SUMMARY

The purpose of these experiments was to determine whether 3 hepatocarcinogens, 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB), 4'-fluoro-4-dimethylaminoazobenzene (4'-F-DAB), and thioacetamide caused similar changes in the nuclear RNA metabolism of precancerous rat liver, and whether these changes could be distinguished from those caused by the weak hepatocarcinogen 4'-methyl-4-dimethylaminoazobenzene (4'-Me-DAB). Carcinogens were administered by intraabdominal injection or by feeding in semisynthetic diets. Their effects on nuclear RNA synthesis and nuclear RNA concentration were compared 5 days after injection or after 2, 4, and 6 weeks of feeding. Nuclei were purified and the RNA fractionated into a slowly labeling non-nucleolar nuclear (NNN) RNA fraction and a more rapidly labeling nucleolar fraction. Synthesis of RNA in these subfractions was determined by incorporation of orotic acid-6-14C and adenine-8-14C. When injected: (a) 3'-Me-DAB decreased incorporation of orotic acid-6-14C into both subfractions and decreased the RNA content of these subfractions; (b) 4'-Me-DAB increased incorporation of orotic acid-6-14C into NNN RNA, but caused no change in the RNA content of either subfraction; (c) thioacetamide increased the RNA content of the nucleolar fraction; and (d) 4'-F-DAB caused no change in either the incorporation of labeled orotic acid or in the RNA content of either subfraction. When RNA synthesis was evaluated by incorporation of adenine-8-14C, injection of 4'-Me-DAB or thioacetamide increased incorporation into NNN RNA, but only thioacetamide increased incorporation into nucleolar RNA. Neither 3'-Me-DAB nor 4'-F-DAB injections altered adenine-8-14C incorporation. In feeding experiments, incorporation of either labeled precursor was decreased in animals fed basal diet and the concentration of RNA in both subfractions declined throughout the 6-week feeding period. After 6 weeks, animals fed 4'-Me-DAB or 3'-Me-DAB showed decreased incorporation of orotic acid-6-14C into NNN RNA, but incorporation into nucleolar RNA was unchanged. Animals fed thioacetamide for 6 weeks showed increased incorporation of labeled orotic acid into nucleolar RNA and these increases were accompanied by increases in the RNA content of this fraction. When RNA synthesis was evaluated by incorporation of adenine-8-14C, animals fed thioacetamide or 4'-F-DAB showed increased incorporation of labeled adenine into NNN RNA, but only those animals fed thioacetamide showed increased incorporation into the nucleolar RNA. It was concluded that, within the parameters of nuclear RNA content and nuclear RNA synthesis, as determined by incorporation of labeled orotic acid or labeled adenine, no common pattern existed between the 2 hepatocarcinogens 3'-Me-DAB and 4'-F-DAB, or between these and thioacetamide. Therefore, it seemed quite likely that changes in these aspects of nuclear RNA metabolism constituted irrelevant steps toward neoplasia in rat liver.

INTRODUCTION

Data from several studies published recently suggested that carcinogenic azo dyes exerted a unique influence on RNA metabolism in rat liver. For instance, Kidson and Kirby (19) reported a decline in the synthesis of mRNA2 among rats fed 4'-F-DAB. Kizer et al. (22) fractionated liver RNA into a rapidly labeling "nucleolar" fraction and a more slowly labeling NNN2 fraction and observed that a single injection of 3'-Me-DAB decreased (a) the RNA content of the nucleolar fraction, and (b) the incorporation of labeled orotic acid into both the nucleolar and NNN subfraction. Injection of the weak hepatocarcinogen 4'-Me-DAB (26) did not cause these effects (22), nor were they seen when 3'-Me-DAB was injected into hypophysectomized rats (20). Since tumor induction would be unlikely in rats fed 4'-Me-DAB (26) or in hypophysectomized rats fed 3'-Me-DAB (15), we were tempted to speculate that losses in RNA content and synthesis after injections of 3'-Me-DAB (22) might be correlated with the onset of malignant neoplasia in rat liver.

