Pyrrolopyrimidine Nucleotides and Protein Synthesis*

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SUMMARY
The interaction of amino pyrrolopyrimidine nucleotides with enzymes catalyzing different steps in the reaction sequence leading to the incorporation of amino acids into polypeptides has been studied. None of the analogue nucleotides could function as the energy source for activation of amino acids or esterification of transfer RNA (tRNA) by mammalian or bacterial enzymes. Toyocamycin 5'-triphosphate and sangivamycin 5'-triphosphate were competitive inhibitors of the reactions catalyzed by mammalian enzymes. Tubercidin 5'-triphosphate, toyocamycin 5'-triphosphate, and sangivamycin 5'-triphosphate were effective substrates for rabbit liver tRNA with adenosine nucleoside terminus pyrophosphorylase; the corresponding nucleosides were incorporated at the 3' termini of tRNA, and the amino acid acceptor and transfer activities of the analogue-containing polynucleotides were studied. tRNA molecules with tubercidin termini were near normal in these respects, whereas the acceptor activities of tRNA molecules with sangivamycin and toyocamycin termini were greatly diminished. The codon properties of several tubercidin-containing polynucleotides were indistinguishable from those of the corresponding polymers containing adenosine.

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Previous work has shown that protein synthesis, like nucleic acid synthesis, is rapidly inhibited when growing mouse fibroblasts are exposed to the antibiotic tubercidin (1). Tu is incorporated efficiently into both RNA and DNA; however, it appears unlikely that this incorporation is directly responsible for the interruption of protein synthesis since concentrations of actinomycin that block RNA synthesis almost completely permit the formation of protein for many hours in the same cells (2). We have therefore investigated the action of Tu on several of the enzymatic steps involved in polypeptide synthesis in extracts of mammalian cells. The results show that Tu can replace adenosine nucleotides in some of these reactions, but do not account for the profound inhibition of protein synthesis found in intact cells. Analogous experiments have been performed with the related compounds toyocamycin and sangivamycin (Fig. 1); the results of these are also presented below.

We have also tested the substrate properties of pyrrolopyrimidine nucleotides with tRNA pyrophosphorylase and have observed that these compounds can be incorporated into the terminal sequence of tRNA.

METHODS AND MATERIALS
All antibiotics were generously provided by Drs. H. B. Wood, R. Engle, and A. Stanley of the Cancer Chemotherapy National Service Center. Tubercidin was also generously provided by Dr. C. G. Smith of the Upjohn Company, Kalamazoo, Michigan, and toyocamycin and sangivamycin by Dr. Theodore Medrek of Chas. Pfizer and Co., Maywood, New Jersey.

tRNA-C-C-A pyrophosphorylase was prepared and assayed exactly as described by Daniel and Littauer (3), and was found to be free of RNase. tRNAs from rat liver, rabbit liver, and Escherichia coli were prepared according to the method of Kirby (4). The RNA was purified chromatographically by the method of Röschenthaler and Fromageot (5), and pyrophosphorylized according to the procedure of Daniel and Littauer (3).

Aminoacyl-tRNA synthetases from rabbit liver were prepared as follows. Livers from freshly killed rabbits were minced and homogenized in a Waring Blender with 2 volumes of 8.4% sucrose in 0.03 M Tris-HCl, pH 7.6. The homogenate was centrifuged at 100,000 × g for 3 hours. The supernatant was dialyzed overnight against 0.001 M Tris-HCl, pH 7.6, then poly (U,A), poly (U,G), and poly (U,Tu), the random copolymers of thymine ribonucleoside and uridine, of adenosine and uridine, of uridine and guanosine, and of uridine and tubercidin, respectively; PCA, perchloric acid; poly Tu, polytubercidin.
chromatographed on a DEAE-cellulose column. The activating enzymes were eluted with 0.3 m Tris-HCl, pH 7.2.

Threonine-activating enzyme from rabbit liver was isolated by the procedure of Lipmann et al. (6). The final fraction, purified 450-fold with respect to the original homogenate, had a specific activity of 1300 mmoles of threonine transferred to RNA per mg of protein in 10 min.

