Biochemical and Pathological Effects of Methylazoxymethanol Acetate, a Potent Carcinogen

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SUMMARY

Methylazoxymethanol acetate is known to induce tumors in rodents. Effects produced by a single nonlethal dose on nucleic acid and protein synthesis have been investigated in rat and mouse liver, small intestine, and kidney, the 3 organs most susceptible to carcinogenesis. The agent induced early inhibition of thymidine incorporation into DNA of rat tissues. Rat liver was most sensitive to this effect and was the only organ to show marked inhibition of RNA and protein synthesis. In mice, liver was more sensitive than small intestine or kidney and exhibited an inhibition of RNA and protein synthesis; there were no changes observed in small intestine or kidney. Pathological effects of methylazoxymethanol acetate have been studied by light and electron microscopy. In rats, 6 hr after treatment there was minimal focal necrosis in liver; duodenum and colon, however, showed marked karyorrhexis in crypt epithelium. Intestinal recovery was evident by 1 week, whereas hepatic nuclear changes, consisting of enlarged and irregular nuclei, were detectable months later. Microsegregation in rat hepatic cell nuclei is evident within 1 hr after treatment, prior to inhibition of nuclear RNA synthesis; the nuclear change persisted for several months. Mouse liver showed necrosis while duodenal changes were minimal. Kidneys of both species appeared normal at all times. The relationships between inhibition of the synthesis of the various macromolecules, the pathological effects, and carcinogenesis are discussed.

INTRODUCTION

In 1963 Laqueur et al. (16) reported that rats fed flour prepared from the nuts of the plant Cycas circinalis developed benign and malignant tumors predominantly of the liver and kidneys. Nishida et al. (20) and Riggs (27) had previously isolated from cycads a glucoside termed cycasin and had reported that the aglycone moiety methylazoxymethanol is linked to glucose in the β-D configuration. Experiments in which cycasin was given by various routes to normal and to germ-free animals revealed that the agent is cleaved by the intestinal flora which possess a β-D-glucosidase, and that the free aglycone methylazoxymethanol is the active carcinogen (13-15, 34). Matsumoto et al. (19) synthesized MAM acetate (Chart 1), which is stable and not dependent upon β-glucosidase cleavage for activity. Single or only a few doses of cycasin, or of the aglycone methylazoxymethanol, are required to induce tumors in rats which are primarily of the kidney, intestinal tract, and liver (11, 15). Aqueous extracts of cycad nut have also been shown to induce tumors of liver and kidneys in mice (21). The present investigation was undertaken to study the acute pathological and biochemical effects produced by MAM acetate in the kidney, small intestine, and liver of mice and rats. It was hoped that the information derived from this study might help to elucidate the initial action of the carcinogen which is requisite for tumor formation.

MATERIALS AND METHODS

MAM acetate was purchased from Mann Research Laboratories, Inc., New York, N. Y. Thymidine-2-14C (53.8 mCi/mmmole), uridine-6-3H (9.34 Ci/mmmole), l-leucine-1-14C (25.6 mCi/mmmole), cytidine-5-3H (26.2 Ci/mmmole), and orotic acid-6-14C hydrate (4.6 mCi/mmmole) were obtained from New England Nuclear Corporation, Boston, Mass.; uridine-6-3H (10.4 Ci/mmmole) was from Tracerlab, Waltham, Mass.; and o-phosphoric acid-32P (1 mCi/ml) was from E. R. Squibb and Sons, New York, N. Y. Immature weanling (50- to 70-g) and young adult (150-g) male Sprague-Dawley rats (CD line) and adult male CD1 mice (25 g) were obtained from Charles River Breeding Laboratories, Brookline, Mass., and given food and water ad libitum.

All solutions for injection were made with 0.9% NaCl solution and administered i.v. either by tail vein or femoral vein, in a volume of 10 ml/kg. Solutions of MAM acetate were prepared immediately before use. Animals given injections of NaCl solution served as controls in all experiments. The animals were killed by either cervical dislocation (mice) or exsanguination from the abdominal aorta while under ether anesthesia (rats).

