2-Acetylaminofluorene and \( N \)-Hydroxy-2-acetylaminofluorene
Inhibition of Incorporation of Orotic Acid-5-\(^3\)H into
Nuclear Ribosomal and Heterogeneous RNA in Normal
and Regenerating Liver

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SUMMARY

The effects of 2-acetylaminofluorene and \( N \)-hydroxy-2-acetylaminofluorene (N-OH-AAF) on the incorporation of orotic acid-5-\(^3\)H into nuclear ribosomal RNA and heterogeneous RNA were evaluated in normal and regenerating liver. The \( N \)-hydroxy metabolite was approximately 8 times more potent than the parent carcinogen on the basis of dose-response studies in partially hepatectomized male rats, while 7-hydroxy-2-acetylaminofluorene was without effect on nuclear RNA. Partially hepatomecetomized male and female animals were more sensitive than sham-operated animals to the inhibitory effects produced by 2-acetylaminofluorene and N-OH-AAF; partially hepatectomized female animals were less responsive than male animals. No changes occurred in the specific radioactivity of uridine triphosphate in the liver at 1 to 4 hr after injection of N-OH-AAF, although a 55% elevation in the concentration of uridine triphosphate occurred at this time. Inhibition of nuclear RNA synthesis by N-OH-AAF also resulted in a concomitant reduction in ribosomal RNA and messenger RNA associated with free and membrane-bound polyribosomes. Experimental evidence is presented which suggests that the inhibitory effects of N-OH-AAF on RNA synthesis is the result of inhibition of RNA polymerase activities associated with the synthesis of nucleolar ribosomal RNA and extranucleolar heterogeneous RNA.

INTRODUCTION

One of the primary macromolecular targets in the liver for the carcinogen, AAF,\(^2\) is RNA. Its mode of reaction with RNA is believed to involve arylationation of position 8 of guanine following metabolic activation of AAF to a more reactive electrophilic ester (21, 22). Binding studies in vivo with radioactively labeled AAF and its metabolite, N-OH-AAF, have shown that these carcinogens bind to RNA in rat liver during the early stages of hepatocarcinogenesis (17) and following their acute administration (1, 18, 20, 21, 25). Binding of AAF to rRNA appears to parallel the susceptibility of the liver to the hepatocarcinogenicity of AAF (21).

Several recent studies have reported AAF and N-OH-AAF inhibition of hepatic RNA synthesis in normal rodent liver (3, 39) as well as in regenerating liver (26). Such effects were ascribed to inhibition of 1 or more rRNA polymerase activities (3, 10, 39). The present experiments were designed to test whether or not the inhibitory effects of AAF and N-OH-AAF were confined to a single species of rRNA and to determine whether their action was potentiated in rapidly dividing liver cells in comparison to normal hepatic tissue. The characterization of the effects of these hepatocarcinogens on nuclear rRNA and HnRNA is described in this report.

MATERIALS AND METHODS

Materials. Orotic acid-5-\(^3\)H (12 Ci/m mole) was purchased from New England Nuclear, Boston, Mass. Di-AAF, 7-OH-AAF, and AF were obtained from Aldrich Chemical Co., Milwaukee, Wis. AAF was from Pfaltz and Bauer, Inc., Flushing, N. Y. N-OH-AAF (m.p. 146–148\(^\circ\)) was synthesized from 2-nitrofluorene by the method of Poirier et al. (36). All other chemicals were of reagent grade and filtered, deionized water was used.

Partial Hepatectomy. Male or female Sprague-Dawley rats (Cherokee Laboratories, Atlanta, Ga.) weighing 125 to 150 g were maintained 2/cage, containing corncob bedding, with alternating periods of 12 hr of darkness and 12 hr of light. Partial hepatectomies and sham operations were performed under ether anesthesia between 2 and 4 p.m., according to the method of Higgins and Anderson (14). All animals were fasted for a period of 20 hr and were allowed access to water ad libitum.

Treatment. AAF and its analogs were dissolved in propylene glycol at a concentration calculated to give the desired dose in a volume of 1.0 ml/200 g body weight. Compounds were injected i.p., and control animals received an equivalent volume of propylene glycol. All treatment periods refer to the total exposure to carcinogen before termination of the
experiment at 20 hr after sham operation or partial hepatectomy.