$^{32}$P-$^{32}$P exchange was measured by the method of Hoagland (7), and aminoacylation of tRNA according to the procedure of Weiss, Acs, and Lipmann (8). Transfer of amino acid from aminoacyl-tRNA into polypeptide was determined by the method of Nathans and Lipmann (9). Homopolytubercidin and random poly (Tu,U) were prepared in unprimed reactions starting with TuDP, with the use of purified polynucleotide phosphorylase of E. coli (the gift of Mr. David Ward). The assays of the coding properties of Tu-containing polymers were performed with the use of the incorporation system from E. coli prepared according to the method of Nirenberg and Matthaei (10).

Radioactive and nonradioactive pyrrolopyrimidine nucleotides were prepared as follows. The nucleoside (either tritiated or not) was converted to the 2',3'-isopropylidene 5'-monophosphate by incubation in $\text{POCl}_3$ (or $\text{P}^{32}\text{POCl}_3$) in acetone (11). The mixture was neutralized, and the isopropylidene group was removed by incubation in 10% acetic acid at 90° for 3 hours. Following isolation of the 5'-monophosphates by chromatography on Dowex 50-H+, the 5'-di- and triphosphates were synthesized by the method of Smith and Khorana (12). TuMP could be converted to SaMP by warming a solution of the nucleotide in 6 N HCl for 20 min at 100°. All pyrrolopyrimidine nucleotides were readily crystallizable from aqueous solutions. Other nucleotides (radioactive or nonradioactive) were obtained from regular commercial sources. Radioactive and nonradioactive amino acids were acquired from New England Nuclear and Mann, respectively.

RESULTS

Effect of TuTP on Amino Acid Activation—As seen in Table I, TuTP cannot replace ATP as an energy source for the aminoacylation of tRNA; TuTP fails to function in this reaction for any of the 16 amino acids present in the $^{14}$C-algal hydrolysate, as well as for lysine and phenylalanine when the latter were tested individually. Similar results were obtained with E. coli enzymes and E. coli tRNA. It has been shown that these activating enzymes catalyze an exchange reaction, dependent on amino acids, between inorganic pyrophosphate and ATP. No such exchange is observed when TuTP replaces ATP with activating enzymes from E. coli or from rat liver. Thus, TuTP is not a substrate for any of the reactions catalyzed by aminoacyl-tRNA synthetases. Nor is TuTP an effective competitive inhibitor of ATP for these enzymes (Table II), since a 10-fold excess of TuTP with respect to ATP does not detectably affect either the rate or the extent of aminoacylation of tRNA by ATP. These observations are noteworthy for several reasons. (a) They imply that TuTP is not bound to any of these activating enzymes, at least not in a manner which influences their catalytic activity. (b) Since none of the amino acid-activating enzymes interacts with TuTP, it appears possible that a single mode of binding of nucleoside triphosphate, involving N-7 of adenine, is common to all of these enzymes. This is...
in contrast to the many enzymes, including several phosphokinases, RNA polymerase, and terminal tRNA-C-C-A pyrophosphorylase (see below), which effectively utilize TuTP in place of ATP. Like TuTP, ToTP is not a substrate for mammalian activating enzymes (Table II); no exchange reaction between ToTP and 32PP is observed in the presence of added amino acids.

Likewise, SaTP is not a substrate for the over-all reaction catalyzed by activating enzymes; nor does SaTP participate in an exchange reaction with 32PP. However, in contrast to TuTP, ToTP and SaTP are competitive inhibitors of ATP both for the aminoacylation of tRNA and for the nucleotide-32PP exchange reaction. The inhibition constants are, for ToTP, $K_i = 3.8 \times 10^{-4}$ M, and, for SaTP, $K_i = 2.6 \times 10^{-4}$ M (Fig. 2). It may be that the nitrogen atom of SaTP and ToTP side chains can partially substitute for N-7 of ATP and so permit some interaction of these nucleotides with the ATP-binding site of activating enzymes.