Precursor Incorporation Studies

The radiolabeled precursors used to measure the synthesis of nucleic acids and protein were injected at vari-

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to a final concentration of 10%. The acid-insoluble
homogenized in iced water with a VirTis homogenizer,
ice-cold water, where they were trimmed, and then
precipitate of each precursor was linear during the pulse
interval was allowed for precursor incorporation (the in
acetate (see the tables and charts for details). After an
ous intervals of time after the administration of MAM
procedure is similar to the method of Schneider (30).
containing degraded nucleic acids, were combined for
anol: ether (3:1). The pellet was heated twice at 90° for
fatted once with absolute ethanol and 3 times with eth-
 pellet was washed several times with 5% TCA and de
and a measured portion of homogenate was added to
TCA, to a final concentration of 10%. The acid-insoluble

Methylazoxymethanol acetate

Chart 1. The structural formula of methylazoxymethanol acetate.

cally with diphenylamine and orcinol as reagents, respect-
ively (30). Deoxyadenosine and adenosine were used as
standards for DNA and RNA determinations, respecti-
ately. Protein content was determined according to
the method of Lowry et al. (17), with bovine serum albumin
as the standard. The results are expressed as dpm/μmole
deoxyribose, dpm/μmole ribose, or dpm/mg protein
for incorporation of thymidine-2-14C or orthophosphoric
acid-32P into DNA, uridine-6-1H, orotic acid-6-14C hy-
drate, or cytidine-5-1H into RNA and leucine-1-14C into
protein, respectively. Since only the purine ribose or de-
oxo-ribose moieties give colorimetric reactions, the results
have been adjusted to include the pyrimidine bases.

Pathology Studies

Light Microscopy. Weanling and young adult rats were
given a single injection of MAM acetate in a dose of 35 or
50 mg/kg, respectively. Approximately equal numbers of
each age group were killed at varying intervals; a total of
4 at 1 hr, 10 at 6 hr, 8 at 24 hr, 6 at 1 week, 6 at 1 month,
4 at 2 months, and 4 at 6 to 7 months. Tissues were im-
mediately fixed in Bouin's solution and paraffin sections
were stained with hematoxylin and eosin. In the older
rats given the higher dose all thoracic and abdominal
viscera, salivary glands, sternum, thyroid, testes, and
skeletal muscle were examined microscopically, while in
the weanling rats only liver, duodenum, colon, kidney,

Samples of acid-containing fractions were added to
liquid scintillator which consisted of 0.52 g POPOP, 26 g
PPO, 416 g naphthalene, 1.2 liters methanol, 2 liters
toluene, and 2 liters dioxane (10). Samples of alkali-
digested protein were added to liquid scintillator which
consisted of 0.1 g POPOP, 8 g PPO, 0.6 liter absolute
ethanol, and 1.4 liters toluene. The radioactivity was de-
termined in a Packard Tri-Carb liquid scintillation spec-
trometer.

The DNA and RNA content of the respective fractions
obtained as described above was determined colorimetri-

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and testes were examined. Fifteen control rats were each given 1 injection of NaCl solution, and 1 or more of these were killed at each of the above indicated time intervals.

**Electron Microscopy.** The ultrastructural studies reported below were concerned primarily with nuclear changes in the hepatocytes of rats within the first 24 hr after treatment with MAM acetate, although a few preliminary observations were made at 1 and 2 months after injection. As in the light microscopic studies both weanling and young adult male rats received a single injection of either 35 or 50 mg/kg, respectively. No differences were observed with regard to age at time of injection or to dose. Eight rats were examined at 6 hr and 6 rats at 24 hr after treatment. In addition, 4 rats were killed within the 1st hr; 1 each at 15 and 30 min and 2 rats at 1 hr. Controls given injections of NaCl solution were also examined: 1 at 30 min, 2 at 6 hr, 4 at 24 hr, and 1 each at 1 and 2 months. Sections of the left lateral lobe of liver were fixed by immersion at room temperature for 1 hr in a mixture of 6% glutaraldehyde and 2% acrolein (28) and postfixed for 1 hr in 2% osmium tetroxide (22) with added sucrose (3). Both solutions were buffered at pH 7.2 with Veronal acetate. Dehydration was carried out through a graded series of alcohols and propylene oxide. Specimens were embedded in Maraglas-Dow epoxy resin 732 (5). Sections were stained with methanolic uranyl acetate (35) and with lead citrate (25) and were examined in a Siemens Elmiskop I electron microscope.