Our findings with 3'-Me-DAB (22) were supported by Sporn and Dingman (32) who showed that injection of 3'-Me-DAB resulted in losses of RNA from a chromatin fraction isolated from rat liver nuclei. Furthermore, they showed that long term

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feeding of 3'-Me-DAB also caused losses of chromatin RNA, but administration of the noncarcinogen 2 Me-DAB, either by injection or ingestion, failed to decrease the chromatin RNA content (32). Their data showing losses of chromatin RNA after short exposures to N-OH-AAF and aflatoxin B₁ (32) also suggested that the ability to alter uniquely nuclear RNA metabolism might be a characteristic shared by diverse hepatocarcinogens.

The purpose of experiments described in this report was to determine whether 3 hepatocarcinogens, 3'-Me-DAB, 4'-F-DAB, and thioacetamide, exerted influences on nuclear RNA metabolism that were similar and whether these effects were unique from those exerted by the weak hepatocarcinogen 4'-Me-DAB. Data are presented which indicate that 3'-Me-DAB did exert unique effects upon nuclear RNA metabolism, but these effects were not seen with the equally strong hepatocarcinogen 4'-F-DAB. The data indicated that within the parameters of nuclear RNA content and nuclear RNA synthesis, as determined by incorporation of labeled orotic acid or adenine, no common pattern existed between the 2 hepatocarcinogens 3'-Me-DAB and 4'-F-DAB or between these and thioacetamide.

MATERIALS AND METHODS

Animals. Female Holtzman rats weighing 150–180 gm were fed Rockland mouse and rat diet when used in injection experiments. Azo dyes were dissolved in 2 ml of corn oil and injected intraabdominally at 250 mg/kg body weight. Each animal received a single intraabdominal injection and was killed by cervical fracture after 5 days. Control animals received oil alone. Thioacetamide was dissolved in 0.9% saline and injected intraabdominally at 50 mg/kg body weight once daily for 5 days. The animals were killed 24 hr after the last injection. Controls received saline alone.

In feeding experiments, azo dyes were added to a semisynthetic diet (9) to give a concentration of 0.06% and thioacetamide was added at 0.066%. Control animals received the basal diet alone. All animals were conditioned 7 days on basal diet before dyes or thioacetamide was added to the diets.

Orotic acid-6-¹⁴C, 3.3 µc/rat, or adenine-8-¹⁴C, 5 µc/rat, was dissolved in 2 ml of saline and injected intraabdominally. Animals were killed 30 min after isotope injection.

Techniques of Tissue Preparation. Isolation and purification of nuclei, subfractionation of nuclear RNA into NNN and nucleolar fractions, and determination of RNA have been described previously (22). The DNA content of nuclei was determined by the method of Burton (5). Isotope concentration is expressed as dpm/mg DNA, with DNA determined by the method of Burton (5). The amount of isotope in each subfraction was determined by liquid scintillation counting in a xylene-dioxane-cellosolve counting cocktail described by Bruno and Christian (4). Quenching was corrected by the channels ratio method of Bush (6).

Source of Chemicals. The azo dyes, 3'-Me-DAB, 4'-F-DAB, and 4'-Me-DAB were synthesized using procedures described by Giese et al. (12). Thioacetamide was purchased from J. T. Baker Company, Phillipsburg, New Jersey. Orotic acid-6-¹⁴C and adenine-8-¹⁴C were purchased from Isotopes, Inc., Burbank, California.
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labeled orotic acid, while RNA concentrations are expressed as μg RNA/mg DNA. Incorporation of labeled orotic acid into both subfractions was decreased by 3'-Me-DAB injections, but was not appreciably altered by 4'-F-DAB injections. Injections of the weak hepatocarcinogen 4'-Me-DAB resulted in increased incorporation into NNN RNA, but incorporation into nucleolar RNA was unchanged. Among the 3 injected azo dyes, only 3'-Me-DAB caused changes in the RNA content. Here, significant losses were observed in both subfractions. This latter result is in accord with reports by Hirota (17) and, more recently, by Sporn and Dingman (32), which described losses of RNA in rat liver following 3'-Me-DAB injections.