**Incorporation of Pyrrolopyrimidine Nucleotides into Terminal Sequence of tRNA**—It has been shown that the nucleoside-terminal sequence of tRNA (pCpCpA) undergoes enzymatic turnover in vivo (13) and in vitro (14), and that the interaction of tRNA with activating enzymes probably requires the intact trinucleotide sequence (14). These facts suggested the possibility that the inhibition of protein synthesis in vivo by Tu and Sa might be caused, at least in part, by substitution of these nucleosides for adenosine in the terminal sequence of tRNA with consequent impairment of tRNA function.

To test for the incorporation of Tu, the experiment described in Table III was performed. It can be seen that: (a) TuTP is a substrate for the specific tRNA pyrophosphorylase, and radioactive Tu is incorporated into tRNA, (b) the incorporation of Tu into pyrophosphorylyzed tRNA, like that of adenosine, is dependent on the presence of CTP, (c) TuTP, like ATP, does not inhibit the incorporation of CMP into pyrophosphorylyzed tRNA, and (d) the amount of Tu which can be incorporated into the terminal sequence of tRNA is virtually equal to that of adenosine. These facts suggest that Tu can be incorporated into the terminal sequence of tRNA at the position normally occupied by adenosine.

More direct evidence in favor of this conclusion is shown in Figs. 3 and 4. For the experiment the results of which are illustrated in Fig. 3, a large scale incubation was performed exactly as described in the legend to Table III, with CTP and TuTP-3H as substrates. The tRNA was isolated and hydrolyzed with RNase, and the hydrolysis products were analyzed by electrophoresis on paper. All the radioactivity released from the RNA migrated with mobility indistinguishable from that of in a liquid scintillation counter. B, SaTP inhibition of ATP pyrophosphate exchange. The reaction mixture contained, in 1.0 ml, sodium pyrophosphate-32P, 3 μmoles; MgCl2, 4 μmoles;KF, 9 μmoles; Tris-HCl, pH 7.8, 100 μmoles; n-threonine, 0.7 μmoles; ATP as indicated; and 2.3 μg of partially purified threonine-activating enzyme (see "Methods and Materials"). The reaction mixture was incubated at 37° for 10 min, and an equal volume of 10% trichloracetic acid was added. The precipitate was removed by centrifugation. To the supernatant were added 10 mg of acid-washed charcoal and 1 ml of 1 M potassium acetate buffer, pH 3.2. After standing in ice for 10 min, the charcoal was collected onto Millipore filters and washed with 50 ml of 5% trichloracetic acid. Dried filters were counted in a gas flow counter. C, ToTP inhibition of aminoacylation. Reaction conditions were as in A, except that ToTP, 0.1 μmole, was used in place of SaTP.
authentic Tu. The Tu had therefore been incorporated into the terminal nucleoside position.

To establish that Tu had been incorporated at a position adjacent to CMP, the experiment was repeated with Tu-P-P-P as substrate. The tRNA was then isolated and hydrolyzed with alkali. Electrophoretic analysis of the resultant degradation products showed that radioactivity was confined to the area corresponding to 2'(3')-CMP. The TuMP had therefore been incorporated adjacent to CMP (Fig. 4).

Toyocamycin and sangivamycin 5'-monophosphates are also incorporated into pyrophosphorylized tRNA (Table IV). As in the case of TuMP and AMP, the incorporation of ToMP and SaMP is CTP dependent. Alkaline hydrolysis of the tRNA incubated with CTP and Tu-P-P-P or Sa-P-P-P yielded 2'(3')-CMP as the sole radioactive nucleotide, a finding consistent with the nucleoside-terminal incorporation of both analogues.

Aminoacylation of tRNA containing Pyrrolopyrimidine Nucleoside Termini—tRNA-containing pyrrolopyrimidine nucleoside termini can serve to esterify amino acids in the presence of homologous activating enzymes. As seen in Table V, with the exception of phenylalanine, the acceptor activity of tRNA for the amino acids tested is quite similar to that of another aliquot of the same tRNA preparation bearing adenosine end groups. The basis of the difference in phenylalanine acceptor activity remains to be analyzed.

Several control experiments were performed to test the possibility that Tu end groups in tRNA might have been replaced or contaminated by adenosine end groups; such replacement might have resulted from enzymatic or polynucleotide impurities introduced during the course of incubation with amino acid-activating enzymes.