**RESULTS**

**Toxicity**

There was a narrow range between the dose of MAM acetate when given i.v., which was lethal within 48 hr, and that which permitted normal survival. In a dose of 35 mg/kg in the mouse was lethal (5/5), 25 mg/kg produced no deaths (0/9). Similar narrow ranges were observed with rats. The following doses, which produced no deaths, were used for both biochemical and pathological studies: adult rat, 50 mg/kg; weanling rat, 35 mg/kg; adult mouse, 25 mg/kg.

**Precursor Incorporation Studies**

In weanling rat liver (Chart 2), thymidine-2-14C incorporation into DNA was inhibited 35% 1 hr after treatment, and 65% from the 3rd to the 24th hr. The ability to synthesize DNA recovered thereafter. RNA synthesis, however, measured by determining the incorporation of orotic acid-6-14C hydrate into nuclear RNA (Chart 3), was not markedly affected until 3 hr after MAM acetate was given and was completely recovered by 24 hr (orotic acid was used as a precursor of RNA synthesis in liver and kidney because of the poor utilization of uridine for RNA synthesis by these organs). The inhibition of leucine-1-14C incorporation into protein, about 60% (Chart 2), appeared to follow more closely the inhibition of RNA synthesis in that it too began to recover 24 hr after treatment.

The effects of MAM acetate upon nucleic acid and protein synthesis in small intestine and kidney were not as marked as that in liver. Thymidine incorporation in weanling rat small intestine (Chart 4) and kidney (Chart 5) was inhibited about 25 and 40%, respectively, although the inhibitions appeared to last longer than that in liver. Inhibition of RNA synthesis in small intestine (Chart 4), approximately 30%, was detectable with uridine-6-3H while RNA synthesis in kidney nuclei using orotic acid-6-14C hydrate was not affected even as late as 24 hr after MAM acetate (Chart 3). Protein synthesis was not affected in either small intestine (Chart 4) or kidney (Chart 5).

To ascertain whether the inhibition of thymidine-2-14C incorporation into DNA was representative of de novo synthesis inhibition and not an artifact of thymidine metabolism, orthophosphoric acid-32P incorporation into DNA of weanling rat liver nuclei was determined. The data in Table 1 indicate that DNA synthesis in liver was inhibited about 80% 1 to 3 hr after MAM acetate, a finding in agreement with the data obtained using thymidine. The lack of inhibition of RNA synthesis in kidney nuclei as determined by orotic acid incorporation may be due to the poorer utilization of this precursor by this tissue as compared to liver (Chart 3). Therefore, nuclear RNA synthesis in kidney was measured with cytidine-5-3H. The data indicated that, as observed with orotic acid, there was no inhibition of RNA synthesis 5 hr after MAM acetate, at a time when inhibition of DNA synthe-
sis was maximal. Cytidine incorporation into liver nuclear RNA was inhibited, however, a result in agreement with that obtained with orotic acid (Table 1).

The incorporation of labeled precursors into their respective macromolecules was also determined in adult rats. The data show (Table 2) that MAM acetate exerts a similar effect in adult rats as in weanling rats. To ascertain whether the adrenal gland played a role in the effects produced by MAM acetate, thymidine-2-'H incorporation into DNA was measured in adrenalectomized female weanling rats. (Animals were allowed NaCl solution and food ad libitum for 3 days prior to the experiment.) Six hr after MAM acetate, 50 mg/kg, thymidine incorporation into DNA of liver, small intestine, and kidney was inhibited 87, 45, and 66% respectively, results which are similar to those obtained in intact animals.

