SUMMARY

We studied the effects of asparagine depletion on macromolecular synthesis in an asparagine-dependent line of the 6C3HED lymphoma maintained in suspension culture. Asparagine was removed from the culture by the asparaginase present in guinea pig serum or by deleting it in formulating the medium. Protein, RNA, and DNA synthesis were measured by the incorporation of labeled precursors and the relative rates of synthesis of the individual types of RNA were estimated by a dual-label technique which involves chromatographic fractionation. Guinea pig serum caused a reproducible sequence of inhibitions of macromolecular synthesis. Protein synthesis declined first with biphasic, exponential kinetics. An initial precipitous decline was followed by a more gradual falling off. Accompanying the second phase was an exponential decline in DNA synthesis with a similar half-life. Dual-label autoradiography showed that the inhibition of DNA synthesis was not caused by blocking of the initiation of S-phase but by slowing the rate of DNA synthesis in each cell with a resultant prolongation of S-phase. Omission of asparagine from the medium resulted in a similar sequence of inhibition of macromolecular synthesis but of more gradual onset and progression. Cycloheximide, a specific inhibitor of protein synthesis, rapidly produced a similar sequence of inhibition of nucleic acid synthesis. The effects of asparaginase on nucleic acid synthesis thus seem to be secondary to the inhibition of protein synthesis.

INTRODUCTION

It is now recognized that certain transplantable tumors are auxotrophic for asparagine. This followed from the observations that normal guinea pig serum had dramatic antitumor activity in vivo for the Gardner lymphosarcoma (6C3HED Asn− lymphoma) (26), and that the effective agent was L-asparaginase (EC 3.5.1.1) (7, 8). Mutant lines of the 6C3HED tumor which were not dependent on exogenous asparagine could be selected by their ability to replicate in vitro in the absence of the amino acid (8). These asparaginase-resistant lines (6C3HED Asn+) have high levels of asparagine synthetase (34, 37), an enzyme which is inducible under conditions of asparagine depletion. Recently, studies have been conducted on the effects of GPS2 (45) and of asparagine deletion (10) on macromolecular synthesis in 6C3HED Asn− cells. Both studies showed a rapid decrease in protein synthesis. A subsequent decrease in precursor incorporation into RNA and DNA were observed by Sobin and Kidd (45), who noted the analogy between this and the cessation of DNA synthesis after the inhibition of protein synthesis in other systems.

Because deprivation of a required amino acid has revealed the presence of important regulatory mechanisms for nucleic acid synthesis in auxotrophic bacteria (29), we have explored the effects of L-asparagine deprivation on nucleic acid metabolism in the 6C3HED lymphoma cell in the hope of observing some similar mechanisms and simultaneously exploring the derangement in macromolecular synthesis caused by L-asparaginase in the sensitive line of lymphoma cells.

MATERIALS AND METHODS

Culture of 6C3HED Lymphoma Cells. Cultures of 6C3HED lymphoma cells were established from the ascitic form of the Gardner lymphosarcoma, which was carried in C3H mice by intraperitoneal injection of 0.4 ml ascites fluid obtained after 1 week of growth. The cells used for culture were obtained after the injection of 0.5 ml sterile 0.02% Versene in 0.9% NaCl solution into the ascites to prevent clotting on harvesting. The cells were then centrifuged out, and the ascites plasma was removed. The cell pellet was resuspended in

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growth medium at a concentration of 1.5 × 10⁶ cells/ml. The cells grew readily in suspension in McCoy's 5A spinner medium (Grand Island Biological Company) supplemented with amino acids at 25% of the concentration present in Eagle's modified medium (15) and 25% fetal calf serum. The cell concentration was maintained between 2 and 6 × 10⁶ cells/ml and the cells were doubled in 25 to 35 hours. The cultures were fed daily by replacing approximately half the culture volume with fresh medium to reduce the cell concentration. The cell line has remained susceptible to the inhibitory effects of GPS asparaginase. Because this line demonstrates an asparagine dependence, in conformity with genetic terminology, it will be referred to as 6C3HED Asn- to distinguish it from the asparaginase-resistant, asparagine-independent variants which occur (6C3HED Asn+) and which have been isolated from this line. (A. M. Fabrizio, in preparation). Cell viability was estimated by eosin exclusion. All cell counts were performed with hemocytometers.