1. The enzyme preparations used for aminoacylation of tRNA were shown to be free of endogenous amino acid acceptor activity and to be incapable of pyrophosphorylation of tRNA-Tu containing 3H-tubercidin end groups.

2. A single activating enzyme specific for threonine, purified

![Fig. 3. Electrophoresis of tRNA-C-C-Tu RNase digest. The reaction mixture contained, in 10.0 ml, 400 μmoles of glycine buffer, pH 9.5, 6 μmoles of MgCl₂, 10 μmoles of P-enolpyruvate, 0.8 mg of P-enolpyruvate kinase, 8 μmoles of [3H]-TuTP (1.93 × 10⁶ cpm per μmole), 8 μmoles of CTP, 0.5 μmole of pyrophosphorylized tRNA, and 8 μg of pyrophosphorylase. After incubation for 45 min at 37°, the reaction mixture was extracted with 90% phenol. The tRNA was precipitated in cold 2% potassium acetate buffer, pH 5.2, with 3 volumes of ethanol and placed in the freezer. After several hours, the material was centrifuged and washed with cold ethanol, and the precipitate was dissolved in 0.04 M citrate buffer, pH 5.2, for 90 min at 30°. The hydrolysate was analyzed by paper electrophoresis in a 0.04 M citrate buffer, pH 5.2, at 300 volts for 18 hours.

![Fig. 4. Nucleotide radioactivity after alkaline hydrolysis of tRNA-C-C-Tu-P-P-Tu. The reaction mixture contained, in 1.0 ml, 40 μmoles of glycine buffer, pH 9.5, 6 μmoles of MgCl₂, 10 μmoles of P-enolpyruvate, 0.4 mg of P-enolpyruvate kinase, 8 μmole of Tu-P-P-P (1.91 × 10⁶ cpm per μmole), 8 μmole of CTP, 0.8 mg of pyrophosphorylized tRNA, and 0.8 mg of pyrophosphorylase. After incubation for 45 min at 37°, the reaction mixture was precipitated with 0.5 N PCA and washed with 0.25 N PCA, alcohol-ether (3:1, v/v), and ether. Thereafter, an aliquot was hydrolyzed with 1.0 ml of 0.5 N KOH for 18 hours at 37°. The hydrolysate was neutralized with cold PCA and centrifuged. The supernatant (0.02 ml) was spotted on Whatman No. 3MM paper and analyzed by paper electrophoresis in 0.04 M citrate buffer, pH 3.5, at 300 volts for 20 hours.]
TABLE IV
Incorporation of To-3H-P-P-P and Sa-32P-P-P into rat liver tRNA

<table>
<thead>
<tr>
<th>RNA</th>
<th>Nonradioactive nucleotide added</th>
<th>Radioactive nucleotide added</th>
<th>Radioactive nucleotide incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA-C-C-To</td>
<td>None</td>
<td>ATP-H</td>
<td>0.151</td>
</tr>
<tr>
<td>RNA-C-C-Sa</td>
<td>CTP</td>
<td>ATP-H</td>
<td>0.81</td>
</tr>
</tbody>
</table>

TABLE V
Amino acid acceptor activity of tRNA-C-C-Tu

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>tRNA-C-C-Tu</th>
<th>tRNA-C-C-A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mpmoles amino acid/mpmoles tRNA</td>
<td>mpmoles amino acid/mpmoles tRNA</td>
</tr>
<tr>
<td>Lysine</td>
<td>29.4</td>
<td>26.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>37.6</td>
<td>84.0</td>
</tr>
<tr>
<td>Valine</td>
<td>53.8</td>
<td>75.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>43.6</td>
<td>62.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>17.7</td>
<td>26.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.71</td>
<td>40.6</td>
</tr>
<tr>
<td>Proline</td>
<td>45.7</td>
<td>37.5</td>
</tr>
<tr>
<td>Serine</td>
<td>87.3</td>
<td>83.5</td>
</tr>
</tbody>
</table>