The effects of 25 mg/kg MAM acetate upon the synthesis of nucleic acids and protein in mouse tissues were also investigated. There appears to be a slight inhibition of thymidine-2-'H incorporation into mouse liver DNA 24 hr after MAM acetate, whereas the effects upon uridine-6-'H and leucine-1-'H incorporation appear earlier and have returned to normal at that time (Chart 6). Orotic acid-6-'H hydrate incorporation into mouse liver nuclei was also determined. There was 52 and 18% inhibition at 2 and 5 hr, respectively. The synthesis of nucleic acids and protein in the small intestine and kidney of the mouse was not affected as late as 24 hr after the administration of MAM acetate.

Chart 4. The effect of MAM acetate, 35 mg/kg, upon DNA, RNA, and protein synthesis in the weanling rat small intestine. At various times after treatment with MAM acetate, thymidine-2-'H (TdR-2-'H) (21 μCi/5 μmoles/kg), uridine-6-'H (UR-6-'H) (500 μCi/25 μmoles/kg), or leucine-1-'H (25 μCi/50 μmoles/kg) was injected for 15-, 10-, or 10-min pulses, respectively. The specific activities were determined as described under "Materials and Methods." See Chart 2 for other details.

Chart 5. The effect of MAM acetate, 35 mg/kg, upon DNA and protein synthesis in weanling rat kidney. See Chart 2 for details.

Pathology Studies

Light Microscopy. Since the pathological effects observed were similar in the weanling and young adult rats, both groups are described together. No abnormalities were present in rats 1 hr after receiving MAM acetate. At 6 hr there was decreased cytoplasmic basophilia of the hepatocytes in all rats. Abnormal nuclei were observed in scattered hepatocytes in 9 of 10 rats. The abnormal nuclei contained irregular clear areas with margined chromatin (Figs. 1 and 2). Only a minority of cells were involved. In addition, karyorrhectic material was present in sinusoids and Kupffer cells. The intestinal crypts in duodenum and colon contained numerous karyorrhectic cells (Fig. 6); the change was more marked in the duodenum. All other tissues were normal.

At 24 hr all rats showed liver changes similar to those described at 6 hr. In addition, the liver of 2 of the 8 rats showed areas of congestion and hemorrhage; 1 of these contained focal collections of lymphocytes and polymorphonuclear leukocytes. The intestinal crypts in duodenum and colon contained numerous karyorrhectic cells (Fig. 6): the change was more marked in the duodenum. All other tissues were normal.

Seven days after treatment the hepatic cells showed greater variation in size of nuclei and density of chromatin pattern than did controls (Fig. 3). Many hepatic cells had enlarged clear areas in the cytoplasm principally in the perinuclear region (found to be glycogen by electron microscopy). In 3 of the 6 rats many hepatic cells showed significant irregularities in size and shape with the result that the normal sinusoidal pattern was distorted. The duodenum of 2 of the rats showed increased numbers of inflammatory cells (mainly lymphocytes and a few polymorphonuclear leukocytes) in the lamina propria and ectasia of crypts. Similar changes were present in the colon of 4 rats. In 2 animals there was a decrease in hematopoietic elements in the red pulp of the
spleen and a slight loss of corticomedullary demarcation in the thymus.

At 1 month, all treated rats had nuclear and cytoplasmic alterations in the liver like those noted at 7 days. There was, however, greater variation in nuclear size and an increase in the number of enlarged nuclei. Cells with increased glycogen deposits occurred in clusters. In 1 rat some of the hepatic cells were also enlarged. Two rats had testicular damage consisting of a few enlarged and multinucleated germinal epithelial cells in some seminiferous tubules. The livers of the 8 rats killed at 2, 6, or 7 months had changes in hepatocytes like those described above at 1 month, but with variations in severity that appeared in this small series to be independent of time. For example, the most abnormal hepatocytes were observed in 2 rats at 2 months (Fig. 4). At 6 and 7 months after receiving MAM acetate, 1 rat had hyperplastic nodules of the liver and an infiltrating adenocarcinoma of the duodenum, another had an in situ adenocarcinoma of the duodenum, and a 3rd had hyperplastic nodules of the liver (Fig. 5).

