The Effects of Amino Acid Starvation on Regulation of Polypeptide Chain Initiation in Ehrlich Ascites Tumor Cells

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The rate of initiation of protein synthesis in Ehrlich ascites tumor cells in culture is reduced by over 60% in the absence of a single essential amino acid. Cell-free extracts prepared from control and amino acid-starved cells retain some of the translational characteristics of these cells and are able to form [40 S-Met-tRNA\(^{\text{Met}}\)] initiation complexes. Studies with inhibitors show that up to 63% of the translation directed by endogenous mRNAs but does not abolish the difference between fed and starved preparations. Mixing experiments have not so far revealed any inhibitor of initiation complex formation in the starved cell extracts.

Correlation with changes in rates of protein synthesis and in polysome profiles suggested that binding of Met-tRNA\(^{\text{Met}}\) to native 40 S subunits could be the rate-limiting step in the sequence of reactions involved in initiation of translation.

These observations were made in intact cells labeled briefly with radioactive methionine in culture. In the analysis of the regulatory mechanisms involved would be greatly facilitated by the preparation of cell-free extracts from fed and starved cells in which the relative activities of the protein synthetic components could be maintained as near as possible to the in vivo state. In this paper, we describe some of the characteristics of such undialyzed, postmitochondrial supernatant systems prepared from Ehrlich cells, including their ability to translate their endogenous mRNAs and to form initiation complexes on native 40 S subunits in vitro. We have also examined the effects of added purified initiation factors on 40 S initiation complexes in an attempt to define more precisely the mechanism whereby amino acid starvation inhibits polypeptide chain initiation.

EXPERIMENTAL PROCEDURES

Materials—The following radiochemicals were obtained from the Radiochemical Centre, Amersham, United Kingdom: L-[\(^{35}\)S]methionine, L-[\(^{14}C\)]leucine, L-[\(^{3}H\)]valine, and L-[\(^{3}H\)]phenylalanine. The specific radioactivities of these materials in cell-free incubations were lowered by mixing with the corresponding endogenous unlabelled amino acids as specified in the figure and table legends. Poly(U) and creatine phosphokinase (EC 2.7.3.2.) were from Sigma Chemical Co.; edeine was purchased from Calbiochem. CTAB\(^{1}\) was a product of BDH Chemicals Ltd.

Buffers—Buffer A: 30 mM Tris-HCl, pH 7.5, and 140 mM KCl. Buffer B: 10 mM K\(^{+}\) Hepes, pH 7.5, 10 mM KCl, 1.5 mM magnesium acetate, and 7 mM 2-mercaptoethanol.

Initiation Factors—Factors eIF-2 and eIF-3 were generously provided by Dr. William Merrick. The eIF-2 was approximately 95% pure (9) and the eIF-3 approximately 80% pure (10). They were stored in small aliquots in liquid nitrogen until use.

Growth of Cells—Ehrlich ascites tumor cells were grown in spinner culture at 37°C in Eagle’s minimal essential medium (3) supplemented with 20 mM Mops (pH 7.35 at 20°C). Cultures were diluted at appropriate intervals to maintain the cell number between 2 X 10\(^{5}\) and 8 X 10\(^{5}\) cells/ml.

Amino Acid Starvation—To make sure that cells were fully fed, additional glucose (1 mg/ml) was added to the cultures approximately 18 h before the cells were harvested. Two hours before harvesting, the cells were also diluted with 50% further fresh warm medium to a cell concentration of approximately 3.5 X 10\(^{5}\)/ml. For harvesting, the cultures were centrifuged for 10 min at 800 X g at room temperature. The cell pellet was rapidly drained and resuspended at a concentration of 2 to 3 X 10\(^{5}\)/ml in warm medium without serum and lacking either lysine or glutamine. The suspended cells were divided into two

\(^1\) The abbreviations used are: CTAB, cetyl trimethyl ammonium bromide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, morpholinopropanesulfonic acid; 40 S, native 40 S ribosomal subunits; eIF, eukaryotic initiation factor.
and cell extracts methods were used for determination of the protein content of cells. The ionic conditions were restored to nearer the physiological values almost identical in any one pair of fed and starved cell preparations. The gradients were centrifuged for 4.5 h at 196,000 g in the

