carcinogen-DNA interactions. If template activity inhibition proves to be selective for carcinogens in their target organ, this parameter may become a valuable accessory method in the identification

of chemical carcinogens or, specifically, which DNA base modifications are relevant to carcinogenesis.

BIBLIOGRAPHY

1. Miller, E.C. and Miller, J.A.: in "The Molecular Biology of Cancer", ed. H. Busch, Academic Press, New York, 377-402, 1974.

2. Heidelberger, C.: Chemical carcinogenesis. Ann. Rev. Biochem. 44: 79, 1975.

3. Boutwell, R.K.: The function and mechanism of promotors of carcinogenesis, CRC Critical Rev. Toxicol. 3: 419, 1974.

4. Slaga, T.J., Bowden, G.T., Shapas, B.G. and Boutwell, R.K.: Macromolecular synthesis following a single application of alkylating agents used as initiators of mouse skin tumorigenesis. Cancer Res. 33: 769, 1973.

5. Boutwell, R.K.: Some biological aspects of skin carcinogenesis. Prog. Exp. Tumor Res. 4: 207, 1964.

6. Peraino, C., Fry, M., Staffeldt, E. and Christopher, J.P.: Comparative enhancing effects of phenobarbital, amobarbital, diphenylhydantoin and dichlorodiphenyltrichloroethane on 2-acetylaminofluorene-induced hepatic tumorogenesis in the rat. Cancer Res. 35: 2884, 1975.

7. Craddock, V.M.: Liver carcinomas induced in rats by a single administration of dimethylnitrosoamine after partial hepatectomy. J. Natl. Cancer Inst. 47: 899, 1971.

8. Armuth, V. and Bereblum, I.: Systemic promoting action of phorbol in liver and lung carcinogenesis in AKR mice. Cancer Res. 32: 2259, 1972.

9. Goodman, L.S. and Gilman, A.: "The Pharmacological Basis of Therapeutics", MacMillan Publishing Co., Inc., 1975.

10. Miller, J.A.: Carcinogenesis by chemicals: An overview - O.H.A. Clowes Memorial Lecture. Cancer Res. 30: 559, 1970.

11. Cramer, J.W., Miller, J.A. and Miller, E.C.: N-Hydroxylation: A new metabolic reaction observed in the rat with the carcinogen 2-acetylaminofluorene. J. Biol. Chem. 235: 885, 1960.

12. Miller, J.A., Cramer, J.W. and Miller, E.C.: The N- and Ring-hydroxylation of 2-acetylaminofluorene during carcinogenesis in the rat. Cancer Res. 20: 950, 1960.

13. Miller, E.C., Miller, J.A. and Hartmann, H.A.: N-Hydroxy-2-acetylaminofluorene: A new metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. Cancer Res. 21: 815, 1961.

14. Irving, C.C. and Veazy, R.A.: Persistant binding of 2-acetylaminofluorene to rat liver DNA in vivo and consideration of the mechanism of binding of N-hydroxy-2-acetylaminofluorene to rat liver nucleic acids. Cancer Res. 29: 1799, 1969.

15. DeBaun, J.R., Miller, E.C. and Miller, J.A.: N-Hydroxy-2-acetylaminofluorene sulfotransferase: Its probable role in carcinogenesis and in protein-(methion-S-yl) binding in rat liver. Cancer Res. 30: 577, 1970.

16. Irving, C.C., Wiseman, R. and Hill, J.T.: Biliary excretion of the O-glucuronide of N-hydroxy-2-acetylaminofluorene by the rat and rabbit. Cancer Res. 27: 2309, 1967.

17. Gutmann, H.R., Malejka-Giganti, D. and Rydell, R.E.: On the correlation between the hepatocarcinogenicity of the carcinogen, N-2-fluorenylacetamide and its metabolic activation by the rat. Cancer Res. 32: 1554, 1972.

18. Miller, E.C., Miller, J.A. and Enomoto, M.: The comparative carcinogenicities of 2-acetylaminofluorene and its N-hydroxy metabolite in mice, hamsters, and guinea pigs. Cancer Res. 24: 2018, 1964.

19. Kriek, E.: Carcinogenesis by aromatic amines. Biochim. Biophys. Acta 355: 177, 1974.

20. DeBaun, J.R., Smith, J.Y.R., Miller, E.C. and Miller, J.A.: Reactivity in vivo of the carcinogen N-hydroxy-2-acetylaminofluorene: Increased by sulfate ion. Science 167: 184, 1970.