Fig. 5. Identification of valyl-tubercidin. tRNA-C-C-3H-Tu (25 mpmoles) was incubated for 15 min at 37° in 1 ml with partially purified amino acid-activating enzymes in the presence of 2 mmoles of ATP, 5 mmoles of P-enolpyruvate, 100 μg of P-enolpyruvate kinase, 4 mmoles of MgCl₂, 100 mmoles of Tris-HCl buffer, pH 7.2, and 1 μC of 3H-valine (136 μC per μmole). The reaction mixture was extracted with phenol, and the aminoacylated RNA was precipitated with alcohol, dissolved in 0.5 ml of water, and dialyzed overnight against water. An aliquot of the dialysate was digested with 100 μg of RNase in the presence of 0.01 m acetate buffer, pH 5.2, for 30 min at room temperature. The digest was analyzed by paper electrophoresis in 0.04 m citrate buffer, pH 3.5, at 300 volts for 16 hours. The spot on the electropherogram corresponding to valyl-tubercidin was eluted and concentrated. An aliquot was re-electrophoresed. Another aliquot was treated at pH 10 for 10 min at 37° and then subjected to paper electrophoresis.

TABLE VI
 incorporation of amino acids from 3C-algal hydrolysate into tRNA-C-C-To and tRNA-C-C-Sa

<table>
<thead>
<tr>
<th>RNA</th>
<th>Incorporation of amino acids from 3C-algal hydrolysate into tRNA-C-C-To and tRNA-C-C-Sa</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA-C-C-A</td>
<td>Radioactivity incorporated (cpm/mpmoles tRNA)</td>
</tr>
<tr>
<td>RNA-C-C-Sa</td>
<td>410</td>
</tr>
<tr>
<td>RNA-C-C-To</td>
<td>375</td>
</tr>
<tr>
<td>RNA-C-C-Sa (zero time)</td>
<td>2</td>
</tr>
<tr>
<td>RNA-C-C-To (zero time)</td>
<td>4</td>
</tr>
</tbody>
</table>

nucleoside end groups of tRNA. However, when dATP was used as the energy source (15) for purified threonine-activating enzyme, the aminoacylation of tRNA Tu proceeded undiminished.

A preparation of tRNA-3H-Tu-14C-valine was treated with RNase, and the digest was analyzed by electrophoresis (16). As seen in Fig. 5, radioactivity was located in a region corresponding to an electrophoretic mobility slightly greater than that of free Tu. This spot is believed to represent Tu-valine; its cationic mobility, which is about double that of adenosyl-valine under the same conditions, is thought to reflect the greater mobility of Tu (at this pH value) as compared with adenosine. This tubercidyl-valine was eluted from the paper with water and the eluate was concentrated. One aliquot of this eluate was reanalyzed by electrophoresis; another aliquot was treated at pH 10 for 10 min at 37° and then subjected to paper electrophoresis.

G. Acs, unpublished results.

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pH 10 for 10 min at 37° and thereafter analyzed by paper electrophoresis. The electropherogram of the eluted and concentrated tRNA-Tu polymerase had the same electrophoretic mobility as that of the original poly(A). The aliquot treated with alkali yielded two discrete radioactive spots, one of them corresponding to "C-valine and the other to "H-tubercidin.

As seen in Table VI, the aminoacylation of tRNA bearing To and Sa, followed by replacement with adenosine termini, restored the acceptor activity of the tRNA to the level found in the original samples containing adenosine.

Transfer into Polypeptide of Amino Acids from Aminoacyl-tRNA-Tu

The aminoacylation of tRNA bearing To and Sa, followed by replacement with adenosine termini, is significantly reduced compared with an aliquot of the same preparation containing adenosine end groups. Although the basis of this difference has not been investigated, it has been established that (a) the total number of To and Sa, and (b) pyrophosphorylase of To and Sa, followed by replacement with adenosine termini, restored the acceptor activity of the tRNA to the level found in the original samples containing adenosine.