**Effects of Fed Azo Dyes.** The foregoing experiments indicated that decreases in RNA concentration and orotic acid incorporation were characteristics unique to animals injected with 3'-Me-DAB. Since these characteristics were not observed among animals injected with 4'-F-DAB, it was important to determine whether the same patterns would be observed when the carcinogens were administered under circumstances known to result in malignant transformations. Arcos et al. (3) determined hepatic tumor incidence as a function of time of feeding a semisynthetic diet containing 0.06% 3'-Me-DAB. They reported that rats fed 2 weeks did not develop liver cancer. Thereafter, the incidence rose sharply, resulting in about 25% incidence after 4 weeks of feeding, and 70–75% after 6 weeks; all animals developed liver cancer after 8 weeks (3). Although it has been well established that thioacetamide was hepatocarcinogenic in the rat (10, 13, 16), careful incidence-time studies apparently have not been published.

We fed the 3 azo dyes or thioacetamide throughout 6 weeks. After 2, 4, and 6 weeks, we determined the extent to which labeled orotic acid was incorporated into the 2 nuclear RNA subfractions and the concentration of RNA in both subfractions. The data are shown in Chart 2. Among animals fed basal diets, incorporation of labeled orotic acid decreased in both subfractions throughout the 6-week period. Decreased incorporation was apparently associated with decreased RNA synthesis, which, as shown in Chart 2, significant losses in RNA content of both subfractions occurred simultaneously. Causes for these losses are unknown to us, but such losses are not without precedent. Sporn and Dingman (32) reported losses of chromatin RNA among basal diet-fed animals and attributed them to aging. Irrespective of whether aging should be implicated here, these losses complicated difficulties in determining the effects of carcinogens on nuclear RNA metabolism.

After 6 weeks, among animals fed 3'-Me-DAB or 4'-Me-DAB, decreased incorporation of orotic acid-6-14C into NNN RNA was observed; however, no similar decreases were observed in the nucleolar fraction. Animals fed thioacetamide showed increased incorporation of labeled orotic acid into nucleolar RNA, and perhaps some increase in incorporation into NNN RNA may have occurred, for decreased incorporation observed at 2 weeks was not sustained at 4 and 6 weeks.

Losses of RNA from the NNN subfraction in animals fed 3'-Me-DAB, 4'-F-DAB, and 4'-Me-DAB was greater than that of animals fed basal diet. Animals fed thioacetamide showed marked increases in nucleolar RNA content as early as 2 weeks, and these increases persisted throughout the feeding period. This result agrees with previous reports concerning increases in nuclear RNA following feeding (13, 25) or injection (1, 21, 23) of thioacetamide.

The decreases in incorporation of labeled orotic acid into NNN RNA after 6 weeks of feeding 3'-Me-DAB may have been associated in part with increases in DNA content (18, 29). This, together with the lack of effect of fed 3'-Me-DAB upon incorporation of labeled orotic acid into nucleolar RNA or upon the content of RNA in either subfraction, led us to conclude that effects of fed 3'-Me-DAB were considerably less severe than effects seen following injection of 3'-Me-DAB. The failure to observe inhibition of labeled orotic acid incorporation into RNA among rats fed 3'-Me-DAB appears to be in accord with Reid's (30) observation that activities of 2 enzymes associated with orotic acid conversion to uridine-5'-phosphate (viz., orotidine-5'-phosphate pyrophosphorylase, EC.2.4.2.10; and orotidine-5'-phosphate decarboxylase, EC.4.1.3.1) were slightly enhanced in rats fed 3'-Me-DAB through 45 days.

Our observations concerning effects of 3'-Me-DAB on nuclear RNA content were not helpful in resolving conflicts among previous reports. For instance, Reid (29), in summarizing the data of half a decade ago, cited reports describing both slight decreases and marked increases. Subsequently, Hirota (18) reported that the nuclear RNA content was unchanged by feeding 3'-Me-DAB, and recently, Sporn and Dingman (32) reported losses. O'Sullivan and Kirby (27) reported that the content of liver RNA was unchanged by feeding 4'-F-DAB; our data on levels of nuclear RNA supported a similar conclusion.

**Incorporation of Labeled Adenine.** At this juncture, there seemed to be no support for the idea that carcinogens exerted unique influences upon the RNA metabolism of rat liver. Effects seen when 3'-Me-DAB was injected were unique indeed compared to those caused by 4'-F-DAB injections. But, when fed, losses seen with 3'-Me-DAB were not sustained, 4'-F-DAB still was ineffective, and thioacetamide showed marked increases in incorporation of labeled orotic acid and in RNA content. From these observations, it was clearly possible that deposition of labeled orotic acid into nuclear RNA was influenced by factors other than those associated with RNA synthesis. To gain further insight, the incorporation of adenine-8-14C was studied. Griffin et al. (14) previously studied incorporation of adenine-8-14C into nuclear RNA of rats after feeding 3'-Me-DAB for about 8 weeks and their data suggested increased incorporation.