The kidney did not show any pathological changes or preneoplastic alterations in any of the 42 rats examined.

Table 1
The effect of MAM acetate upon the synthesis of DNA and RNA in the weanling rat liver

<table>
<thead>
<tr>
<th>Time after MAM (hr)</th>
<th>Pulse time (hr)</th>
<th>Control</th>
<th>Treated</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthophosphoric acid-32P incorporation into DNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1</td>
<td>1047</td>
<td>151</td>
<td>37</td>
</tr>
<tr>
<td>1.0</td>
<td>2</td>
<td>1054</td>
<td>130</td>
<td>12</td>
</tr>
<tr>
<td>1.0</td>
<td>2</td>
<td>1040</td>
<td>256</td>
<td>25</td>
</tr>
<tr>
<td>Cytidine-5'-H incorporation into RNA (specific activity X 10^3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
<td>142</td>
<td>45</td>
<td>32</td>
</tr>
</tbody>
</table>

In a limited study with mice, selected tissues were studied at 6 and 24 hr following a single administration of 25 mg/kg MAM acetate. In mice killed at 6 hr, diminished cytoplasmic basophilia was present in central zone hepatocytes; in addition, a few necrotic hepatocytes were seen immediately adjacent to central veins in nearly all central zones. In the duodenum, a few karyorrhectic crypt cells were present in a minority of crypts. The kidneys were normal. Of 4 mice killed at 24 hr, 2 had central necrosis in liver, involving one-fourth to one-half of each lobule. In the other 2 mice, only a few necrotic cells were present around central veins in all lobules. In the duodenum of the 24-hr mice a few karyorrhectic cells were present in the majority of crypts. The kidneys were normal.

Electron Microscopy. While this study was in progress, Ganote and Rosenthal (7) described hepatic changes in young male Osborne-Mendel rats that had received 60 to 110 mg/kg MAM acetate i.p. and had been killed at 2, 6, 12, and 24 hr. Our studies, confirming and extending theirs, are summarized below.

As early as 15 min, and persisting until 24 hr, most hepatocyte nuclei contained nucleolar plaques. These were round discrete, electron-dense bodies composed of fibrillar or granular material (Figs. 7, 8, and 9). Similar structures have been described as "spotted" nucleoli in tumor cells (2) and as nucleolar plaques (26) or microspherules (37) following administration of actinomycin D. At 15 and 30 min in the present studies, the presence of plaques was the only nucleolar change. At 1 hr there was, in addition, an irregular separation of nucleolar components with loss of skein-like arrangement (Fig. 8). At 6 hr, segregation of nucleolar components appeared (Fig. 9). Some nucleoli at this time had a reticulated pattern composed of fibrillar and granular components (Fig. 10). At 24 hr some nucleoli consisted of a central granular mass with peripheral extensions of fibrillar material (Fig. 11). At 1 hr and thereafter, the nucleolar alterations were present in less than one-half of the hepatocytes.

Occasional necrotic cells were present at 6 and 24 hr (see "Results, Light Microscopy"). These were characterized by finely granular homogenous nucleoplasm with a few varied electron-dense structures, not other-
deposits in cytoplasm in some cells. In the 2 rats killed at 2 months hepatocytes were noted which contained a fibrillar mass formed a cap around the periphery (Fig. 2). Whorls of smooth endoplasmic reticulum were noted in hepatocytes 24 hr after treatment, confirmed the findings noted above (Fig. 2). Whorls of smooth endoplasmic reticulum were noted in hepatocytes 24 hr after treatment; these changes were similar to those described by Ganote and Rosenthal (7).

Preliminary observations of 4 rats, 2 killed at 1 and 2 at 2 months after treatment, confirmed the findings noted by light microscopy, i.e., nuclear enlargement, irregular chromatin patterns, and increased amounts of glycogen deposits in cytoplasm in some cells. In the 2 rats killed at 2 months hepatocytes were noted which contained structures considered to be abnormal nucleoli. Some of the nuclei containing these bodies also had in addition a normal or an enlarged nucleolus. The ring-like abnormal structures were composed of granular material containing vacuoles (Figs. 13, 14, and 15). In one instance a fibrillar mass formed a cap around the periphery (Fig. 13).

