A Dissociative Effect of Puromycin on the Pathway of Protein Synthesis by Ehrlich Ascites Tumor Cells*

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Puromycin is a broad spectrum antibiotic for various microorganisms, parasitic worms, and some transplanted animal tumors (2). It has been shown to interfere with adaptive enzyme formation (3) and protein synthesis (4) in bacteria and also to inhibit incorporation of labeled amino acids into protein by a cell-free system of liver ribosomes (5), Escherichia coli ribosomes (9), and thymus nuclei (7). We find it to be a potent inhibitor of protein synthesis by intact Ehrlich ascites tumor cells and of hemoglobin synthesis by rabbit reticulocytes. Low concentrations of puromycin dissociated the labeling of soluble protein from that of ribosomal protein in the Ehrlich ascites tumor cell, but not in the reticulocyte. In this communication we describe some of the characteristics of the initial lesion.

EXPERIMENTAL PROCEDURE

Materials—Puromycin hydrochloride was a gift of Dr. B. L. Hutchings, Lederle Laboratories, Pearl River, New York. The aminonucleoside and the leucine analogue of puromycin, in which the L-O-methyltyrosine moiety of the antibiotic is replaced by L-leucine, was a gift of Dr. J. L. Goldman of the same institution. L-Valine, L-phenylalanine, and L-leucine, uniformly labeled with carbon 14, were obtained from the Nuclear-Chicago Corporation, Des Plaines, Illinois. The uniformly labeled L-lysine·HCl and DL-valine-1-C14 were obtained from the Volk Radiochemical Company, Chicago, Illinois. DL-Valine-1-C14 was mixed with nonradioactive DL-valine to prepare solutions of appropriate concentration and specific activity. Adenine-8-C14, 100 μg per mg, was purchased from Schwarz BioResearch Inc., Mount Vernon, New York. The amount of radioactive amino acid added or its concentration, and the extent of its radioactivity, are given in individual protocols.

Incubation and Isolation Procedures—Ehrlich ascites cells were obtained and prepared for incubation as previously described (8). In those cases where the effect of puromycin or its analogues on amino acid incorporation into total cell protein was determined, the same incubation and isolation procedure was followed. When cell fractionation procedures were performed, the cells (2 ml packed cell volume) were suspended in 4 ml of buffer (8) and added to 60-ml flasks containing 2 ml of additional solutions in buffer distributed in the main compartment and side arm. After temperature equilibration for 7 minutes, the contents of the side arm, puromycin and radioactive amino acid, were tipped into the main compartment and the incubation carried out for the required time. The flasks were then removed, shaken in ice until their contents reached 0-1°C, and the supernatant fluid removed by centrifugation in a refrigerated centrifuge at the above temperature. The cells were lysed with 10 volumes of cold distilled water, and the high sodium chloride procedure of Littlefield and Keller (9) for isolation of ribosomes and soluble protein was followed. All protein fractions were washed and plated for counting as reported earlier (8). Ribonucleic acid was isolated by extraction with 10% sodium chloride at 100°C for 30 minutes, as described by Hoagland et al. (10). One aliquot was plated on copper planchets and counted, and another was assayed spectrophotometrically.

Rabbit reticulocytes were prepared and incubated in buffer (11, 12) without amino acid supplements or iron. The flasks were shaken in ice following the incubation period, and the cells with their medium suspended in cold isotonic sucrose. The lysis of cells, differential centrifugation of components, and chromatography of hemoglobin were also previously described (11). The ribosome fraction was washed with buffer (Medium A of Keller and Zamecnik (13)) before isolation and counting of protein. Counting was performed with a Nuclear-Chicago gas flow counter containing a Micromil window.

RESULTS

Inhibition of Protein Synthesis by Puromycin in Ehrlich Ascites Cell—Low concentrations of puromycin inhibited the incorporation of radioactive amino acids into total cell protein (Fig. 1). This inhibition was markedly specific, for the aminonucleoside was inactive and the leucine analogue inhibited by 50%, only at a 40-fold higher concentration than the antibiotic. Inhibitions of protein synthesis of over 90% were obtained without interfering with oxygen uptake or adenine-8-C14 incorporation into ribosomal or soluble ribonucleic acid. Similar results have been reported in Pseudomonas fluorescens (4).