21. Irving, C.C., Janss, D.H. and Russell, L.T.: Lack of N-hydroxy-2-acetylaminofluorene sulfotransferase activity in the mammary gland and zymbals gland of the rat. Cancer Res. 31: 387, 1971.

22. Miller, J.A. and Miller, E.C.: The presence and significance of bound aminoazo dyes in the livers of rats fed p-dimethylaminobenzene. Cancer Res. 7: 468, 1947.

23. Miller, J.A. and Miller, E.C.: The carcinogenic aminoazo dyes. Adv. Cancer Res. 1: 339, 1953.

24. Jungmann, R.A. and Schweppe, J.S.: Binding of chemical carcinogens to nuclear proteins of rat liver. Cancer Res. 32: 952, 1972.

25. Lotlikar, P. and Paik, W.: Binding of carcinogenic aromatic amines to rat liver nuclear acidic proteins in vivo. Biochem. J. 124: 443, 1971.

26. Pitot, H.C. and Heidelberger, C.: Metabolic regulatory circuits and carcinogenesis. Cancer Res. 23: 1694, 1963.

27. Miller, E.C. and Miller, J.A.: Mechanisms of chemical carcinogenesis: Nature of proximate carcinogens and interactions with macromolecules. Pharmacol. Rev. 18: 805, 1966.

28. Miller, J.A. and Miller, E.C.: The metabolic activation of carcinogenic aromatic amines and amides. Progr. Exper. Tumor Res. 11: 273, 1969.

29. Grantham, P.H.: Metabolism of fluorene-9-^{14}C in rats, guinea pigs and rabbits. Biochemistry 2: 610, 1963.

30. Irving, C.C. and Williard, R.F.: On the in vivo protein binding of 1-hydroxy-2-acetylaminofluorene-1-^{14}C in the rat. Cancer Res. 24: 77, 1964.

31. Miller, E.C., Juhl, V. and Miller, J.A.: Nucleic acid guanine: Reaction with the carcinogen N-acetoxy-2-acetylaminofluorene. Science 153: 1125, 1966.

32. Fink, L.M., Nishimura, S. and Weinstein, I.B.: Modifications of ribonucleic acid by chemical carcinogens. I. In vitro modification of transfer ribonucleic acid by N-acetoxy-2-acetylaminofluorene. Biochemistry 9: 496, 1970.

33. Matsushima, T. and Weisburger, J.H.: Inhibitors of chemical carcinogenesis as probes for molecular targets: DNA as a decisive receptor for metabolites from N-hydroxy-N-2-fluorenylacetamide. Chem.-Biol. Interactions 1: 211, 1969/1970.

34. Marroquin, F. and Farber, E.: The binding of 2-acetylaminofluorene to rat liver ribonucleic acid in vivo. Cancer Res. 25: 1262, 1965.

35. Irving, C.C., Veazy, R.A. and Williard, R.F.: On the significance of the binding of 2-acetylaminofluorene and N-hydroxy-2-acetylaminofluorene to rat liver nucleic acid in vivo. Cancer Res. 27: 720, 1967.

36. Kriek, E., Miller, J.A., Juhl, V. and Miller, E.C.: 8-(N-2-Fluorenylacetamido)-guanosine, an arylamidation reaction product of guanosine and the carcinogen N-acetoxy-N-2-fluorenylacetamide in neutral solution. Biochemistry 6: 177, 1967.

37. Irving, C.C. and Veazy, R.A.: Differences in the binding of 2-acetylamionfluorene and its N-hydroxy metabolite to liver nucleic acids of male and female rats. Cancer Res. 31: 19, 1971.

38. Zieve, F.J. and Gutmann, H.R.: Reactivities of the carcinogens N-hydroxy-2-fluorenylacetamide and N-hydroxy-3-fluorenylacetamine, with tissue nucleophiles. Cancer Res. 31: 471, 1971.

39. Brookes, P. and Lawley, P.D.: Evidence for the binding of polynuclear aromatic hydrocarbons to the nucleic acids of mouse skin, relation between carcinogenic power of hydrocarbons and their binding to deoxyribonucleic acid. Nature 202: 781, 1964.