GPS was obtained from Dr. Helga Suld who ascertained that its asparaginase level was 2.61 i.u./ml before and 2.55 i.u./ml after heat inactivation of nonspecific factors (in which form it was used) for 30 min at 56°. The method of assay has been previously described (48). Cycloheximide was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Measurement of Rates of Synthesis of Macromolecules. Experiments were of two basic types. First, for the determination of the rate of total protein and total ribonucleic acid synthesis or total nucleic acid synthesis the incorporation (during a prescribed interval) of uniformly labeled leucine-¹⁴C (Nuclear-Chicago, Des Plains, Ill. 6.6 mCi/mmole), uridine-5-³H or cytidine-5-³H (Nuclear-Chicago, 1 to 5 Ci/mmole), respectively, at 37° into the acid-insoluble components of a given number of cells was used. Where the temporal sequence of changes in these parameters was to be determined 2 suspended cell cultures, the control and the experimental, were set up in spinner flasks. Duplicate 1-ml aliquots were taken from the suspensions at the required periods and incubated at 37° in stoppered 10-ml Erlenmeyer flasks with the 2 labeled precursors, with shaking for the requisite interval. At the end of the incubation the flasks were chilled in ice, diluted with 5 ml ice-cold balanced saline and the cells filtered onto cellulose acetate membranes (pore size, 5 μ). The residue in the flasks was rinsed onto the membranes with another 5-ml aliquot of the cold balanced saline. The membranes were washed free of acid-soluble materials with 2-ml volumes ice-cold 5% trichloroacetic acid in succession, placed in a scintillation vial, and digested overnight with 0.5 ml Nuclear-Chicago solubilizer to render the incorporated isotopes into a toluene-soluble form. Radioactivity was determined in a liquid scintillation counter after the addition of a scintillation mixture (Spectrafluor, Nuclear-Chicago) as previously described (18). Quench correction and cross-contamination of isotopes was determined with the channel ratios method with an external standard; polynomial curve fitting to the standards and the calculations were performed with an IBM 360/30 computer.

Blank values for the membranes were determined by refiltering the medium from a sample through another membrane and processing it in the same fashion as the membrane supporting the cells. Cell-associated counts were more than 100 times greater than these background values, which were, however, duly subtracted.

Second, for the determination of the relative rates of synthesis of the individual nucleic acid species between 2 different cultures the recently described dual-label method (18) was used. Briefly, the 2 cultures to be compared (e.g., asparagine depleted and control) were divided into 2 aliquots of 5 ml each (2 to 5 × 10⁶ cells/ml). Unlabeled cytidine (to a final concentration of 10 µg/ml) was added to all aliquots and then to one of each pair cytidine-5-³H (5 Ci/mmole) and to the other cytidine-¹⁴C (6.8 mCi/mmole to 0.5 µCi/ml) was added. The aliquots were placed in a metabolic shaker at 37° for 40 min. At the end of this time the suspensions were chilled and the cells were removed by centrifugation (1000 × g, 3 min). The cell pellets were lysed into the usual solution of sodium dodecyl sulfate, and the ¹⁴C-labeled control was mixed with the ³H-labeled experimental lysate (Set I) and vice versa (Set II). The 2 mixed lysates were then deproteinized, the nucleic acids were precipitated with ethanol, washed, and analyzed by methylated albumin-Kieselguhr chromatography as detailed elsewhere (18). The experimental results are expressed as the rate of synthesis of a particular nucleic acid species in the treated (e.g., asparagine depleted) cells as a percentage of its rate in the control cells. The calculations depend only on the ³H/¹⁴C ratios of the peak fractions of the separated nucleic acids and provide a value analogous to a geometric mean of the 2 separately analyzed groups (Sets I and II). The values were calculated with the formula

\[
100 \times \sqrt{\left(\frac{[3H/14C]}{[14C/3H]}\right) \times \left(\frac{[14C/3H]}{[3H/14C]}\right)}.
\]

The method minimizes column-to-column variation and eliminates any procedural differences in handling the specimens which might otherwise influence the analysis of the nucleic acids from each group. It also cancels out any bias the cells have in handling the 2 different isotopes.