Dissociation of Labeling of Ribosomal from Soluble Protein—When puromycin in low concentration and a C14-labeled amino acid were added to an Ehrlich ascites cell suspension at 22°C, a marked inhibition of the labeling of ribosomal protein was observed. As is shown in Fig. 2, this inhibition of incorporation into ribosomal protein was attended by a stimulation in the labeling of the soluble protein. Incorporation into ribosomal protein reached 10% of the control without decreasing the initial rate of incorporation into soluble protein (Fig. 2). Similar results were obtained with leucine-C14 and valine-C14. At 5 ×
Fig. 1. Inhibition by puromycin of valine incorporation into total Ehrlich ascites cell protein. The cells (0.17 ml) were incubated in buffer (1.8 ml) at 37° with L-valine-C\(^4\), 1 \times 10^{-8} \text{ M}, 7.9 \times 10^{4} \text{ c.p.m.}, and puromycin as indicated. The uninhibited control incorporated 1.8 \mu\text{oles} \text{ valine per g of protein during the 15-minute incubation period.}

Fig. 2. Dissociation by puromycin of phenylalanine labeling of soluble protein from its incorporation into ribosomal protein. Ehrlich ascites cells (2 ml) were incubated in 6 ml of buffer for 5 minutes at 22° with L-phenylalanine-C\(^4\), 50 \mu\text{moles}, 3.9 \times 10^{4} \text{ c.p.m., and puromycin as indicated.} 10^{-4} \text{ M puromycin, the incorporation of phenylalanine into ribosomal protein was inhibited to one-third the control value, whereas the labeling of the soluble protein was doubled. This concentration of antibiotic was therefore used for subsequent studies. The antibiotic at this concentration had no effect on the incorporation of leucine into soluble ribonucleic acid, which was isolated after a 5-minute incubation by the method of Hoagland et al. (10), although it severely inhibited incorporation into ribosomal protein.

Kinetics of Dissociation—This apparent inconsistency in the currently accepted precursor role of ribosomes in the labeling of soluble protein made it desirable to determine the duration of stimulation of labeling of the soluble protein in the presence of puromycin. As is shown in Fig. 3, a net stimulation could be observed up to 25 minutes of incubation, but an inhibited rate of incorporation of valine into soluble protein was evident much earlier. It is also apparent from Fig. 3 that the stimulation occurring during the early period of the incubation is a result of the elimination of a lag phase in the labeling of soluble protein.

Fractionation of Soluble Protein—To ascertain whether the soluble protein labeled during the early phases of puromycin stimulation was similar to that formed in the absence of this antibiotic, it was subjected to a simple fractionation technique. The data in Table I show that the pH 4 precipitate of protein labeled in the presence of puromycin was enriched in incorporated valine and suggest that the soluble protein labeled in the presence of puromycin is not identical with that formed in its absence.

Dissociation of Incorporation of Valine, Lysine, and Phenylalanine—Puromycin inhibited the incorporation of several amino acids into ribosomal protein without interfering with labeling of soluble protein. The data in Table II show this phenomenon for valine, lysine, and phenylalanine.

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**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control</th>
<th>With puromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total soluble protein</td>
<td>29.3</td>
<td>63.8</td>
</tr>
<tr>
<td>pH 4 precipitate</td>
<td>32.8</td>
<td>85.6</td>
</tr>
<tr>
<td>1% trichloroacetic acid precipitate</td>
<td>25.2</td>
<td>41.2</td>
</tr>
<tr>
<td>5% trichloroacetic acid precipitate</td>
<td>33.2</td>
<td>40.7</td>
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</tbody>
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Delayed Addition of Puromycin—If the cells were first incubated with radioactive valine until the labeling of ribosomal protein had reached a steady state, addition of puromycin resulted in a precipitous drop in the specific activity of this fraction and a slight rise in the labeling of the soluble protein (Fig. 4). This indicates that puromycin does not prevent the release of preformed labeled protein at the ribosomal site.