Incubation Complex Formation in Cell Extracts—Incubations were carried out under the conditions used for measurement of cell-free protein synthesis (see above). After 2 min at 30°C in the presence of [35S]methionine (190 to 200 μCi/ml), together with a known concentration of nonradioactive methionine provided by the endogenous pool, the reaction was stopped by rapid cooling in a -10°C alcohol bath and the incubation mixtures (100 to 120 μl) layered on 12.5-ml 20 to 50% sucrose gradients in low pH buffer (0.1 mM sodium cacodylate, final pH 6.6, 100 mM KCl, and 5 mM magnesium acetate) (13). The gradients were centrifuged for 4.5 h at 196,000 × g in the Beckman SW41 rotor. Approximately 20 fractions per gradient were collected into 1-ml aliquots of 0.5 mM sodium acetate, pH 5.0, containing 0.5 mg of carrier yeast RNA. The fractions were precipitated by addition of 1-ml aliquots of 2% CTAB (14) and radioactivity determined per A260 unit of cell extract.

Estimation of Protein Concentrations—Previously established methods were used for determination of the protein content of cells and cell extracts (16).

RESULTS

Protein Synthesis in Intact Cells—When Ehrlich ascites tumor cells are resuspended in lysine-free medium and incubated at 37°C, their rate of protein synthesis immediately falls by approximately 60% relative to control cells. Refeeding of the missing amino acid restores protein synthesis to its normal rate within 10 min (4). We have measured the true rate of protein synthesis in intact cells by incubating them with increasing concentrations of radioactive precursor ([35S]valine (Fig. 1). These conditions flood the intracellular pools and establish a specific activity close to that of the extracellular amino acid (18-20). We have calculated the rates of protein synthesis in incubated intact fed cells under these circumstances to be 0.035 and 0.075 mg of protein/mg of existing protein/h, respectively. The decrease in protein synthetic rate caused by amino acid deprivation is accompanied by extensive loss of polysomes and a reduction in the average number of ribosomes per polysome (2-8). This indicates a fall in the rate of polypeptide chain initiation relative to the rates of elongation and termination.

Protein Synthesis in Cell Extracts—Postmitochondrial supernants prepared from homogenized Ehrlich cells synthesized protein in vitro for 15 to 20 min at 30°C when supplemented with energy sources and the appropriate salts. In extracts from fed and starved cells, up to 63% of the amino acid incorporation directed by endogenous mRNA is due to reincorporation of pre-existing polypeptide chains in vitro, as judged by sensitivity to the antibiotic edeine (21) and to homopolyribonucleotide inhibitors of initiation (22). The remainder represents synthesis of pre-existing polypeptide chains by ribosomes “run-off” (Table I). It was, therefore, possible to investigate the effects of amino acid starvation on both the initiation of translation and the completion of nascent chains in vitro. As shown in Table I, glutamine or lysine deprivation of the cells

1 The rate of protein synthesis (k,) is calculated from the rate of valine incorporation (A) in disintegrations per min/mg of protein/h, the specific activity of the extracellular valine (B) in disintegrations per min/mmol, and the valine content of total Ehrlich cell protein (C) (400 nmol/mg of protein) using the following equation:

Fig. 1. Protein synthesis in fed and lysine-starved intact Ehrlich cells. Cells were incubated in 1-ml aliquots at a concentration of 2.3 X 10^6/ml in the presence (○—○) or absence (□—□) of lysine, with the indicated concentrations of [3H]valine (specific activity 1.67 Ci/mmol). After 20 min, protein was precipitated with 1 ml of 10% trichloroacetic acid and pelleted by centrifugation. Precipitates were dissolved in 0.3 M NaOH and incubated at 37°C for 1 h. Protein was then reprecipitated with 10% trichloroacetic acid and redissolved in 0.1 M NaOH. Aliquots of the final solution were taken for measurement of protein (16) and for measurement of radioactivity by liquid scintillation counting, using “Tritosol” scintillant mixture as described previously (17). Efficiency of counting for [H] was 25%.
Regulation of Polypeptide Initiation by Amino Acids

