of chromophores, both intrinsic and extrinsic, in a variety of systems of biological interest.


**Correction:** In the article “Crystal and Molecular Structure of N,N'-Diethyl-N,N'-Diphenylurea”, by Ganis, P., Avitabile, G., Benedetti, E., Pedone, C. & Goodman, M., which appeared in the September 1970 issue of *Proc. Nat. Acad. Sci. USA* 67, 426-433, the following correction should be made in Table 2, p. 428: The z coordinate of atom N1 is 0.3517, not 0.3317.

**Correction.** In the article “DNA and Gene Therapy: Transfer of Mouse DNA to Human and Mouse Embryonic Cells by Polyoma Pseudovirions,” by Qasba, P. K. & Aposhian, H. V., which appeared in the October 1971 issue of *Proc. Nat. Acad. Sci. USA* 68, 2345-2349, several corrections should be made. On p. 2345, column 2, line 10 (from the top) should read: “10-40% sucrose gradient, containing...” not “10-20%...” Due to editorial errors made in the PROCEEDINGS office, the following corrections should be made. The last line of the legend to Table 4, p. 2347, should read: “the nuclear fraction of 6.3 x 10^6 infected cells was used for each hybridization.” In Table 6, p. 2349, the right-center column head should read “% Total cellular radioactivity.”

**Correction.** In the article “Regulation of the Nucleolar DNA-Dependent RNA Polymerase by Amino Acids in Ehrlich Ascites Tumor Cells”, by Franze-Fernández, M. T. & Pogo, A. O., which appeared in the December 1971 issue of *Proc. Nat. Acad. Sci. USA* 68, 3040-3044, the following corrections should be made. In the legend to Table 1, p. 3042, reference “(18)” should read “(13)”. On p. 3043, eleven lines from the bottom, right-hand column, the sentence beginning “Assumptions b and c suggest” should read “Assumptions a and b suggest”.

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Regulation of the Nucleolar DNA-Dependent RNA Polymerase by Amino Acids in Ehrlich Ascites Tumor Cells
(nuclear ribosomal RNA precursor/cytoplasmic ribosomal RNA/nuclear rapidly labeled RNA)

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Communicated by Alfred E. Mirsky, September 23, 1971

ABSTRACT Experiments were performed to ascertain the degree to which the amount of amino acids might be one of the regulatory factors that control the activity of the nucleolar RNA polymerase. Assays of the enzymatic activity were done with isolated nuclei from cells incubated with low and high concentrations of amino acids. Soon after the cells were exposed to a medium enriched in amino acids, a rapid increase of nucleolar RNA polymerase activity occurred. A similar result was obtained in cells incubated with lower concentrations of amino acids. However, the rate of ribosomal RNA synthesized was regularly much higher in cells incubated in a medium enriched with amino acids than in a medium low in amino acids. Apparently, the amino acids only controlled ribosomal RNA synthesis. Thus, neither maturation, processing, and transport of nuclear precursors into cytoplasmic ribosomal RNA, nor the synthesis of rapidly labeled RNA was affected.

The factors controlling the synthesis of ribosomal RNA (rRNA) in eukaryotic cells are not well understood. The absence of amino acids in the incubation medium have been reported to cause a marked reduction in the rate of appearance of newly formed [3H]uridine-labeled ribosomal subunits in the cytoplasm of Landschütz cells (1). However, neither the time at which the first subunit appeared nor the relative rate of transport of preformed ribosomal subunits from nucleus to cytoplasm was affected. These observations suggested that amino acids may exert the effect at or before the stage when 45S rRNA precursor was cleaved to form 18S and 28S rRNA. Studies in HeLa cells also indicated that the synthesis and maturation of 45S rRNA are depressed in the absence of protein synthesis (2, 3) and also when the cells are cultured in a valine-deprived medium (4). Nevertheless, Smulson (5) failed to find any difference in the RNA polymerase (EC 2.7.7.6) activity of isolated nuclei of HeLa cells incubated in the presence or absence of amino acids in the incubation medium. The latter author, however, did not indicate which RNA polymerase activity he measured.

Taking advantage of the fact that it is now possible to select conditions for assaying different RNA polymerases in isolated, intact nuclei (6-11), we have explored the likelihood that the amount of amino acids in the incubation medium might be one of the regulatory factors that control nucleolar RNA polymerase activity (polymerase I; see ref. 12). Our results showed that the activity of this enzyme was controlled by the amounts of amino acids in the incubation medium.