The incorporation of adenine-8-14C into the 2 nuclear RNA subfractions, 5 days after a single injection of 3'-Me-DAB, 4'-F-DAB, or 4'-Me-DAB and after 5 once-daily injections of thioacetamide, is shown in Chart 3. In the NNN RNA fraction, 4'-Me-DAB and thioacetamide injections increased the incorporation of labeled adenine, while in the nucleolar RNA fraction, only thioacetamide injections caused increases. When the azo dyes or thioacetamide were fed throughout 6 weeks, incorporation of labeled adenine occurred as depicted.
Chart 2. Comparison of orotic acid-6-14C incorporation and RNA concentration in NNN RNA and nucleolar RNA subfractions after 2, 4, or 6 weeks of feeding azo dyes or thioacetamide. Azo dyes, 3'-Me-DAB (3M), 4'-F-DAB (4F), and 4'-Me-DAB (4M), were added to a semisynthetic diet (9) to give a concentration of 0.06%. Thioacetamide (TA) was added to this diet to give a concentration of 0.066%. Controls received the basal diet (B) alone. After 2, 4, or 6 weeks of feeding, orotic acid-6-14C (3.3 μC/rat) was injected intraperitoneally and allowed to incorporate 30 min. All other experimental details were as described in Chart 1. Points represent averages of values from 6-12 animals. Asterisks (*) denote values different from corresponding basal control values at a probability level of 0.10% or greater.
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Adenine-8-14C Incorporation (dpm/mg DNA)

Chart 3. Incorporation of adenine-8-14C into the RNA of nuclear subfractions after injecting azo dyes or thioacetamide. Azo dyes were injected as described in Chart 1. Thioacetamide (50 mg/kg body weight) was injected intraabdominally once daily for 5 days. Control animals received the saline vehicle alone. After 5 days or 24 hr after the 5th thioacetamide injection, adenine-8-14C (5 /¿e/rat in 2 ml of 0.9% saline) was injected intraabdominally and allowed to incorporate for 30 min. All other experimental procedures were as described in Chart 1. Bars represent averages ± S.E. of values from 7 or 8 animals. Shaded bars indicate values which differed from appropriate control values at a probability level of 0.10% or greater.

Adenine-8-14C Incorporation ( dpm/mg DNA )

Chart 4. Incorporation of adenine-8-14C into the RNA of nuclear subfractions after feeding azo dyes or thioacetamide for 2, 4, or 6 weeks. Compounds were fed exactly as described in Chart 2. At the end of each feeding interval, adenine-8-14C (5 /¿e/rat in 2 ml of 0.9% saline) was injected intraabdominally and allowed to incorporate for 30 min. All other experimental details were as described in Chart 1. Points represent averages of values from 7-12 animals. Asterisks (*) indicate values different from basal control values at a probability level of 0.10% or better.

Comparison of Effects of the 3 Carcinogens on Nuclear RNA. A graphical comparison of the effects of the 3 carcinogens on incorporation of orotic acid-6-14C or adenine-8-14C into NNN RNA is shown in Chart 5. Values depicted for fed groups in Chart 4. As was observed in experiments with labeled orotic acid, incorporation of adenine-8-14C into NNN RNA of rats fed the basal diet decreased throughout the 6-week period; however, decreases were not observed in the nucleolar fraction. The only carcinogen to exert pronounced effects upon incorporation of adenine-8-14C was thioacetamide, which increased incorporation particularly in the nucleolar RNA subfraction.