For labeling of nuclear rRNA and HnRNA, orotic acid-5-3H was diluted with 0.9% NaCl solution and allowed to incorporate for 15 min after i.p. injection. Cytoplasmic rRNA and mRNA were labeled similarly using an incorporation period of 1 hr.

Determination of the Specific Activity of UTP. Animals were given injections of orotic acid-5-3H and were killed 15 min later by decapitation. Livers were perfused with 50 ml of ice-cold 0.9% NaCl solution and homogenized in 3 volumes of ice-cold 0.25 N perchloric acid. The perchloric acid homogenates were centrifuged at 500 X g at 4°, and the supernatant fluid was neutralized with 5 N KOH, with phenol red as an internal indicator. The precipitated KC104 was removed by centrifugation after it had been kept on ice for 30 min. The neutralized supernatants were chromatographed on Dowex 1-X8-formate columns by the method of Hurlbert et al. (15) as previously described (5).

Preparation of Nuclei and Polyribosomes. Nuclei were isolated from livers perfused with 50 ml of ice-cold 0.25 M sucrose-5 mM MgCl2, according to the procedure of Higashikagawa et al. (13). Free and membrane-bound polyribosomes were prepared by centrifugation on discontinuous sucrose gradients, as previously described (8). Total polyribosomes were prepared in a similar manner except that the 10,000 X g supernatant fluid was treated with deoxycholate to a final concentration of 1% before being placed on the sucrose gradient.

Preparation of Nuclear and Cytoplasmic RNA. Nuclear rRNA and HnRNA were extracted from isolated nuclei using the differential pH extraction procedure (Method 1) of Edmonds and Caramella (4). Cytoplasmic rRNA and mRNA-enriched fractions were extracted in an analogous manner. Electrophoresis of RNA fractions on 2.4% polyacrylamide gels was carried out according to the method of Loening (24) as previously described (6). Samples were monitored for radioactivity in a Beckman LS-335 liquid scintillation system as described previously (5).

### RESULTS

Effect of AAF and N-OH-AAF on Incorporation of Orotic Acid-5-3H into nRNA. A number of analogs of AAF were tested for their effects in vivo on the incorporation of orotic acid-5-3H into nuclear rRNA and HnRNA from regenerating liver (Table 1). N-OH-AAF was found to be the most potent analog producing a 2.4- to 11-fold greater inhibitory effect on rRNA and HnRNA than the other compounds tested at an equimolar dose. Of particular interest was the relative inactivity of 7-OH-AAF compared to that of the N'-hydroxy derivative. AAF produced an equipotent effect on both rRNA fractions while Di-AAF and AF were marginally effective in inhibiting HnRNA.

To characterize further the parent carcinogen, AAF, and its N'-hydroxy metabolite, dose-response studies were carried out in both sham-operated and partially hepatectomized male rats (Chart 1). While sham-operated animals were not affected by administration of AAF (40 mg/kg) partially hepatectomized rats exposed to this compound for 2 hr showed a dose-related decrease in incorporation of labeled precursor into rRNA and HnRNA. In contrast, a pronounced decrease in labeling of nRNA and in particular rRNA resulted from treatment of sham-operated animals with N-OH-AAF. Administration of N-OH-AAF also caused a greater inhibition of nRNA synthesis in partially hepatectomized animals when compared to the effects in animals similarly treated with AAF; 40% inhibition of nRNA synthesis occurred with AAF (40 mg/kg) versus N-OH-AAF (5 mg/kg).