MATERIALS AND METHODS

Ehrlich ascites tumor cells were inoculated into the abdominal cavity of mice and harvested after 4-7 days, washed twice with the incubation medium at room temperature, and then incubated at a concentration of 1-2 X 10⁶ cells/ml of medium.

Incubation Medium. Eagle’s minimum essential medium was prepared as follows: 10 ml of 10 times concentrated phosphate-buffered saline (pH 7.2) (Dulbecco’s medium), 10 ml of nondialysed bovine serum, 1 ml of 100 times concentrated vitamin mixture, and 0.3 g of glucose. Amino acids were added in the form of 1 ml of nonessential amino-acid mixture (100 times concentrated) and 2 ml of essential amino-acid mixture (50 times concentrated). Distilled water was added to make a final volume of 100 ml. Penicillin was added to a final concentration of 50 units/ml and streptomycin to 50 µg/ml. The pH of the medium was adjusted to 7.3. Eagle’s medium without amino acids replaced the amino-acid mixtures with distilled water.

Cell Fractionation. About 7 X 10⁶ cells were poured onto 250 ml of crushed frozen saline solution (0.9% NaCl), sedimented by centrifugation, and washed with 25 ml of a cold solution containing 0.3 M sucrose–1.5 mM MgCl₂–10 mM Tris·HCl buffer (pH 7.9)†. The washed cells were resuspended in 25 ml of a cold solution containing 10 mM Tris·HCl buffer (pH 7.9)–10 mM KC{l}–1 mM MgCl₂ and allowed to swell for 10 min at 4°C. These cells were then resuspended in 12 ml of a lytic solution containing 0.3 M sucrose–1.5 mM MgCl₂–0.3% Triton X-100–10 mM Tris·HCl buffer (pH 7.9) and disrupted by three strokes with a Teflon homogenizer. The disrupted cells were fractionated by differential centrifugation at 800 x g for 6 min into a crude nuclear pellet and a cytoplasmic supernatant. The cytoplasmic fraction was used to extract rRNA (vide infra). The crude nuclear fraction was washed with 10 ml of a solution containing 0.3 M sucrose–4 mM MgCl₂–10 mM Tris·HCl buffer (pH 7.9) and finally resuspended in the medium to be used for the RNA polymerase assay. All of these procedures were performed at 4°C.

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† To whom requests for reprints should be addressed.
‡ All pH values were determined at 23°C.
RNA Polymerase Assay. RNA polymerase was assayed by measurement of the incorporation of [3H]GMP and [3H]UMP into RNA. Aliquots of the crude nuclear fraction containing 80-90 μg of DNA were added to the assay mixture (final volume 0.25 ml) containing 20 mM Tris·HCl buffer (pH 8.0, at 23°C)-60 mM NaCl-4 mM MgCl₂-1 mM dithioerythritol-4 mM phosphoenolpyruvate-1 μg of pyruvate kinase-0.2 μmol each of ATP, CTP, UTP, and [3H]GTP (specific activity 100 Ci/mol). Unless otherwise indicated, incubation was for 15 min at 37°C. The reaction was stopped by addition of 300 μg of GTP or UTP, and 7 ml of 10% cold trichloroacetic acid containing 40 mM sodium pyrophosphate. The precipitate was collected by centrifugation and washed three times with 7 ml of the cold trichloroacetic acid-pyrophosphate solution. The final precipitate was dissolved in 0.4 ml of concentrated formic acid and the radioactivity was counted as described (9). All of the assays were performed in duplicate or triplicate.

Isolation and Characterization of Labeled RNA. Radioactive rRNA was obtained from cells incubated for 90 min in the presence of 5 μCi/ml of [3H]uridine (specific activity 27 Ci/mmole). Cell fractionation was performed as described before and the RNA from the cytoplasmic fraction was extracted with the sodium dodecyl sulfate-phenol method at room temperature (13). Radioactive 45S rRNA precursor was obtained from cells that were pulse-labeled for 25 min with 5 μCi/ml of [3H]uridine. The total cellular RNA was extracted with the hot sodium dodecyl sulfate method (14). The extracted RNA was dissolved in 0.1 M NaCl-50 mM acetate buffer (pH 5.0); aliquots were layered on a 15-30% sucrose gradient in the same buffer and centrifuged as explained in Fig. 1. The gradients were fractionated in samples of 0.4 ml that were collected in vials, 15 ml of Bray's solution was added, and counting was performed in a Beckman scintillation counter.