### Table I

<table>
<thead>
<tr>
<th>Cell extract</th>
<th>Precursor</th>
<th>Edeine sensitive (pmol/Am2)</th>
<th>Edeine resistant</th>
<th>Poly(r1) sensitive</th>
<th>Poly(r1) resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>[3H]Phe</td>
<td>144</td>
<td>100</td>
<td>131</td>
<td>113</td>
</tr>
<tr>
<td>Starved for Gln</td>
<td>[35S]Met</td>
<td>31 (78)</td>
<td>25 (75)</td>
<td>12 (91)</td>
<td>44 (61)</td>
</tr>
<tr>
<td>Fed</td>
<td>[3H]Phe</td>
<td>24</td>
<td>22</td>
<td>29</td>
<td>17</td>
</tr>
<tr>
<td>Starved for Gln</td>
<td>[3H]Phe</td>
<td>11 (54)</td>
<td>7 (68)</td>
<td>11 (62)</td>
<td>7 (59)</td>
</tr>
<tr>
<td>Fed</td>
<td>[3H]Leu</td>
<td>33</td>
<td>36</td>
<td>22</td>
<td>47</td>
</tr>
<tr>
<td>Starved for Lys</td>
<td>[3H]Leu</td>
<td>23 (30)</td>
<td>22 (39)</td>
<td>15 (32)</td>
<td>30 (36)</td>
</tr>
</tbody>
</table>

**TABLE II**

**Amino acid content of extracts from fed and amino acid-starved Ehrlich cells**

<table>
<thead>
<tr>
<th>Cell extract</th>
<th>Methionine</th>
<th>Leucine</th>
<th>Phenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>0.28</td>
<td>1.19</td>
<td>1.69</td>
</tr>
<tr>
<td>Starved for Gln</td>
<td>1.07</td>
<td>2.26</td>
<td>1.48</td>
</tr>
<tr>
<td>Fed</td>
<td>0.60</td>
<td>1.30</td>
<td>1.05</td>
</tr>
<tr>
<td>Starved for Lys</td>
<td>1.02</td>
<td>2.33</td>
<td>1.65</td>
</tr>
<tr>
<td>Fed</td>
<td>0.98</td>
<td>2.38</td>
<td>1.44</td>
</tr>
<tr>
<td>Starved for Lys</td>
<td>1.26</td>
<td>3.01</td>
<td>1.81</td>
</tr>
</tbody>
</table>

Results in substantial inhibition of the inhibitor-sensitive (initiation) and inhibitor-resistant (run-off) components of protein synthesis in extracts. The latter effect correlates with the reduction in the proportion of ribosomes in polysomes resulting from starvation (2–8).

In order not to overlook any possible influence of low molecular weight constituents in eliciting the effects of amino acid deprivation on protein synthesis in vitro, we have not routinely subjected the cell-free preparations to dialysis or Sephadex treatment before use. Correction for differences in amino acid content have been made by direct amino acid analyses of the extracts. These measurements revealed substantial increases in the concentrations of several amino acids (other than the missing one) in the starved cell systems (Table II).

We have also investigated the capacity of extracts from fed and starved cells to translate exogenous mRNAs, with or without elimination of endogenous mRNA by micrococcal nuclease treatment. However, a considerable variation in the abilities of different preparations from fed or starved cells to respond to added mRNAs has precluded a meaningful analysis of changes in this translational activity which may arise as a result of nutritional deprivation.