DISCUSSION

In the present studies, inhibition of DNA synthesis (as measured by changes in thymidine and orthophosphate incorporation) was observed in rat liver, small intestine, and kidney within the first few hr following the administration of MAM acetate. The ability of MAM acetate to induce tumors in rats has been reported by others (14, 15), and we have observed the development of adenocarcinomas of the rat duodenum and hyperplastic nodules of the rat liver 6 to 7 months after a single administration of this agent. In mice, however, we found no inhibition of DNA synthesis, and there have been no reports of tumors in this species following MAM acetate. In our laboratory, we have not observed tumors in mice as late as 1 year after a single i.v. administration of the agent. The results suggest, therefore, that the initial actions of MAM acetate which result in early inhibition of DNA synthesis may also be responsible for nuclear changes in rat liver which persisted for several months after treatment, and for the subsequent development of tumors. Whether the cells showing late nuclear changes are those which were originally affected or cells in which the response was delayed is difficult to say at this time.

DNA synthesis in rat kidney was less sensitive to the inhibitory effects of MAM acetate than was that of liver and did not show any pathological effects throughout the course of this study. Tumors which have been observed in rat kidney (15) were the result of multiple injections of MAM acetate.

The inhibition of DNA and RNA synthesis in rat liver may be the result of a change in DNA template activity because of methylation of the guanine moieties in DNA by MAM acetate (18, 33) with the subsequent loss of inhibitory effects of MAM acetate than was that of liver and did not show any pathological effects throughout the course of this study. Tumors which have been observed in rat kidney (15) were the result of multiple injections of MAM acetate.

The lesser effects of MAM acetate upon the synthesis of DNA in rat small intestine and kidney as compared to liver and the finding that similar degrees of inhibition of the synthesis of DNA in the 2 former tissues are associated with different effects upon RNA synthesis may be a reflection of less uptake of the carcinogen by these tissues, lower amounts of methylation of the nucleic acids, or different sensitivities of the polymerases to the methylated DNA (18, 33). It has been observed that RNA of liver is methylated to a greater extent after cycasin than is that of kidney or ileum (33) and the same may be true for DNA. The different rates of proliferation between the small intestine and kidney may also be a factor. Although the inhibition of thymidine incorporation is less pronounced in small intestine and kidney than that produced in liver, it is of a longer duration.

The liver, which is less proliferative than small intestine, was more sensitive to inhibition of DNA synthesis by MAM acetate. We have explored this phenomenon somewhat further and have found that 3 hr after MAM acetate, at a time when liver DNA synthesis was inhibited 70%, the inhibition of DNA synthesis in thymus, spleen, and testis was 50%. These last-mentioned tissues showed no pathological effects.

In both small intestine and kidney, however, in contrast to liver, no effect upon leucine incorporation into protein could be observed. Shank and Magee (33) have reported that cycasin inhibits leucine incorporation into protein by MAM acetate; these changes were similar to those described by Ganote and Rosenthal (7).
protein of liver but not of kidney or ileum, data which are similar to those obtained in these experiments with MAM acetate. Since the inhibition by cycasin began 5 hr after administration, they postulated that metabolism of cycasin to MAM was responsible for the delay in onset. The earlier effect by MAM acetate on leucine incorporation into liver protein in these experiments is consistent with their hypothesis.

The inhibition of protein synthesis in rat liver may be due to methylation of RNA (18, 33), resulting in a template which is less efficient to direct the synthesis of protein or because of disaggregation of polysomes and depletion of rough endoplasmic reticulum which have been observed ultrastructurally by Ganote and Rosenthal (7) and in our laboratory as well. Furthermore, polysome dissociation into monomer and dimer units has been reported following the administration of MAM and cycasin (32). The administration of aqueous extracts of Macrozamia communis, a plant which contains the same aglycone as present in cycasin, produces an inhibition of leucine incorporation in vitro in rat liver microsomal preparations (9). The addition of synthetic polyribonucleotide to these microsomal preparations produced a stimulation of amino acid incorporation into acid-insoluble material (9). This suggests that fewer polysome units were present in treated animals and allowed for greater binding of synthetic template in vitro.