RESULTS

RNA synthesis in Ehrlich cells

Ehrlich cells were incubated with or without the addition of amino acids. Eagle's medium without amino acids contains a relatively small amount of amino acids that are present in the nondialysed bovine serum.§ After 60 min, [3H]uridine was added and incubation was continued for 25 or 90 min. Fig. 1A illustrates the incorporation of [3H]uridine into the 18S and 28S rRNA species after 90 min of labeling. The results showed a preferential labeling of the 18S as compared with the 28S rRNA in both conditions of cell incubation (25 or 90 min at 37°C). These observations were similar to those reported for HeLa cells and indicated that the 40S ribosomal subunit appears in the cytoplasm before the 60S subunit (15). The incorporation of [3H]uridine into RNA was much higher in cells incubated with a large excess of amino acids than in a medium low in amino acid content. Furthermore, the ratio of the rates of incorporation of [3H]uridine into the two RNA species was not affected by the amount of amino acids in the media. Fig. 1B illustrates the incorporation of [3H]uridine into 45S rRNA after 25 min of labeling. The results showed that more labeled 45S rRNA accumulated in cells incubated in a medium enriched with amino acids than in a medium low in amino acid content.

These observations were similar to those reported for Landshütz cells (1) and strongly suggest that amino acids did not alter the processing of the 45S rRNA precursor. The effect of amino acids on 45S nuclear rRNA precursor, and on the 18S and 28S cytoplasmic rRNA species is similar. Thus, the difference in incorporation into cytoplasmic rRNA is not demonstrably due to altered maturation, processing, or transport of the nuclear precursors to cytoplasmic rRNA.

Table 1 lists the amounts of radioactivity incorporated into RNA species other than 18, 28 and 45 S. This experiment was done by addition of actinomycin D (0.04 μg/ml) to the incubation medium, in order to suppress rRNA synthesis. The results showed that cells incubated with or without amino acids had similar amounts of [3H]uridine incorporated into RNA species other than the 45S rRNA. Furthermore, no difference in [3H]uridine RNA was detected at various times of cell incubation in media with different concentrations of amino acids.

The low accumulation of 45S rRNA in cells incubated with a small amount of amino acids could thus be caused by its rapid degradation, by inhibition of the RNA polymerase which is involved in rRNA synthesis, or by less template availability for the transcription of the ribosomal cistrons. Since activation of a ribonuclease that specifically digests 45S rRNA seemed very unlikely and extremely difficult to explore, the activity of the RNA polymerase was examined. Nucleolar

§ The incorporation of [3H]-labeled leucine in cells that were incubated in this condition was only 25% of that obtained when amino acids were added to the medium.
RNA polymerase was assayed from isolated nuclei that were obtained from cells incubated in various amounts of amino acids.

DNA-dependent RNA polymerase activity in Ehrlich cell nuclei

As performed under the conditions described in Table 2, the RNA polymerase assay was inhibited by actinomycin D, which showed its DNA dependence. The reaction was dependent upon the presence of all four ribonucleotide triphosphates. The uptake of nucleotides into RNA proceeded optimally at pH 7.9 and was Mg++ dependent. At a low salt concentration and 4 mM Mg++, the reaction was slightly inhibited by α-amanitin. This drug only inhibits RNA polymerase II (11). A slight inhibition was observed in nuclei obtained from cells incubated for 30 or 150 min with low or high concentrations of amino acids (Table 2). The incorporation of UMP and GMP into the newly synthesized RNA was what would be expected for rRNA synthesis, with the assumption that in 45S RNA, there are about 66% guanylic and cytidilic acids, compared to 34% adenylc and uridylic acids. These results were consistent with previous observations (7, 9) that incubation of isolated nuclei with a low salt concentration and Mg++ mainly assayed the RNA polymerase, which synthesized rRNA (nucleolar RNA polymerase or polymerase I; see ref. 11). The incorporation of GMP into RNA was consistently higher in nuclei obtained from cells incubated in a medium enriched with amino acids than in a medium low in amino-acid content.

The increase of the nucleolar polymerase activity could be caused by an increase in the rate of transcription or in the number of growing RNA chains. In the experiment described in Fig. 2, it is shown that the kinetics of GMP incorporation had a similar time course in nuclei isolated from cells incubated in media with high or low amino-acid content. However, the rate of GMP incorporation and final plateau level were less in nuclei isolated from cells incubated in a medium with low amounts of amino acids. The reaction stopped very soon in both cases, which suggests that elongation and termination of ribonucleotide chains were the only RNA polymerase activities that the isolated nuclei had (8).