Effect of Puromycin on Cells Incubated at 37°—The previous data are concerned with the effect of puromycin on the incorporation process at 22°; at this temperature stimulation of labeling of soluble proteins by the antibiotic is due to the elimination of a lag phase. Although no such lag phase exists in the labeling of soluble protein at 37°, a similar, although less marked stimulation occurs (Table III). The dissociation in labeling of soluble protein from ribosomal protein at an early time interval is also apparent, as well as the primary lesion at the ribosomal site, which is present without significant inhibition of labeling of the total cell protein. The data in Fig. 5 indicate that this dissociation continued beyond the first few minutes of incubation.

Similar results were obtained when leucine-C14 was used. However, when the leucine analogue of puromycin was substituted for the antibiotic, it had no effect on the incorporation of leucine into either protein fraction, even when present at a concentration three times that of puromycin. These results are not in accord with the view (5) that puromycin acts as an anti-

## Table II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Ribosomal protein</th>
<th>Soluble protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>With puromycin</td>
</tr>
<tr>
<td></td>
<td>c.p.m./mg protein</td>
<td>c.p.m./mg protein</td>
</tr>
<tr>
<td>Valine</td>
<td>314</td>
<td>85</td>
</tr>
<tr>
<td>Lysine</td>
<td>406</td>
<td>135</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>631</td>
<td>217</td>
</tr>
</tbody>
</table>

**FIG. 4.** Release of labeled protein from ribosomes upon addition of puromycin: •—• with, ○—○ without addition of puromycin. Conditions of incubation were as described in Fig. 3.

Puromycin Inhibition of Protein Synthesis by Reticulocytes—Rabbit reticulocytes readily incorporate amino acids into soluble protein from which hemoglobin can be isolated chromatographically. The ribosomes of these cells have been shown to contain precursors to hemoglobin synthesis (11, 12). Protein synthesis by these cells was not affected by puromycin as it was in the Ehrlich ascites tumor. Instead, when reticulocytes were incubated at 22°, the labeling of all cell proteins was immediately inhibited (Fig. 6). At 37°, incorporation into total soluble protein and hemoglobin was inhibited, and a small increase in the labeling of the ribosomal protein was observed (Fig. 7). This is essentially the inverse phenomenon to that found with the tumor cells and similar to a block observed in the presence of a valine antagonist (12).

## DISCUSSION

The results indicate that puromycin is a potent inhibitor of protein synthesis by the Ehrlich ascites cell and that it also has a profound effect on the synthetic mechanism at concentrations too low to elicit a significant decrease in amino acid incorporation into total cell protein. At these concentrations, a primary lesion at the ribosomal site is apparent. The linear rate of
incorporation of amino acids into soluble protein in the presence of puromycin indicates that the antibiotic does not function through a sudden and permanent disruption of the integrity of the synthetic site of the ribosome. However, the stimulation in labeling of soluble protein through the elimination of a lag phase may be interpreted in terms of continuous interference with the growth of a peptide chain and the premature release of labeled protein from the synthetic site of the ribosome (Fig. 8). The synthetic site functioning in the presence of puromycin may be only loosely associated with the ribosome, for incorporation into soluble protein continues when that into ribosomal protein is inhibited by 90% (Fig. 2).

A similar lesion is not seen with the rabbit reticulocyte, which, on incubation with puromycin at 37°, gives an incorporation pattern apparently inverse to that of the tumor cell. The inhibition mechanism, however, may be similar, with the exception that the initial release of incomplete protein by the reticulocyte ribosomes results in a permanent lesion which prevents the continuous incorporation and release as seen with the Ehrlich ascites tumor cells. Such a puromycin-induced release of protein from a cell-free system of reticulocyte ribosomes has been reported by Morris and Schweet (14).

**SUMMARY**

Puromycin is a potent inhibitor of protein synthesis by Ehrlich ascites tumor cells and rabbit reticulocytes in vitro. With the former cell type, puromycin inhibits incorporation of amino acids into ribosomal protein when present at a concentration too low to inhibit labeling of total cell protein. Under these conditions, a stimulation of the labeling of soluble protein takes place. When the incubation is carried out at 22°, this stimulation can be shown to be due to the elimination of a lag phase in the labeling of soluble protein.

The soluble protein labeled in the presence of puromycin is not the same as that formed in its absence. It is formed over a period of time at a nearly constant rate. The results suggest that puromycin causes the continuous premature release of labeled protein from the synthetic site of the ribosome.

**REFERENCES**

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