Among data recorded following either injection or ingestion of 3'-Me-DAB, there was no evidence that this azo dye inhibited incorporation of adenine-8-14C. Since fed 3'-Me-DAB exerted no appreciable influence, inhibition in the deposition of labeled orotic acid into nuclear RNA was uniquely associated with injected 3'-Me-DAB. Reasons for this inhibition must remain speculative; however, the interconversion of orotic acid to uridine-5'-phosphate would seem to be a likely locus. Reid (30) reported that, although the activities of these liver enzymes were enhanced during the first 5 to 45 days of 3'-Me-DAB feeding, activities declined below control levels after 80 days. Thus, one might rationalize the differences in responses to fed or injected 3'-Me-DAB in the present study on the basis of differences in the concentration and mode of administered 3'-Me-DAB.

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Among fed animals, those receiving 4'-F-DAB also incorporated greater amounts of labeled adenine.

Effects of the 3 carcinogens on incorporation of orotic acid-6-14C and adenine-8-14C into nucleolar RNA are compared in Chart 6. When orotic acid was the labeled precursor, injected 3'-Me-DAB inhibited incorporation, but ingested 3'-Me-DAB was not inhibitory. In contradistinction, fed thioacetamide stimulated incorporation of labeled orotic acid whereas injected thioacetamide exerted no measurable effect. When adenine was the labeled precursor, among the 3 carcinogens only thioacetamide was effective. Whether fed or injected, incorporation of adenine-8-14C was increased by thioacetamide.

Effects of the 3 carcinogens on the concentration of RNA in the nuclear subfraction are compared in Chart 7. Among these, only 3'-Me-DAB significantly altered the concentration of NNN RNA, and then only when injected. On the other hand, the RNA content of the nucleolar fraction was appreciably affected by these carcinogens. When administered by injection, 3'-Me-DAB decreased while injected thioacetamide increased the amount of nucleolar RNA; 4'-F-DAB was without effect. When the carcinogens were fed, all three caused increases in nucleolar RNA; the greatest increase was seen in the groups fed thioacetamide. Here, observations with 3'-Me-DAB and 4'-F-DAB possibly were conservative due to increases in DNA content (18, 27), but it should be remembered that, with both of these carcinogens, the nucleolar RNA content appeared to be conserved while significant losses occurred among control animals (Chart 2).

Data graphically depicted in Charts 5, 6, and 7 facilitated direct comparison between the effects of 3 hepatocarcinogens on two aspects of nuclear RNA metabolism. Similarities between effects exerted by these hepatocarcinogens were absent...
in all cases except one; all three carcinogens, when fed, caused increases in nucleolar RNA. But for this exception, the data were compatible with a concept of carcinogenesis voiced by Gelboin and Bates (11) who stated that "... the multiplicity and heterogeneity of causal agents clearly indicate that initial cellular sites of action of the carcinogen in each case may be quite different."

The methodology used by Sporn and Dingman (32) to isolate chromatin RNA (8) was clearly different from that used by Kono (24) and by us, and probably differed from that used by Rabbi et al. (28). Therefore, the possibility cannot be eliminated that our failure to observe losses of nuclear RNA correlated with carcinogenicity was associated with experimental procedure. On the other hand, it is possible that their correlation resulted from a fortuitous selection of carcinogens. Two isolated observations lend particular support to this possibility: (a) Rabbi et al. (28) fed AAF and observed losses of RNA but, under the same conditions, they observed increases in RNA upon feeding DAB, and (b) in our work reported here, injected 3'-Me-DAB caused significant losses of nuclear RNA but injected 4'-F-DAB was ineffectual, whereas injected thioacetamide caused profound increases in nuclear RNA.

Reid (29) defined criteria for recognizing biochemical lesions associated with neoplasia from among those irrelevant. The work described here represents application of one criterion, viz., criterion 2 dealing with effects of diverse hepatocarcinogens and noncarcinogens. Clearly, no correlation existed either in patterns of RNA synthesis, as adjudged by incorporation of 2 labeled precursors or by changes in RNA content. The closest approach to correlation was the observation that the 2 azo dye carcinogens and thioacetamide caused increases in nucleolar RNA when fed. But, the weak carcinogen 4'-Me-DAB also caused similar increases (Chart 2) and Kono (24), using methodology identical to ours, reported losses of nucleolar RNA during DAB feeding. It would appear, therefore, that changes in these two aspects of nuclear RNA metabolism constitute irrelevant steps toward neoplasia.

REFERENCES


Effects of Hepatocarcinogens on the Synthesis and Content of Rat Liver Nuclear RNA

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