Experiments assessing the temporal effects of AAF and N-OH-AAF on nRNA synthesis were carried out over a constant 20-hr period following sham operation or partial hepatectomy. In all instances, the time after exposure to carcinogen refers to the time preceding the termination of the experiment at 20 hr after operation. Maximum inhibition of the incorporation of orotic acid-5-3H into nRNA resulted when treatment with either AAF (Chart 2) or N-OH-AAF (Chart 3) was extended to 2 hr. The main distinctions between the parent hepatocarcinogen and its N'-hydroxy metabolite

### Table 1

Effect of AAF analogs on hepatic RNA from partially hepatectomized male rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>rRNA</th>
<th>HnRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/A240</td>
<td>%</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>66,400 ± 6,200</td>
<td>100</td>
</tr>
<tr>
<td>N-OH-AAF</td>
<td>3,800 ± 500</td>
<td>6a</td>
</tr>
<tr>
<td>AAF</td>
<td>40,500 ± 6,700</td>
<td>61b</td>
</tr>
<tr>
<td>Di-AAF</td>
<td>46,400 ± 4,900</td>
<td>70b</td>
</tr>
<tr>
<td>AF</td>
<td>44,800 ± 5,500</td>
<td>67b</td>
</tr>
<tr>
<td>7-OH-AAF</td>
<td>56,400 ± 5,100</td>
<td>85</td>
</tr>
</tbody>
</table>

a Statistically significant difference (p < 0.001) versus propylene glycol-treated controls.

b Statistically significant difference (p < 0.05) versus propylene glycol-treated controls.
Hepatocarcinogens and nRNA

Chart 1. Dose-response of AAF and N-OH-AAF on hepatic nRNA from sham-operated (SHAM) and partially hepatectomized (PH) male rats. Animals were exposed for 2 hr to a single i.p. injection of AAF (1, 10, 20, or 40 mg/kg) or N-OH-AAF (1, 5, 20, or 40 mg/kg) beginning at 18 hr after sham operation or partial hepatectomy. Orotic acid-5-3H (600 µCi/kg) was injected i.p. 15 min before animals were killed at 20 hr. The nRNA fractions were prepared as described in "Materials and Methods." Points, mean ± S.E. of determinations from 4 to 5 animals. Values are expressed as percentages of either sham-operated or partially hepatectomized controls treated with propylene glycol. The values (mean ± S.E.) for control activities (cpm/A260) for 8 sham-operated animals were 30,400 ± 3,300 for rRNA and 4,000 ± 500 for HnRNA. The values (mean ± S.E.) for control activities for 15 partially hepatectomized animals were 70,300 ± 5,500 for rRNA and 9,700 ± 800 for HnRNA.

were in the extent and duration of inhibition during the test period. Sham-operated animals were not affected by AAF while the maximum inhibition achieved with this compound in partially hepatectomized animals was 40% (Chart 2). Furthermore, the inhibition was of a transient nature with the specific activity of nRNA approaching control values after 12 hr. In contrast, treatment with N-OH-AAF produced a pronounced inhibition of nRNA synthesis in both sham-operated and partially hepatectomized rats with the effect being greater in the latter group of animals and remaining essentially unchanged throughout a 12-hr period (Chart 3). Sham-operated animals tended to show a partial recovery after 12 hr.

One obvious point to be answered in studies of this type is whether or not the effect of a carcinogen, particularly when administered i.p., is due to interference with the absorption of radioactive precursor in the peritoneum and hence, the specific radioactivity of the precursor in the liver. Therefore, studies were carried out to determine the concentration and specific radioactivity of UTP, the immediate RNA precursor derived from orotic acid, during varying times after injection of N-OH-AAF (40 mg/kg) into partially hepatectomized animals (Chart 4). It was found that no significant change in the specific radioactivity of UTP resulted at 1 to 4 hr after administration of N-OH-AAF while a pronounced fall in specific radioactivity occurred after 12 hr. The latter effect was found to be due to an impaired uptake of radioactive precursor. Of particular significance was the 55% increase in liver concentration of UTP 1 to 4 hr following treatment of partially hepatectomized animals with the N-hydroxy analog. This observation suggests that the impairment of incorporation

Chart 2. Time course of inhibition by AAF of hepatic nRNA from sham-operated (SHAM) and partially hepatectomized (PH) male rats. Animals received a single i.p. injection of AAF (40 mg/kg) at 1.2, 4, or 12 hr before being killed at 20 hr after sham operation or partial hepatectomy. Orotic acid-5-3H (600 µCi/kg) was injected i.p. 15 min before animals were killed at 20 hr. The nRNA fractions were prepared as described in "Materials and Methods." Points, mean ± S.E. of determinations from 4 to 5 animals. Values are expressed as percentages of either sham-operated or partially hepatectomized controls treated with propylene glycol. The values (mean ± S.E.) for control activities (cpm/A260) for 15 sham-operated animals were 41,400 ± 4,000 for rRNA and 5,500 ± 500 for HnRNA. The values (mean ± S.E.) for control activities for 15 partially hepatectomized animals were 74,300 ± 6,000 for rRNA and 13,000 ± 1,300 for HnRNA.
of orotic acid into nRNA occurs at the level of ribonucleoside triphosphate polymerization.