40. Calburn, N.H. and Boutwell, R.K.: The binding of β-propriolactone and some related alkylating agents to DNA, RNA and protein of mouse skin, relation between tumor-initiating power of alkylating agents and their binding to DNA. Cancer Res. 28: 653, 1968.

41. Matsushima, T., Kobuna, I. and Sugimura, T.: In vivo interaction of 4-nitroquinoline-1-oxide and its derivatives with DNA. Nature 216: 508, 1967.

42. Kriek, E.: Persistant binding of a new reaction product of the carcinogen N-hydroxy-N-2-acetylaminofluorene with guanine in rat liver DNA in vivo. Cancer Res. 32: 2042, 1972.

43. Kriek, E.: Difference in binding of 2-acetylamionfluorene to rat liver deoxyribonucleic acid and ribosomal ribonucleic acid in vivo. Biochim. Biophys. Acta 161: 273, 1968.

44. Kapuler, A.M. and Michelson, A.M.: The reaction of the carcinogen N-acetoxy-2-acetylaminofluorene with DNA and other polynucleotides and its stereochemical implications. Biochim. Biophys. Acta 232: 436, 1971.

45. Kriek, E. and Reitsema, J.: Interaction of the carciongen N-acetoxy-N-2-acetylaminofluorene with polyadenylic acid: Dependence of reactivity on conformation. Chem.-Biol. Interactions 3: 397, 1971.

46. Levine, A.F., Fink, L.M., Weinstein, I.B. and Grunberger, D.: Effect of N-2-acetylaminofluorene modification on the conformation of nucleic acids. Cancer Res. 34: 319, 1974.

47. Fuchs, R. and Duane, M.: Physical studies on deoxyribonucleic acid after covalent binding of a carcinogen. Biochemistry 11: 2659, 1972.

48. Fuchs, R. and Duane, M.: Physical basis of chemical carcinogenesis by N-2-fluorenylacetamide derivatives and analogs. FEBS Letters 34: 295, 1974.

49. Fuchs, R., Lefevre, J.F., Pouyet, J. and Duane, M.: Comparative orientation of the fluorene residue in native DNA modified by N-acetoxy-N-2-acetylaminofluorene and two 7-halogeno derivatives. Biochemistry 15: 3347, 1976.

50. Nelson, J.H., Grunberger, D., Cantor, C.R. and Weinstein, I.B.: Modification of ribonucleic acid by chemical carcinogens. IV. Circular dichroism and proton magnetic resonance studies of oligonucleotides modified with N-2-acetylaminofluorene. J. Mol. Biol. 62: 331, 1971.

51. Troll, W., Rinde, E. and Day, P.: Effect of N-7 and C-8 substitution of guanine in DNA on Tm, bouyant density and RNA polymerase priming. Biochim. Biophys. Acta 174: 211, 1969.

52. Fuchs, R. and Duane, M.: Dynamic structure of DNA modified with the carcinogen N-acetoxy-N-2-acetylaminofluorene. Biochemistry 13: 4435, 1974.

53. Epstein, S.M., McNary, J., Bartus, B. and Farber, E.: Chemical carcinogenesis: Persistence of bound forms of 2-fluorenylacetamide. Science 162: 907, 1968.

54. Szafarz, D. and Weisberger, J.H.: Stability of the binding of label from N-hydroxy-N-2-fluorenylacetamide to intracellular targets, particularly DNA in rat liver. Cancer Res. 29: 962, 1969.

55. Cerutti, P.A.: Excision repair of DNA base damage. Life Sciences 15: 1567, 1974.

56. Lieberman, M.W., Baney, R.N., Lee, R.E., Sell, S. and Farber, E.: Studies on DNA repair in human lymphocytes treated with proximate carcinogens and alkylating agents. Cancer Res. 31: 1297, 1971.

57. Strauss, B.S.: Repair of DNA in mammalian cells. Life Sciences 15: 1685, 1975.

58. Painter, R.B.: DNA damage and repair in eucaryotic cells. Genetics 78: 139, 1974.

59. Lieberman, M.W. and Poirier, M.C.: Base pairing and template specificity during DNA repair synthesis in human mouse cells. Biochemistry 13: 5384, 1974.