The RNA:DNA ratio of weanling rat liver nuclei decreases 35% 5 hr after MAM acetate, at a time when orotate incorporation into RNA appears to be maximally inhibited. The ratio of RNA:DNA in kidney nuclei is not decreased. The lowered ratio in liver reflects either less RNA per nucleus, more DNA per nucleus, or a combination of both. Hoch-Ligeti et al. (12) have found similar data in rats 4 hr after feeding 1 g fresh cycad husk (equivalent to 2 mg cycasin); in agreement, we have found the lowered ratio to be due to less total RNA per nucleus.

Alterations in nucleolar structure have been observed with other hepatocarcinogens. Aflatoxin, 3'-methylidemethylaminoazobenzene, lasiocarpine, and tannic acid, which produce macrosegregation (24, 36); and dimethylaminoazobenzene, which produces microsegregation (36) of nucleolar components, also inhibit RNA synthesis (6, 24, 39). The effects on RNA synthesis were studied, however, at a time when nucleolar changes were already present and the temporal relationship between inhibition of synthesis and nucleolar segregation was not defined.

Actinomycin D, which is not a hepatocarcinogen, inhibits RNA synthesis and simultaneously induces changes in nucleolar structure such as macrosegregation [nucleolar capping (26)] in all the hepatocytes (8). The nucleolar effects observed with MAM acetate occurred earlier than the decrease in RNA synthesis and persisted for longer than 24 hr, by which time the synthesis of RNA had already recovered. In view of these findings, it would appear that the phenomena of RNA synthesis inhibition and nucleolar segregation are independent.

The fact that the hepatocarcinogens discussed above and MAM acetate, as well as noncarcinogenic actinomycin D, bind to DNA has suggested that the alteration of nucleolar structure and inhibition of RNA synthesis are related to an effect upon the DNA template (8). Because of the temporal differences in onset and in recovery following MAM acetate, it may be possible to determine which of the 2 phenomena is more sensitive to alteration of the DNA template. Furthermore, the presence of nuclear abnormalities in liver which persist for months after a single injection of MAM acetate may permit the study of the process of carcinogenesis within single cells from the time of administration of the carcinogen to the eventual appearance of neoplasia. Such studies are currently under investigation. It is also of interest that retorsine, a senecio alkaloid, is capable of producing carcinogenic and nuclear effects similar to those produced by MAM acetate after a single injection (1).

Many of the present findings with MAM acetate have also been observed in studies with dimethylnitrosamine, another potent carcinogen which is chemically similar to MAM acetate and has been postulated to react similarly in vivo (33). Other carcinogens which are closely related to MAM acetate because of the presence of nitroso- or azoxy-constituents are streptozotocin (23) and elaiomycin (31), respectively, and each of these are naturally occurring and capable of producing tumors upon single or few administrations. The azoxy- or nitroso-containing agents are of great interest because of their potential hazard to man. Their marked potency, relatively simple chemical structures, and ease of administration make them unique tools for the study of carcinogenesis.

**ADDENDUM**

Since the acceptance of this manuscript, we have found that rats which receive a single i.v. injection of MAM acetate, 35 mg/kg, develop tumors of the colon and kidney 6 to 9 months later. These tumors are in addition to the duodenal tumors and hyperplastic nodules mentioned in the text. The findings are consistent with the suggestion, as discussed above, that the rapidly developing, although transient, effects of MAM acetate which produce inhibition of DNA synthesis in these organs may also be responsible for subsequent tumor formation. In accord with this relationship is the recent finding of Hirono et al. (Cancer Res, 29: 1658-1662, 1969) that cycasin-treated newborn mice develop hepatomas predominantly, but few tumors of the intestine and kidney. In our studies (see text), mouse liver, but not duodenum and kidney, was sensitive to induction of early biochemical and pathological changes by MAM acetate.