Effect of AAF and N-OH-AAF on Polyribosomal RNA. In order to determine whether the inhibition of nRNA by the hepatocarcinogens resulted in a preferential effect on cytoplasmic RNA, rRNA- and mRNA-enriched fractions were isolated from free, membrane-bound and total polyribosomes after treatment of partially hepatectomized animals with AAF and N-OH-AAF (Table 2). It can be seen that the reduction in incorporation of orotic acid-5-3H into rRNA and mRNA-enriched fractions of the various polyribosome fractions paralleled the reduction previously observed for nuclear rRNA and HnRNA (Chart 1). No differential effect of N-OH-AAF on free or membrane-bound polyribosomal RNA was noticed.

Effect of AAF and N-OH-AAF on Female Partially Hepatectomized Rats. One characteristic of AAF-induced hepatocarcinogenesis is its sex specificity, with male rats showing the greatest susceptibility (12, 30, 32). Studies of the effect of AAF and N-OH-AAF on nRNA obtained from sham-operated female animals showed that a comparable degree of inhibition could be obtained in comparison to sham-operated male rats but that nRNA obtained from N-OH-AAF-treated partially hepatectomized female animals exhibited a 2- to 3-fold greater specific radioactivity versus controls than did RNA preparations from partially hepatectomized male rats (Table 3).

Effect of Pretreatment with Phenytoin or 3-Methylcholanthrene. The metabolic hydroxylation of AAF to the more potent carcinogen, N-OH-AAF, is believed to occur via the mixed-function oxidase enzymes in the liver (29, 37). Therefore, an assessment was made of the effect of AAF on nRNA following pretreatment of partially hepatectomized rats with phenobarbital or 3-methylcholanthrene. As shown in Table 4, no significant potentiation of AAF inhibition of nuclear rRNA and HnRNA was induced by pretreatment with the 2 inducing agents. In fact, significantly less inhibition occurred after injection of a dose of AAF which normally produced 40% inhibition of both rRNA and HnRNA (Chart 1). Also noted was the lower incorporation of orotic acid-5-3H into RNA in control animals pretreated with either phenobarbital or 3-methylcholanthrene.

Electrophoretic Patterns of nRNA. Disc gel electrophoresis of nuclear rRNA and HnRNA extracted from control and N-OH-AAF-treated animals indicated that the effect of the carcinogen was associated with depression of the synthesis of 45 S rRNA precursor and not merely impairment of the processing of high-molecular-weight precursors to 28 S and 18 S.
Hepatocarcinogens and nRNA

S rRNA (Chart 5A). The electrophoretic pattern of HnRNA showed a particularly pronounced inhibition of incorporation of orotic acid-5-3H into 24 S and 28 S HnRNA (Chart 5B).

DISCUSSION

The mechanism by which the hepatocarcinogens, AAF and N-OH-AAF, produce their biochemical lesions and, subsequently, their carcinogenicity is believed to involve arylamination of DNA or RNA (20, 21) or both. Arylamide adducts of deoxyguanosine can result in transcriptional modification of the template activities of polynucleotides (9) via changes in their conformational properties (23, 33). In at least one instance, this has resulted in interruption in RNA chain elongation followed by reaction of rat liver DNA with N-acetoxy-AAF (40). Of particular relevance to the present study is the high reactivity of polyadenylic acid with N-acetoxy-AAF (23). The regulatory function of polyadenylation of nuclear HnRNA in the processing of mRNA (38) would provide a critical macromolecular target for the arylamide carcinogens which in turn could interrupt the normal flow of genetic information needed for normal phenotypic expression. Inhibition of nuclear rRNA and HnRNA and the subsequent diminution of cytoplasmic rRNA and mRNA-enriched fractions by AAF and N-OH-AAF indicate that it is possible to perturb the normal precursor-product relationships in the nucleus required for processing cellular RNA.