60. Lieberman, M.W. and Poirier, M.C.: Intragenomal distribution of DNA repair synthesis: Repair in satellite and mainband DNA in cultured mouse cells. Proc. Nat. Acad. Sci. U.S.A. 71: 2461, 1974.

61. Robbins, J.H., Kraemer, K.H., Lutzner, M.A., Festoff, B.W. and Coon, H.G.: Xeroderma pigmentosum: An inherited disease with sun sensitivty, multiple cutaneous neoplasms and abnormal DNA repair. Ann. Int. Med. 80: 221, 1974.

62. Lehmann, A.R., Kirk-Bell, S., Arlett, C.F., Paterson, M.C., Lohmann, P.H.M., de Weerd-Kastelein, E.A. and Bootsma, D.: Xeroderma pigmentosum cells with normal levels of excision repair have a defect in DNA synthesis after UV-irradiation. Proc. Nat. Acad. Sci. U.S.A. 72: 219, 1975.

63. Lehmann, A.R.: Postreplication repair of DNA in ultraviolet-irradiated mammalian cells. J. Mol. Biol. 66: 319, 1972.

64. Higgins, N.P., Kato, K. and Strauss, B.: A model for replication repair in mammalian cells. J. Mol. Biol. 101: 417, 1976.

65. Goth, R.A. and Rajewsky, M.F.: Persistance of 06-ethylguanine in rat brain DNA: Correlation with nervous system-specific carcinogenesis by ethylnitrosourea. Proc. Nat. Acad. Sci. U.S.A. 71: 639, 1974.

66. Frei, J.V. and Lawley, P.D.: Methylation of DNA in various organs of C57Bl mice by a carcinogenic dose of N-methyl-N-nitrosourea and stability of some methylation products up to 18 hours. Chem.-Biol. Interactions 10: 413, 1975.

67. Glazer, R.I., Glass, L.E. and Menger, F.M.: Modifications of hepatic ribonucleic acid polymerase activitys by N-hydroxy-2-acetylamionfluorene and N-acetoxy-2-acetylaminofluorene. Mol. Pharmacol. 11: 36, 1975.

68. Zieve, F.J.: Inhibition of rat liver RNA polymerase by the carcinogen N-hydroxy-2-fluorenylacetamide. J. Biol. Chem. 247: 5987, 1972.

69. Grunberger, G., Yu, F.L., Grunberger, D. and Feigelson, P.: Mechanism of N-hydroxy-2-acetylaminofluorene inhibition of rat hepatic ribonucleic acid synthesis. J. Biol. Chem. 248: 6278, 1973.

70. Glazer, R.I.: Inhibition by N-hydroxy-2-acetylaminofluorene of incorporation of (5-^{3}H) orotic acid into RNA of two classes of nuclei from normal and regenerating liver. Biochim. Biophys. Acta 361: 361, 1974.

71. Herzog, J., Serroni, A., Briemeister, B.A. and Farber, J.L.: N-Hydroxy-2-acetylaminofluorene inhibition of rat liver RNA polymerases. Cancer Res. 35: 2138, 1975.

72. Glazer, R.I., Nutter, R.C., Glass, L.E. and Menger, F.M.: 2-Acetylaminofluorene and N-hydroxy-2-acetylaminofluorene inhibition of incorporation of orotic acid-5-^{3}H into nuclear ribosomal and heterogenous RNA in normal and regnerating liver. Cancer Res. 34: 2451, 1974.

73. Yu, F.L. and Grunberger, D.: Multiple sites of action of N-hydroxy-2-acetylaminofluorene in rat hepatic nuclear transcription. Cancer Res. 36: 3629, 1976.

74. Glazer, R.I.: Alterations in the transcriptional capacity of hepatic DNA-dependent RNA polymerase I and II by N-hydroxy-2-acetylaminofluorene. Cancer Res. 36: 2282, 1976.

75. Troll, W., Belman, S., Berkowitz, E., Chmielwicz, Z.F., Ambrus, J.L. and Bardos, T.J.: Differential responses of DNA and RNA polymerase to modifications of the template rat liver DNA caused by the action of the carcinogen 2-acetylaminofluorene in vivo and in vitro. Biochim. Biophys. Acta 157: 16, 1968.

76. Adams, M.P. and Goodman, J.I.: RNA synthesis and RNA polymerase activity in hepatic nuclei isolated from rats fed the carcinogen 2-acetylaminofluorene. Biochem. Biophys. Res. Comm. 68: 850, 1976.