**Formation of Initiation Complexes on Native 40 S Subunits**—It was previously shown that inhibition of initiation in amino acid-starved Ehrlich cells is associated with a decrease in the number of native 40 S ribosomal subunits carrying initiator Met- tRNA$^{Met}$ in vitro (8). We have investigated whether such an effect can also be seen in vitro, using extracts from fed and starved cells. During a brief incubation with [35S]methionine, radioactivity becomes associated with the 40 S$\mathrm{M}$ peak, as well as with polysomes carrying nascent poly-

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*Fig. 2. Formation of [40 S-Met-tRNA$^{Met}$] initiation complexes in extracts from fed and lysine-starved Ehrlich cells. Extracts from fed and starved cells were incubated for 2 min with [35S]methionine (100 μCi/ml) under conditions of protein synthesis ("Experimental Procedures"). The incubations were rapidly cooled and then layered on sucrose gradients in low pH buffer ("Experimental Procedures"). After centrifugation, the gradients were fractionated, the fractions precipitated with CTAB, and radioactivity determined as described under "Experimental Procedures." a, Fed cell extract; b, lysine-starved cell extract. ---, Optical density at 260 nm; ○--○, radioactivity (counts per minute per fraction)."
order to determine whether this inhibitor is active, equal
volumes of fed and starved cell extracts were incubated
for 2 min with \([^{35}S]\)methionine. However, the level
of labeled 40 S 1 complexes formed was not less than
that expected from the mean of the values given by the
two samples individually (Table IV). Indeed, the fed cell extracts
appeared to stimulate the starved ones to some extent. Thus,
at least under these conditions, there is no dominant inhibitor
in the starved cell-free system acting at this step in initiation.
It should be noted, however, that these results do not exclude
the possibility that there is a ribosomal bound inhibitor in the
starved system which is unable to act on the fed cell initiation
complexes.

Reversal of the Starvation-induced Inhibitions — Since the
extracts prepared from fed and amino acid-starved Ehrlich
cells have been subjected to the minimum of manipulations
before assay, it seemed possible that the content and activity
of both small molecules and macromolecular components
might reflect the behavior of the respective intact cells. Lysine
rapidly restores the rate of protein synthesis in intact starved
cells (data not shown) and so we also attempted to reverse
the effects of lysine deprivation by adding back this amino
acid in vitro. However, this procedure failed to produce any
significant effect on protein synthesis directed by endogenous
mRNA in either fed or starved cell extracts, even though these
cell-free systems are capable of reinitiating translation
under the conditions used. Furthermore, exogenous lysine did
not stimulate formation of [40 S-Met-tRNA\(^{35}\text{S}\)] initiation
complexes in a starved cell extract under the standard assay
conditions used throughout this work (Table V). In contrast
to these observations, restoration of normal lysine supply to
intact cells not only reactivated protein synthesis in vitro but
also reversed the impairment of [40 S-Met-tRNA\(^{35}\text{S}\)] complex
formation and of endogenous amino acid incorporation in vitro by 60 and 100%, respectively (Table V).

Since amino acid starvation results in impairment of 40 S
initiation complex formation, we have examined whether either
of the initiation factors eIF-2 and eIF-3, which are
found in these complexes (28–30), can reverse the inhibitory
effects in vitro. Factor eIF-2 has either no effect or slightly
inhibits initiation complex formation in fed cell extracts; in
marked contrast to this, eIF-2 can stimulate [40 S-Met-tRNA\(^{35}\text{S}\)] formation in starved cell extracts as much as
3-fold (Table VI). Fig. 3a shows the eIF-2 concentration
dependence of the latter effect. It can be seen that higher
concentrations of the factor are less effective in stimulating
complex formation. Translation of endogenous mRNA is also
stimulated by eIF-2 in starved cell extracts (Fig. 3b) but not in
fed cell extracts (data not shown). Initiation factor eIF-3 is
also stimulatory for [40 S-Met-tRNA\(^{35}\text{S}\)] complex formation
(up to 3.5-fold) but almost equally in fed and starved cell
extracts (Table VI). This effect may be at least partially due
to its ability to generate more 40 S ribosomal subunits by
dissociation of 80 S monomers. In contrast, eIF-2 stimulates
initiation complex formation without causing major changes in
the concentration of 40 S subunits in starved extracts
(data not shown). Interestingly, exogenous eIF-3 does not
stimulate protein synthesis in vitro with either kind of cell
extract. Also, the effects of eIF-2 and eIF-3 on initiation
complex formation were not additive, suggesting that eIF-3
might prevent further stimulation by added eIF-2 (Table VI).