The time course of the augmentation of nucleolar RNA polymerase activity of isolated nuclei is shown in Fig. 3. Soon after the cells were exposed to a medium with high concentrations of amino acids, a rapid increase in activity of the enzyme occurred. After about 20 min of incubation, this activity levelled off, but a second burst of enzymatic activity was detected after 60 min of incubation. A similar pattern of increased enzymatic activity was seen in cells incubated with low or high concentrations of amino acids.

Table 1. Incorporation of [3H]uridine into RNA in cells incubated with low amounts of actinomycin D

<table>
<thead>
<tr>
<th>Medium</th>
<th>Medium minus amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9,340</td>
</tr>
<tr>
<td>1.5</td>
<td>11,000</td>
</tr>
<tr>
<td>2.5</td>
<td>8,320</td>
</tr>
</tbody>
</table>

All amounts are cpm, incorporated in 10-min pulse. The cells were incubated in the presence of 0.04 μg/ml of actinomycin D as described in Methods. At the times indicated, triplicate samples containing 1.2 × 10⁶ cells were pulsed for 10 min with 5 μCi/ml of [3H]uridine. The cells were harvested, washed twice with cold saline, and precipitated with 7 ml of 10% trichloroacetic acid containing 0.2 μM/ml of cold uridine. The precipitate was collected by filtration and washed four times with 7 ml of 10% trichloroacetic acid. The Millipore filters were placed in vials, the precipitate was dissolved in 0.4 ml of concentrated formic acid and counted as described (18).

Fig. 2. Kinetics of the RNA polymerase reaction in isolated nuclei of Ehrlich ascites cells. The cells were incubated in Eagle's medium with or without amino acids for 40 min. At that time, the nuclei were isolated and the RNA polymerase activity was measured as described in Methods. O—O, nuclei from cells incubated in Eagle's medium. Δ—Δ, nuclei from cells incubated in Eagle's medium without amino acids.

Fig. 3. Kinetics of RNA polymerase activation by amino acids. RNA polymerase activity of nuclei isolated from Ehrlich ascites cells that were incubated for different times in Eagle's medium with (——) and without (---) amino acids. At the times indicated, the nuclei were isolated and the RNA polymerase activity was measured as described in Methods. The results are expressed in percent of RNA polymerase activity. 100% is the RNA polymerase activity in the nonincubated cells.
low concentrations of amino acids. However, the amount of 
GMP incorporated into RNA was consistently higher in cells 
incubated in a medium enriched with amino acids than in a 
medium low in amino-acid content. The nonlinear nature of 
the elevation in enzymatic activity that occurred during 
incubation with amino acids indicates the complexity of this 
phenomenon and suggests that more than one metabolic 
process may be involved.

**DISCUSSION**

The results of this study bear upon the role of amino acids 
in controlling the activity of nucleolar RNA polymerase and 
upon the broader question of regulation of rRNA synthesis in 
eukaryotic systems. To our knowledge, this is the first 
demonstration that amino acids can regulate nucleolar RNA 
polymerase in animal cells.

The data presented in this paper clearly indicate that 
varying the amount of amino acids in the culture medium 
neither affects the processing of ribosomal precursor nor the 
assembly of the ribosomal subunits. However, the nucleolar 
RNA polymerase is highly sensitive to these changes. Ap-
parently, this seems to be a rather selective action, because 
at least the amount of heterogenous nuclear RNA synthesized 
in a 10-min pulse was not affected (see Table 1).

It has been reported that 45S rRNA synthesis is only 
slightly depressed in puromycin-treated or methionine-
starved HeLa cells (16, 17). On the other hand, it has been 
reported that cycloheximide strongly depresses rRNA syn-
thesis in HeLa cells (18) and rat liver (19, 20). In line with 
the latter report, we find that when cells are incubated in an 
enriched amino-acid medium, the presence of puromycin 
prevents an increase in RNA polymerase activity (un-
published results). The fact that puromycin prevents the 
increase in the RNA polymerase activity that is induced by 
amino acids indicates that protein synthesis is involved in the 
control of rRNA synthesis.

There is a clear-cut difference between the effect of the 
inhibition of protein synthesis by antibiotics and that by low 
centersations of amino acids, on the repression of rRNA 
synthesis. Thus, when protein synthesis is inhibited by anti-
biotics, in addition to depression of rRNA synthesis, there is 
a profound inhibition of ribosome maturation (16–18). On 
the other hand, when protein synthesis is depressed by 
cultivating the cells in a medium low in amino acids the 
maturaon and processing of the ribosomal precursor are not 
affected. This indicates that amino acids affect the synthesis 
of rRNA in a more selective way than puromycin or cyclo-
heximide.