77. Zieve, F.J.: Effects of the carcinogen N-acetoxy-2-fluorenlacetamide of the template properties of deoxyribonucleic acid. Mol. Pharmacol. 9: 658, 1973.

78. Millette, R.L. and Fink, L.M.: The effect of modification of T7 DNA by the carcinogen N-2-acetylaminofluorene: Termination of transcription in vitro. Biochemistry 14: 1426, 1975.

79. Farber, E.: Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylaminofluorene and 3'-methyl-4-dimethylaminoazobenzene. Cancer Res. 16: 142, 1956.

80. Reuber, M.D.: Development of preneoplastic and neoplastic lesions in the liver in male rats given 0.025 percent N-2-fluorenyldiacetamide. J. Natl. Cancer Inst. 34: 697, 1965.

81. Svoboda, D. and Higginson, J.: A comparison of ultrastructural changes in rat liver due to chemical carcinogens. Cancer Res. 28: 1703, 1968.

82. Flaks, B.: Changes in the fine structure of rat hepatocyptes during the early phases of chronic 2-acetylaminofluorene intoxication. Chem.-Biol. Interactions 2: 129, 1970.

83. Walker, P.R. and Potter, V.R.: Isozyme studies on adult regenerating, precancerous and developing liver in relation to findings in hepatomas. Adv. Enzyme Regul. 10: 339, 1972.

84. Sneider, T.W., Bushnell, D.E. and Potter, V.R.: The distribution and synthesis of DNA in two classes of rat liver nuclei during azo dye-induced hepatocarcinogenesis. Cancer Res. 30: 1867, 1970.

85. Farber, E.: Carcinogenesis-cellular evolution as a unifying thread: Presidential address. Cancer Res. 33: 2537, 1973.

86. Bushnell, D.E., Whittle, E.D. and Potter, V.R.: Differential utilization of pyrimidines for RNA synthesis in two classes of rat liver nuclei. Biochim. Biophys. Acta 179: 497, 1969.

87. Johnston, I.R., Mathius, A.P., Pennington, F. and Ridge, D.: The fractionation of nuclei from mammalian cells by zonal centrifugation. Biochem. J. 109: 127, 1968.

88. Kaufmann, E.N., Sidransky, N. and Shinozuka, H.: Incorporation of ^{14}C-orotic acid into RNA of different sized nucleoli of rat livers following partial hepatectomy or after force-feeding or threonine-devoid diet. Exp. Cell. Res. 55: 130, 1969.

89. Chambon, P.: in "The Enzymes", ed. P.D. Boyer, Academic Press, New York, 261-330, 1974.

90. Herzog, J. and Farber, J.: Inhibition of rat liver RNA polymerases by action of the methylating agents dimethylnitrosoamine in vitro and methyl methanesulfonate in vitro. Cancer Res. 36: 1761, 1976.

91. Yu, F.-L. and Feigelson, P.: Cortisone stimulation of nucleolar RNA polymerase activity. Proc. Nat. Acad. Sci. U.S.A. 68: 2177, 1971.

92. Meisler, A.L. and Tropp, B.E.: Studies on ribonucleic acid synthesis in nuclei isolated from rat liver. Biochim. Biophys. Acta 174: 476, 1969.

93. Novello, F. and Stirpe, F.: Experimental conditions affecting RNA synthesis in isolated rat liver nuclei. Biochem. J. 112: 721, 1969.

94. Yu, F.-L.: An improved method for the quantitative isolation of rat liver nuclear RNA polymerases. Biochim. Biophys. Acta 395: 329, 1975.

95. Goodman, J.I. and Potter, V.R.: The effect of 5-fluorodeoxyuridine on the synthesis of deoxyribonucleic acid pyrimidines in precancerous rat liver. Mol. Pharmacol. 9: 297, 1973.

96. Axel, R., Cedar, H. and Felsenfeld, G.: Synthesis of globin ribonucleic acid from duck reticulocyte chromatin in vitro. Proc. Nat. Acad. Sci. U.S.A. 70: 2029, 1973.

97. Marmur, J.: A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3: 208, 1961.

98. Hyman, R.W. and Davidson, N.: Kinetics of the in vitro inhibition of transcription by actinomycin. J. Mol. Biol. 50: 421, 1970.