As a consequence of the differential stimulatory effect
of eIF-2 and the general stimulatory effect of eIF-3 added
in vitro, a 60 to 70% inhibition of [40 S-Met-tRNA\(^{35}\text{S}\)] complex
formation and of endogenous amino acid incorporation
in extracts from fed or amino acid-starved cells

<table>
<thead>
<tr>
<th>Pairs of cell extracts</th>
<th>[40 S-Met-tRNA(^{35}\text{S})] (pmol/A(_{450}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>0.034</td>
</tr>
<tr>
<td>Starved for Lys</td>
<td>0.011 (68)</td>
</tr>
<tr>
<td>Fed</td>
<td>0.094</td>
</tr>
<tr>
<td>Starved for Lys</td>
<td>0.022 (77)</td>
</tr>
<tr>
<td>Fed</td>
<td>0.077</td>
</tr>
<tr>
<td>Starved for Lys</td>
<td>0.022 (71)</td>
</tr>
<tr>
<td>Fed</td>
<td>0.044</td>
</tr>
<tr>
<td>Starved for Lys</td>
<td>0.022 (50)</td>
</tr>
<tr>
<td>Fed</td>
<td>0.021</td>
</tr>
<tr>
<td>Starved for Gln</td>
<td>0.014 (33)</td>
</tr>
</tbody>
</table>

TABLE IV

<table>
<thead>
<tr>
<th>[40 S-Met-tRNA(^{35}\text{S})] initiation complexes in mixed extracts from fed and amino acid-starved cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>2</td>
</tr>
</tbody>
</table>

TABLE V

<table>
<thead>
<tr>
<th>[40 S-Met-tRNA(^{35}\text{S})] initiation complex formation and protein synthesis in extracts from lysine-starved cells; effect of restoration of lysine in vivo and in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
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<tr>
<td>------------</td>
</tr>
<tr>
<td>1</td>
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<td></td>
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<tr>
<td>2</td>
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</tbody>
</table>
TABLE VI

| Experiment | Cell extract | eIF-2 | eIF-3 | [40 S-Met-tRNA\textsuperscript{Met}] \textsuperscript{\textdagger} | Inhibition by starvation pmol/A\textsubscript{iso} %
<table>
<thead>
<tr>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fed</td>
<td>–</td>
<td>–</td>
<td>0.077</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fed</td>
<td>+</td>
<td>–</td>
<td>0.077</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fed</td>
<td>+</td>
<td>+</td>
<td>0.158</td>
<td></td>
</tr>
</tbody>
</table>
|            | Starved      | –     | –     | 0.022             | 71%
|            | Starved      | +     | –     | 0.072             | 6%
|            | Starved      | +     | +     | 0.077             | 51%
| 2          | Fed          | –     | –     | 0.130             |
|            | Fed          | +     | –     | 0.080             |
|            | Fed          | +     | +     | 0.188             |
|            | Starved      | –     | –     | 0.047             | 64%
|            | Starved      | +     | –     | 0.078             | 3%
|            | Starved      | +     | +     | 0.090             | 52%
|            | Starved      | +     | +     | 0.069             | 43%

The changes in the overall rate of protein synthesis which occur in cultured cells in response to variations in nutritional conditions (1-8) constitute a clear example of regulation of translation at the cytoplasmic level. These changes and the mechanism by which they occur are, in many ways, analogous to the influence of heme supply on protein synthesis in rabbit reticulocytes (31) and reticulocyte lysates (14, 23-26). The effects of amino acid deprivation and refeeding on amino acid incorporation in intact cells are rapid and occur independently of any changes in RNA synthesis, at least in the short term (3, 5-7). The major consequence of amino acid deficiency is a block in polypeptide chain initiation, leading to disaggregation of polysomes (2-8). Messenger RNA accumulates in the form of free mRNP particles, which can be recruited back into polysomes on refeeding (6, 32), or if the rate of polypeptide chain elongation is slowed by cycloheximide (33, 34).