Transfer into Polypeptide of Amino Acids from Aminoacyl-tRNA-Tu

The ability of aminoacyl-tRNA-Tu to function as a precursor for polypeptide synthesis was tested as shown in Table VII. The transfer enzyme preparation used in this experiment had been assayed for the presence of both valyl-tRNA synthetase and tRNA-C-C-A pyrophosphorylase, neither of To and Sa, followed by replacement with adenosine termini, restored the acceptor activity of the tRNA to the level found in the original samples containing adenosine.

Transfer into Polypeptide of Amino Acids from Aminoacyl-tRNA-Tu

The aminoacylation of tRNA bearing To and Sa, followed by replacement with adenosine termini, restored the acceptor activity of the tRNA to the level found in the original samples containing adenosine.

Transfer into Polypeptide of Amino Acids from Aminoacyl-tRNA-Tu

The aminoacylation of tRNA bearing To and Sa, followed by replacement with adenosine termini, restored the acceptor activity of the tRNA to the level found in the original samples containing adenosine.

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Transfer into Polypeptide of Amino Acids from Aminoacyl-tRNA-Tu

The aminoacylation of tRNA bearing To and Sa, followed by replacement with adenosine termini, restored the acceptor activity of the tRNA to the level found in the original samples containing adenosine.

{}
of which was detectable. Thus the incorporation into poly-
peptide of 14C-valine from valyl-tRNA-Tu indicates that this
aminoacyl-tRNA-Tu can perform the transfer function, albeit
somewhat less efficiently than tRNA-A.

Coding Properties of Tuber&cin Polymers—By means of E.
coli polynucleotide phosphorylase, homo- and heteropolymers of
TuMP have been synthesized and tested for the ability to
promote polyptide synthesis in a cell-free system. The
activity of Tu polymers under these conditions is illustrated in
Figs. 6 and 7 and Table VIII. Homopolytubercidin, like
polyadenylic acid, functions as a template for the enzymatic
synthesis of polylysine. The coding properties of copolymers of
TuMP and UMP are indistinguishable from those of poly
(U,A); Tu functions effectively as a template for the polymeriza-
tion only of those amino acids the incorporation of which into
polypeptide is normally directed by codons containing adeno-
sine.

Polymers of ToMP and SaMP have not been prepared, since
ToDP is not a substrate for E. coli polynucleotide phosphorylase
and SaDP is a competitive inhibitor of the enzyme.3

Discussion
The experimental data in this paper supplement previous
findings that aminopyropuridimidine nucleotides can substitute
for adenine nucleotides in a wide variety of reactions. Thus,
Tu replaces adenosine efficiently as a substrate for mammalian
terminal tRNA-C-C-A pyrophosphorylase; the presence of Tu
at the nucleoside terminus of tRNA is compatible with normal
tRNA function (a) as a substrate for the same pyrophos-
phorylase, (b) in esterifying amino acids, and (c) in transferring amino
acids into polypeptides. Similarly, To and Sa nucleotides are
incorporated enzymatically into the terminal position of tRNA;
however, these two nucleosides do not permit normal tRNA
function in terms of the esterification of amino acids. It has
not been established whether the reduced aminoacylation of
tRNA-To and tRNA-Sa involves all tRNA species and amino
acids equally, and this phenomenon requires further study.

In view of the large and abnormal cyano and carboxamide
groups introduced into tRNA by incorporation of ToMP and
SaMP, respectively, it is not surprising that the function of the
polynucleotide is adversely affected.

None of the pyropropyrimidine nucleoside triphosphates can
replace ATP as the energy source for amino acid activation,
and the additional fact that TuTP does not inhibit the reaction
implies that TuTP does not bind to the enzyme in the vicinity
of the active site. This suggests that the binding of ATP to
amino acid-activating enzymes is mediated in part by an inter-
action involving N-7 of adenine. The finding that SaTP and
ToTP function as competitive inhibitors of ATP in this reaction
is not necessarily inconsistent with such an interpretation, since
the carboxamid nitrogen atom and nitrile group may be favor-
able oriented for the formation of a relatively weak, abnormal,
and catalytically ineffect complex of these nucleotides with the
enzymes at the ATP-binding site. It may be noted that the
affinity of the enzyme for inhibitory analogues is substantially
lower than that for substrate ATP.

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forming the coding experiments with polytubercidin.

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