99. Cedar, H.: Transcription of DNA and chromatin with calf thymus RNA polymerase B in vitro. J. Mol. Biol. 95: 257, 1975.

100. Cedar, H. and Felsenfeld, G.: Transcription of chromatin in vitro. J. Mol. Biol. 77: 237, 1973.

101. Simpson, R.T.: Distribution of RNA polymerase binding sites in fractionated chromatin. Proc. Nat. Acad. Sci. U.S.A. 71: 2740, 1974.

102. Stein, G.S., Mans, R.J., Gabbay, E.J., Steink, J.L., Davis, J. and Adawadkar, P.D.: Evidence for fidelity of chromatin reconstitution. Biochemistry 14: 1859, 1975.

103. Chiu, J.F., Wang, S., Fjugitani, H. and Hnilica, L.S.: DNA-binding chromosomal nonhistone proteins. Isolation, characterization and tissue specificity. Biochemistry 14: 4552, 1975.

104. Hill, B.T. and Baserga, R.: Changes in the number of binding sites for RNA polymerase in chromatin of WI-38 fibroblasts stimulated to proliferate. Biochem. J. 141: 27, 1974.

105. Cedar, H., Solage, E. and Zurucki, F.: Control of RNA synthesis by chromosomal proteins. Nucleic acids Res. 3: 1659, 1976.

106. Meilhac, M. and Chambon, P.: Animal DNA-dependent RNA polymerases: Initiation sites on calf thymus DNA. Eur. J. Biochem. 35: 454, 1973.

107. Gilmour, R.S. and Paul, J.: Tissue-specific transcription of the globin gene in isolate chromatin. Proc. Natl. Acad. Sci. U.S.A. 70: 3440, 1971.

108. Berg, D., Barrett, K. and Chamberlin, M.: Purification of two forms of E. coli RNA polymerase and sigma component. Methods in Enzymol. 24: 506, 1971.

109. Burgess, R.R.: A new method for the large scale purification of E. coli desoxyribonucleic acid-dependent ribonucleic acid polymerase. J. Biol. Chem. 244: 6160, 1969.

110. Ceriotti, G.: A microchemical determination of deoxyribonucleic acid. J. Biol. Chem. 198: 297, 1952.

111. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265, 1951.

112. Blobel, G. and Potter, V.R.: Distribution of radioactivity between the acid-soluble pool and the pools of RNA in the nuclear nonsedimentable and ribosome fractions of rat liver after a single injection of labelled orotic acid. Biochim. Biophys. Acta 166: 48, 1968.

113. Winters, M.A. and Edmonds, M.: A poly (A) polymerase from calf thymus. J. Biol. Chem. 248: 4763, 1973.

114. Domingo, E., Escarmis, C. and Warner, R.C.: Transcription of Azotobarter phage DNA; salt-independent equilibrium between steps in initiation. J. Biol. Chem. 250: 2872, 1975.

115. Escarmis, C., Domingo, E. and Warner, R.C.: Temperature and salt effects on the formation of preinitiation complexes between RNA polymerase and phage DNA. Biochim. Biophys. Acta 402: 261, 1975.

116. Dawson, K.M.: Time course of the effects of AAF on mouse liver nuclei acid synthesis and its modification by inhibitors. Chem.-Biol. Interactions 5: 153, 1972.

117. Yu, F-L.: Increased levels of rat hepatic free and engaged RNA polymerase activities during liver regeneration. Biochem. Biophys. Res. Comm. 64: 1107, 1975.

118. Organtini, J.E., Joseph, C.R., Farber, J.L.: Increases in the activity of solubilized rat liver nuclear RNA polymerases following partial hepatectomy. Arch. Biochem. Biophys. 170: 485, 1975.

119. Schwartz, L.B. and Roeder, R.G.: Purification and subunit structure of deoxyribonucleic acid-dependent ribonucleic acid polymerase I from the mouse myeloma, MOPC 315. J. Biol. Chem. 249: 5898, 1974.

120. Yu, F-L.: Two functional states of the RNA polymerase in rat hepatic nuclear and nucleolar fractions. Nature 251: 344, 1974.

121. Ligo, S., Sagher, D., Fang, S-M.: Isolation of chromatin-free RNA polymerase from mammalian cell nuclei. Nature 220: 1336, 1968.