The present report indicates that partially hepatectomized male and female animals were more sensitive than normal animals in their responsiveness to the effects of AAF and N-OH-AAF on RNA synthesis. The only difference discerned in experiments utilizing both sexes of animal was the lesser inhibition of nRNA by N-OH-AAF in partially hepatectomized female rats. Otherwise, the response or lack of response to the carcinogen was similar in both sexes. This observation is in apparent disagreement with that of Zieve (39) who found that nRNA polymerase activities were unaffected after treatment of female rats with N-OH-AAF (30 mg/kg) although male animals responded to the inhibitory effects of this hepatocarcinogen. Since there were differences in the strain of animals used in these experiments, it is possible that differences in the metabolism of N-OH-AAF might account for alterations in the net concentration of inhibitory metabolite present in the target tissue.

The relative refractoriness of normal liver may be the result of 1 or more factors such as (a) the amount of liver mass exposed to carcinogen, (b) differences in metabolism of the carcinogen, and (c) the susceptibility of DNA and RNA during the proliferative phase of tissue regeneration. Although there is no quantitative information regarding tissue concentrations of AAF in normal versus regenerating liver, the temporal, dose-response, and sensitivity differences between RNA and HnRNA of the 2 tissues preclude this possibility as the entire explanation. Marsh and Drabkin (26) found that, although partially hepatectomized rats responded to N-OH-AAF with a comparable degree of inhibition of total RNA, sham-operated animals were not affected. In contrast, incubation of tissue slices of normal and regenerating liver with N-OH-AAF
Table 3

Effect of AAF and N-OH-AAF on hepatic nRNA from sham-operated and partially hepatectomized female rats

Animals were exposed for 2 hr to a single i.p. injection of propylene glycol, AAF (40 mg/kg) or N-OH-AAF (20 mg/kg), beginning at 18 hr after sham operation or partial hepatectomy. Orotic acid-5-3 H (600 µCi/kg) was injected i.p. 15 min before animals were killed at 20 hr. The nRNA fractions were prepared as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Treatment</th>
<th>rRNA cpm/A260</th>
<th>%</th>
<th>HnRNA cpm/A260</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propylene glycol: sham</td>
<td>73,400 ± 12,300b</td>
<td>100</td>
<td>5,100 ± 1,100b</td>
<td>100</td>
</tr>
<tr>
<td>AAF; sham</td>
<td>50,000 ± 5,200</td>
<td>78</td>
<td>4,300 ± 900b</td>
<td>84</td>
</tr>
<tr>
<td>Propylene glycol: PH</td>
<td>118,000 ± 11,700</td>
<td>100</td>
<td>11,800 ± 800b</td>
<td>100</td>
</tr>
<tr>
<td>AAF; PH</td>
<td>74,000 ± 14,000c</td>
<td>63</td>
<td>6,200 ± 600d</td>
<td>53</td>
</tr>
<tr>
<td>Propylene glycol: PH</td>
<td>135,000 ± 9,400</td>
<td>100</td>
<td>14,900 ± 1,400</td>
<td>100</td>
</tr>
<tr>
<td>N-OH-AAF; PH</td>
<td>51,600 ± 8,100d</td>
<td>38</td>
<td>7,000 ± 700d</td>
<td>47</td>
</tr>
</tbody>
</table>

| a| Sham, sham operation; PH, partial hepatectomy. |
| b| Mean ± S.E. of determinations from 4 to 6 animals. |
| c| Statistically significant difference (p < 0.05) versus propylene glycol-treated controls. |
| d| Statistically significant difference (p < 0.001) versus propylene glycol-treated controls. |

Table 4

Effect of pretreatment with phenobarbital or 3-methylcholanthrene on the action of AAF on hepatic nRNA from partially hepatectomized male rats