In the work described here, we have adopted the approach of preparing crude extracts from fully fed and amino acid-starved cells in the hope that such extracts will retain many of the properties of, and the differences between, the cells from which they are derived. This appears to be the case, at least qualitatively, with respect to protein synthesis in vitro directed by endogenous mRNA and with respect to formation of initiation complexes on native 40 S ribosomal subunits. However, it must be recognized that the initial rates of translation in extracts incubated at 30°C are only approximately 14% of the rates in the intact cells at 37°C (Fig. 1). Calculation of the levels of [40 S-Met-tRNA\textsuperscript{Met}] initiator complexes formed in vitro in fed Ehrlich cell extracts indicates that up to 16% of 40 S\textsubscript{0} subunits carry Met-tRNA\textsuperscript{Met}. The corresponding values for starved cell extracts are one-third to one-half of this level. These figures are comparable with the values of 5% and 10.7% of 40 S\textsubscript{0} estimated to exist as initiation complexes in lysine-starved and fed intact cells respectively (8), suggesting that loss of ability to form [40 S-Met-tRNA\textsuperscript{Met}] complexes is not the reason why the extracts are limited in protein synthetic activity, especially in the case of fed cell systems.

We have shown previously (8) that amino acid or glucose starvation of Ehrlich cells inhibits polypeptide chain initiation in vivo at the level of [40 S-Met-tRNA\textsuperscript{Met}] initiation complexes. This result has been confirmed and extended in the present study by virtue of the fact that the extracts from fed and amino acid-starved cells maintain a differential ability to form and/or break down these complexes and to initiate translation on endogenous mRNA in vitro. The fact that initiation factor eIF-2 stimulates formation of complexes on 40 S\textsubscript{0} subunits and enhances endogenous protein synthesis in extracts of starved but not fed cells strongly implicates this factor in the mechanism of nutritional control of protein synthesis. By analogy with the role of eIF-2 in the regulation of reticulocyte protein synthesis by heme (23-26) and other agents (35-37), it may be proposed that nutritional deprivation might activate an inhibitor which modifies the activity of the initiation factor, perhaps by phosphorylation (26, 37). We have previously identified an activity which may constitute such an inhibitor in extracts from Ehrlich cells (27). Nevertheless, the present experiments do not provide any evidence that this inhibitor is involved in the response to amino acid deprivation unless its activity is rapidly reversed upon mixing fed and starved cell extracts or remains tightly bound to a component (e.g. ribosomal subunits) from the starved cells.

Initiation factor eIF-3, which together with eIF-2 is found in association with 40 S\textsubscript{0} complexes in eukaryotic cells (28-30), also stimulates [40 S-Met-tRNA\textsuperscript{Met}] formation in extracts of Ehrlich cells but to almost the same extent in fed extracts as in starved ones. This suggests that the availability of this factor may be limiting for 40 S initiation complex formation whether or not the cells have been subjected to amino acid deprivation. Exogenous eIF-3 markedly increases the concentration of 40 S\textsubscript{0} subunits (by about 7-fold) but we calculate that only a small proportion of these subunits bind Met-tRNA\textsuperscript{Met} in vitro. The limitation in initiation activity in the starved extracts does not prevent the eIF-3-dependent stimulation of [40 S-Met-tRNA\textsuperscript{Met}] formation but still results in a 52% reduction in the concentration of these complexes.
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relative to that in fed cell extracts. The apparent ability of eIF-3 to prevent any additional eIF-2-catalyzed stimulation in starved cell-free systems (Table VII), together with the observation that less than 20% of 40 S s carry [Met-tRNA<sup>[Met]</sup>] in any case (8), suggests that the regulation of this stage of initiation is very complex and may involve further components (38-40).

The biochemical mechanisms by which the protein-synthesizing machinery of Ehrlich and other cells recognizes a con-}
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