**ACKNOWLEDGMENTS**

We wish to thank Dr. Frederick S. Phillips for his advice and cooperation during the course of this study, Miss Maggie Byler for preparation of the samples for electron microscopy, and Mr. John Hlinka and Mr. William Matz for the photomicrographs and electron micrographs, respectively. During the course of this study, Miss Jane Sodergren also was very helpful.
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Figs. 1 to 6. Paraffin sections stained with hematoxylin and eosin.

Figs. 7 to 15. Thin sections from rat liver fixed in glutaraldehyde, postfixed in osmium, embedded in Maraglas, and stained with uranyl acetate followed by lead citrate. All rats received a single i.v. injection of MAM acetate at the indicated dosage.

Fig. 1. Liver of adult rat given an injection of NaCl solution and killed 1 month after injection. Hepatocyte nuclei vary only slightly in size and configuration and have a uniform chromatin distribution. \( \times 340 \).

Fig. 2. Liver of rat killed 6 hr after receiving MAM acetate, 35 mg/kg. Note that several nuclei have relatively clear nucleoplasm and prominent nuclear membranes. \( \times 340 \).

Fig. 3. Liver of rat killed 7 days after MAM acetate, 50 mg/kg. Hepatocyte nuclei vary in size and have irregular chromatin pattern. Enlarged clear areas around nuclei represent glycogen. \( \times 340 \).

Fig. 4. Liver of rat killed 2 months after MAM acetate, 50 mg/kg. This is representative of most marked nuclear abnormalities. \( \times 340 \).

Fig. 5. Liver of rat killed 7 months after MAM acetate, 50 mg/kg. One of several hyperplastic nodules. \( \times 130 \).

Fig. 6. Duodenum of rat killed 6 hr after MAM acetate, 35 mg/kg showing karyorrhexis in the crypts. \( \times 340 \).

Fig. 7. Liver of rat killed 15 min after MAM acetate, 35 mg/kg. Dense nucleolar plaques were present in the nucleolus which otherwise appeared normal. Similar plaques were present at later times (see Figs. 8 and 9). \( \times 31,000 \).

Fig. 8. Liver of rat killed 1 hr after MAM acetate, 50 mg/kg. The nucleolus shows a loss of skein-like arrangement, separation of granular and fibrillar components, and plaques. \( \times 43,000 \).

Fig. 9. Liver from a rat killed 6 hr after MAM acetate, 35 mg/kg. Segregation of nucleolar components is apparent with fibrillar material in the upper part of the nucleolus and granular component below. Nucleolar plaques are also present. \( \times 17,500 \).

Fig. 10. Liver from a rat killed 6 hr after MAM acetate, 50 mg/kg. This nucleolus has a reticulated configuration (see Ref. 7, Figs. 5 and 6). \( \times 5800 \).

Fig. 11. Liver from a rat killed 24 hr after MAM acetate, 35 mg/kg. In this nucleolus, fibrillar material forms peripheral strands around a central granular mass (see Ref. 7, Fig. 7). \( \times 21,000 \).

Fig. 12. Liver from a rat killed 24 hr after MAM acetate, 35 mg/kg. Example of necrotic cell (near center) seen at 6 and 24 hr after treatment (see Fig. 2). Relatively homogenous nucleoplasm with condensed chromatin at nuclear envelope. Lipid and dense bodies are present in cytoplasm. Adjacent cells show little change. \( \times 3500 \).

Fig. 13. Liver from a rat killed 2 months after MAM acetate, 50 mg/kg. One enlarged nucleolus is below. At upper right is an abnormal nucleolar-like accumulation of granular material with vacuole, and semicircular cap of fibrillar material. \( \times 19,000 \).

Fig. 14. Liver from same rat as in Fig. 13. Granular mass with vacuoles. \( \times 6600 \).

Fig. 15. Liver of rat given MAM acetate, 50 mg/kg, and killed 2 months later. Nucleolus at upper left appears normal. At right is an abnormal nucleolar-like mass with vacuolated granular material. \( \times 10,500 \).
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