122. Turner, M.K. and Reid, E.: An anomalous effect of ethionine on ribonucleic acid synthesis. Nature 203: 1174, 1964.

123. Reuber, M.D.: Various degrees of susceptibility of different stocks of rats to N-2-fluorenyldiacetamide hepatic carcinogenesis. J. Natl. Cancer Inst. 57: 111, 1976.

124. Bucher, N.L.R.: Regeneration of mammalian liver. Intern. Rev. Cytol. 15: 245, 1963.

125. Mohla, S., DeSombre, E.R. and Jensen, E.V.: Tissue-specific stimulation of RNA synthesis by transformed estradiol-receptor complex. Biochem. Biophys. Res. Comm. 46: 661, 1972.

126. Metzger, G. and Duane, M.P. In vitro binding of N-acetoxy-2-acetylaminofluorene to DNA in chromatin. Cancer Res. 35: 2732, 1975.

127. Grantham, P.H., Mohan, L., Yanamoto, R.S., Weisburger, E.K. and Weisburger, J.H.: Alteration of the metabolism of the carcinogen N-2-fluorenylacetamide by acetanilide. Tox. Appl. Pharmacol. 10: 118, 1968.

128. Hopp, M.L., Matsumoto, M., Wendell, B., Lee, C. and Oyasu, R.: Suppressive role of indole on 2-acetylaminofluorene hepato-toxicity. Cancer Res. 36: 234, 1976.

129. Mohan, L.C., Grantham, P.H., Weisburger, E.K., Weisburger, J.H. and Idoine, J.B.: Mechanisms of the inhibitory action of p-hydroxy-acetanilide on carcinogenesis by N-2-fluorenylacetamide or N-hydroxy-N-2-fluorenylacetamide. J. Natl. Cancer Inst. 56: 763, 1976.

130. Jackson, C.D. and Irving, C.C.: Sex differences in cell proliferation, and N-hydroxy-2-acetylaminofluorene sulfotransferase levels in rat liver during 2-acetylaminofluorene administration. Cancer Res. 32: 1590, 1972.

131. Morais, R.A., Poirier, L.A. and Dupuis, C.: Inhibition of mitochondrial 5' endonuclease activity by carcinogenic amines and their N-oxidized derivatives. Chem.-Biol. Interactions 5: 391, 1972.

132. Rahman, Y.E., Cerny, E.A. and Peraino, C.: Studies on rat liver ribonucleases in developing, 2-acetylaminofluorene fed and partially hepatectomized rats. Biochim. Biophys. Acta 178: 68, 1969.

133. Irving, C.C., Jackson, C.D. and Russell, L.T.: Changes in liver sulfotransferase levels during hepatocarcinogenesis by 2-acetylaminofluorene. Fed. Proc. 30: 1254, 1971.

134. Debaun, J.R., Miller, E.C., Miller, J.A.: N-hydroxy-2-acetylaminofluorene sulfotransferase: its probable role in carcinogenesis and in protein-(methion-S-yl) binding in rat liver. Cancer Res. 30: 577, 1970.

135. Matsushima, T., Grantham, P.H., Weisburger, E.K. and Weisburger, J.H.: Phenobarbital-mediated increase in ring- and N-hydroxylation of the carcinogen N-2-fluorenylacetamide, and decrease in amounts bound to liver DNA. Biochem. Pharmacol. 21: 2043, 1972.

136. Bartsch, H., Dworkin, C., Miller, E.C. and Miller, J.A.: Formation of electrophilic N-acetoxyarylamines in cytosols from rat mammary gland and other tissues by transacetylation from the carcinogen N-hydroxy-4-acetylaminobiphenyl. Biochim. Biophys. Acta 304: 42, 1973.

137. King, C.M.: Mechanism of reaction, tissue distribution and inhibition of arylhydroxamic acid acyltransferase. Cancer Res. 34: 1503, 1974.

138. Gutmann, H.R. and Erickson, R.R.: The conversion of the carcinogen N-hydroxy-2-fluorenylacetamide to o-aminodphenols by rat liver in vitro. An inducible enzymatic reaction. J. Biol. Chem. 244: 1729, 1969.

139. Eker, P.: Effects of the carcinogen urethane on nuclear RNA polymerase activities. Eur. J. Cancer 11: 493, 1975.