One must then ask whether amino acids control the nucleolar 
RNA polymerase activity directly, or indirectly by their effect 
on protein synthesis. If the control is indirect, then it must be 
only for proteins involved in the function of the ribosomal 
geneome.

Three mechanisms by which amino acids may control the 
synthesis of rRNA can be postulated: (a) an effect on 
the function or synthesis of some factor(s) necessary for the 
binding of the enzyme to the template, (b) alteration of the 
availability of the DNA template that codes for the ribosomal 
cistrons, and (c) control of the amount of RNA polymerase, 
either by affecting the synthesis of the enzyme or some of its 
subunits. The latter postulate assumes that the amount of 
RNA polymerase is the limiting factor within the cells. How-
ever, this seems unlikely because recent observations obtained 
in *Xenopus laevis* embryos demonstrated that no correlation 
exists between the levels of enzymatic activity and the amount 
and type of RNA synthesis (21). Assumptions b and c suggest 
that amino acids act as metabolites to alter the function of the 
ribosomal genome. The similarity between this amino-
acid control of nucleolar RNA polymerase and stringent 
control of rRNA synthesis in bacterial cells makes it at-
ttractive to assume that a similar phenomenon underlies both 
processes. In view of the difficulty in obtaining a mutation of 
the genetic locus that controls rRNA synthesis in eukaryotic 
cells, such a conclusion seems to be premature. Nevertheless, 
the possibility of having temperature-sensitive mutants of 
DNA-dependent RNA polymerase in yeast (A. O. Pogo and

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**Table 2. Properties of the DNA-dependent RNA polymerase reaction in isolated nuclei of Ehrlich ascites tumor cells**

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Incubation time (min)</th>
<th>Antibiotics* (µg)</th>
<th>pmol of nucleotides incorporated/mg of DNA</th>
<th>Ratio of UMP to GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eagle’s</td>
<td>150</td>
<td>30 α-Amanitin</td>
<td>2500</td>
<td>0.39</td>
</tr>
<tr>
<td>Eagle’s</td>
<td>150</td>
<td>100 Actinomycin D</td>
<td>2200</td>
<td>0.39</td>
</tr>
<tr>
<td>Eagle’s</td>
<td>150</td>
<td>30 α-Amanitin</td>
<td>50</td>
<td>0.39</td>
</tr>
<tr>
<td>Eagle’s without amino acids</td>
<td>150</td>
<td></td>
<td>630</td>
<td>0.39</td>
</tr>
<tr>
<td>Eagle’s without amino acids</td>
<td>150</td>
<td></td>
<td>20</td>
<td>0.39</td>
</tr>
<tr>
<td>Eagle’s</td>
<td>35</td>
<td>30 α-Amanitin</td>
<td>970</td>
<td>0.39</td>
</tr>
<tr>
<td>Eagle’s</td>
<td>35</td>
<td>20 α-Amanitin</td>
<td>980</td>
<td>0.39</td>
</tr>
<tr>
<td>Eagle’s</td>
<td>35</td>
<td>30 α-Amanitin</td>
<td>980</td>
<td>0.39</td>
</tr>
<tr>
<td>Eagle’s without amino acids</td>
<td>35</td>
<td></td>
<td>620</td>
<td>0.39</td>
</tr>
<tr>
<td>Eagle’s without amino acids</td>
<td>35</td>
<td></td>
<td>620</td>
<td>0.39</td>
</tr>
<tr>
<td>Eagle’s</td>
<td>40</td>
<td></td>
<td>1550</td>
<td>0.39</td>
</tr>
<tr>
<td>Eagle’s without amino acids</td>
<td>40</td>
<td></td>
<td>750</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Incubation of the cells, isolation of the nuclei and assay of the RNA polymerase were performed as described in Methods. When the incorporation of UMP was measured, the [3H]GTP was replaced with [3H]UTP in the reaction.

* Total amount was added in a final volume of 0.25 ml (Methods).
M. Shalem, unpublished results) promises to be a useful system to clarify this important point and to understand the problem of ribosomal RNA regulation in eukaryotic cells.

We thank Miss Margalith Shalem for technical assistance and Dr. Robert Hirsch for help in preparing the manuscript. This work was supported by grant no. HE-09011 from the National Institutes of Health: Health, Heart, and Lung Institute, and Division of Research Resource.