When the cell concentration differed between the 2 groups (as in Tables 3 and 4) the relative rates were corrected for this difference by multiplying the relative rates by: viable cell concentration of control sample/viable cell concentration of treated sample (cf. Ref. 18).

The variation in the method stems from 2 sources: random counting errors and cell counting and sampling errors. The first leads to an error which is expressed in the tables as the 95% confidence interval about the least certain value, which was for the nucleic acid sample which provided the fewest counted disintegrations. The formula used for calculating the 95% confidence interval of the product \([\left(\frac{3H/14C}{14C/3H}\right)] \times \left(\frac{[14C/3H]}{[3H/14C]}\right)\) was

\[
a = \sqrt{2} \left(\frac{1}{H_1} + \frac{1}{C_1} + \frac{1}{H_2} + \frac{1}{C_2}\right)
\]

where \(H_1\), \(C_1\), \(H_2\), \(C_2\) are the means of the counts recorded for the duplicate aliquots for \(^3\)H and \(^{14}\)C in the 2 sets of columns. Because the relative rates of synthesis are the
where $h_1$, $h_2$, $c_1$, $c_2$, are the actual number of disintegrations counted in the samples from the 2 sets of tubes (i.e., not means of duplicates as for the other nucleic acid fractions). It represents a maximal variation for evaluating the significance of difference between the values for the individual nucleic acid species within an experiment. The second source of variation leads to a factor of uncertainty which affects all the values to the same extent and produces a 95% confidence interval of approximately 10% for the overall difference between treated and control group.

For assessment of the rate of maturation of rRNA in relation to the rate of transcription of rRNA, we made use of the fact that MAK chromatography crudely separates the species of rRNA, which elute in the order 18 S, 18 S, and 28 S and high molecular weight precursors (Q$_2$-RNA) (17). The resolution is poor, but by analysis of each successive tube and determination of the relative rate of isotope incorporation into the broad classes of mature and precursor RNA, an approximate estimation of the rate of maturation can be made. Tubes from the elution of Set I and Set II nucleic acids resulting from the dual-label design (see above), which corresponded to one another on the basis of their position in relation to the absorbance marker profile of the rRNA, were matched. The relative rate of synthesis of RNA in that region of the rRNA elution was calculated from the $^3$H/$^14$C ratios of the matching tubes, yielding a value $R_f$ for Tube t. With the short labeling periods used in this study the DNA-like RNA which elutes with salt contributed a significant amount of the isotope present in the rRNA zone. Because D-RNA was less responsive to the changes being studied the contamination reduced the real magnitude of the alteration in rates of synthesis of rRNA. As can be seen in Chart 1 some isotope was incorporated into RNA in the sample treated with actinomycin D (which suppressed rRNA synthesis), which eluted with the absorbance peak of rRNA, in front of the Q$_2$-RNA peak. It has previously been shown that this material resembles D-RNA in composition rather than rRNA (12). The actual amount of isotope in D-RNA in each tube across the rRNA zone of the control cell elution can be calculated if the assumption is made that actinomycin D treatment does not alter the relative amounts of D-RNA eluting as Q$_2$-RNA and tenaciously bound D-RNA. In this regard there is no significant difference in the relative amounts of Q$_2$-RNA and TD-RNA from HeLa cells labeled in the presence of the same concentration of actinomycin D for periods ranging from 20 min to 2 hr (K. A. O. Ellem and S. L. Rhode, unpublished results). The formula giving the proportion ($p_f$) of isotope present in D-RNA in a Tube t in the rRNA zone of an elution is: $p_f = d_f / d_p$, where $d_p$ is the amount of isotope in the TD-RNA fraction which is used as a measure of the total D-RNA fraction because it is relatively uncontaminated with isotope in other nucleic acid moieties (17) and $d_f$ is the total amount of isotope in Tube t. $d_{p}$ is the ratio of D-RNA in a tube of corresponding position, relative to the A$_{260}$ profile, to the TD-RNA of the actinomycin D-treated sample in Chart 1. The values for $p_f$ are listed in Table 1 and these are similar to values calculated for HeLa cells. The relative rates of isotopic incorporation into rRNA for each tube were then corrected for the contaminating D-RNA by using the easily derived formula

$$R_f = \left(\frac{R_{RTD}}{R_{TD}}\right) \left(\frac{1 - p_f}{1 - p_{RTD}}\right)$$

where $R_{RTD}$ is the uncorrected relative rate of RNA synthesis in Tube t and $R_{TD}$ is the relative rate of TD-RNA synthesis.