Animals were pretreated for 3 days with either phenobarbital (100 mg/kg/day) or 3-methylcholanthrene (40 mg/kg/day) diluted in 0.9% NaCl solution or corn oil, respectively. Following pretreatment, animals were exposed for 2 hr to a single i.p. injection of AAF (40 mg/kg) or propylene glycol vehicle beginning at 18 hr after partial hepatectomy. Orotic acid-5-3 H (600 µCi/kg) was injected i.p. 15 min before animals were killed at 20 hr.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>rRNA cpm/A260</th>
<th>%</th>
<th>HnRNA cpm/A260</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital; propylene glycol</td>
<td>22,700 ± 3,800d</td>
<td>100</td>
<td>8,000 ± 400</td>
<td>100</td>
</tr>
<tr>
<td>Phenobarbital; AAF</td>
<td>14,800 ± 2,500</td>
<td>65</td>
<td>6,700 ± 1,400</td>
<td>84</td>
</tr>
<tr>
<td>3-Methylcholanthrene; propylene glycol</td>
<td>32,200 ± 5,500</td>
<td>100</td>
<td>4,800 ± 600</td>
<td>100</td>
</tr>
<tr>
<td>3-Methylcholanthrene; AAF</td>
<td>36,400 ± 4,900</td>
<td>113</td>
<td>3,700 ± 500</td>
<td>77</td>
</tr>
</tbody>
</table>

| a| Mean ± S.E. of determinations from 4 animals. |

produced identical inhibitory responses for RNA synthesis in both tissues (19). In agreement with our studies, equal inhibitory effects by AAF and N-OH-AAF were observed for nuclear and cytoplasmic RNA synthesis in vivo in normal liver (3, 39); however, differential inhibition by N-OH-AAF of nucleolar and nucleoplasmic RNA polymerase activities has also been reported (10). The latter disparity may be a function of the dose of inhibitor as reported by Zieve (40). Nevertheless, our study clearly indicates that both nucleolar and extranucleolar RNA are affected by hepatocarcinogens and that elevation of endogenous levels of UTP in the liver following administration of N-OH-AAF indeed suggests disruption of ribonucleotide polymerization as the locus of the inhibitory effect. Whether the inhibition is due to a direct effect by the carcinogen on the polymerase enzyme as suggested by Zieve (39) or a result of the interaction of N-OH-AAF with nucleolar DNA as proposed by Grunberger et al. (10) remains to be resolved. In vitro experiments with the activated carcinogen, N-acetoxy-AAF, have suggested precedence for both proposals via direct acetylation of enzyme protein (2) or DNA template modification (40). With regard to liver regeneration, the latter alternative appears to be particularly intriguing. It has been shown that the conformational characteristics of single-stranded polynucleotides appear to facilitate reaction with carcinogens in contrast to the lesser reactivity of double-stranded oligomers (23). This suggests that exposure of conformationally altered transcribed regions of chromatin to the proximate carcinogen during the replicative phase of hepatic proliferation might result in a greater susceptibility to modification of biological activity.

Ring- and N-hydroxylation of AAF occurs via the cytochrome P-450 mixed-function oxidase system (29, 37). The pivotal role that hydroxylation of AAF plays in carcinogenesis is exemplified by the reduction in AAF-induced carcinogenicity by treatment of animals with phenobarbital (35) or 3-methylcholanthrene (16, 27, 31). This effect is presumably associated with a reduction in the concentration of reactive metabolites of AAF resulting in their decreased binding to...
Structure-activity experiments have established an order of carcinogenicity for several analogs of AAF. Di-AAF (34), AAF (12, 30, 32), and AF (32) are generally equipotent with the latter compound showing a greater latency for tumor formation (32). N-OH-AAF is considerably more potent than AAF (12, 30) but, in contrast, 7-OH-AAF appears to be noncarcinogenic (11). The present study of these compounds to determine their ability to interfere with the fabrication of nRNA has established an order of potency with N-OH-AAF > AAF > AF = Di-AAF > 7-OH-AAF. These data agree closely with the observations of Marsh and Drabkin (26) for assessing the effects of AF, AAF, and N-OH-AAF on inhibition of incorporation of uridine into total hepatic RNA.

Although it would be highly speculative to attempt to extrapolate these observations to actual carcinogenic events, these experiments and previous studies (5-7) demonstrate that partially hepatectomized animals can serve as a model with which to probe and amplify the interactions of hepatocarcinogens with macromolecules in the target tissue.

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