**Autoradiography:** After exposure of the cells to labeled precursor they were centrifuged, washed, fixed in ethanol: acetic acid (3 : 1), and air dried on glass slides. The slides were then coated with NTB-3 emulsion, exposed, developed, and stained with Giemsa. When $^3$H and $^14$C were both present in a specimen the slides were further treated by coating the first developed emulsion with celloidin to filter out $^3$H disintegrations and developed. This method shows $^14$C labeling in the second emulsion layer and $^3$H plus $^14$C in the first layer (2).

**RESULTS**

**Effects of GPS on Total Macromolecular Synthesis.** Sobin and Kidd (45) studied the effects of normal GPS on macromolecular synthesis of 6C3HED Asn$^-$ cells growing *in vivo* in the ascites form. To confirm their overall conclusions we have studied the effects of GPS on 6C3HED Asn$^-$ cells growing *in vitro* in greater detail because of the greater reproducibility of the *in vitro* system. The overall rates of protein, RNA, and DNA synthesis were estimated by measuring the amount of leucine-$^14$C, uridine-$^5$H, and thymidine-$^3$H, respectively, incorporated into aliquots of a suspension culture during a definite interval of incubation after various periods of time had elapsed after the addition of GPS to the culture. In Chart 2A a comparison of the effects of GPS on protein and RNA synthesis over a 16-hr period is illustrated. Two aliquots were taken from a culture 4 hr after routine medium replenishment. To one of them GPS was added to a final concentration of 2%. At intervals thereafter, duplicate one 1-ml aliquots were removed from each and the rates of protein and RNA synthesis measured by the simultaneous incorporation of leucine-$^14$C (0.5 $\mu$Ci/ml) and uridine-$^5$H (1 $\mu$Ci/ml) for 1 hr. The acid-soluble isotope was removed and the cells were solubilized and counted as in "Materials and Methods." The rate of incorporation into the GPS-treated cells is expressed as a percentage of the control cell rate. The dramatic fall in leucine-$^14$C incorporation into protein within the first 2 hr after adding the GPS was characteristically biphasic. The inhibition of RNA synthesis was more gradual and was not obviously biphasic. Because the effects of adding GPS was precipitous a more detailed study was made of macromolecular synthesis in the first 2 hr of GPS action. Charts 2B and 2C show a comparison of DNA, RNA, and protein synthesis in cells taken from a culture 24 hr after refedding in the presence and in the absence of 2% GPS. Protein synthesis was most rapidly affected by GPS so that its rate
K. A. O. Ellem, A. M. Fabrizio, and L. Jackson

TUBENUMBER

Chart 1. MAK elution profiles of a mixture of nucleic acids from 6C3HED lymphoma cells labeled for 40 min with cytidine-\(^3\)H (control cells) and cytidine-\(^14\)C (cells exposed to actinomycin D 0.03 µCi/ml showing selective inhibition of isotope incorporation into Q1-RNA and rRNA. For simplicity the tRNA and the DNA eluted from this column are omitted. The portions of the chromatogram shown are the ribosomal RNA complex (Tubes 30 to 40), and the elution of tD-RNA with a convex gradient of guanidine thiocyanate (from 0 to 6M, at 35°) followed by a wash with 6M guanidine thiocyanate at 80° (tubes 65 to 90) (8). The absorbances across the ribosomal peak, \(--\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\·
When DNA is the primary target of the effects under study, the effect is measured as a percentage of the control rate, during the 40 min after the elapsed hours of asparagine depletion. The determinations were made by the dual-label technique and MAK chromatography as described in the text.

Inhibition of nucleic acid synthesis by omission of asparagine from the medium of 6C3HED cells

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Hours after depletion of asparagine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>tRNA</td>
<td>95.3%</td>
</tr>
<tr>
<td>tRNA + 5 S RNA</td>
<td>98.3%</td>
</tr>
<tr>
<td>L-1 RNA</td>
<td>97.0%</td>
</tr>
<tr>
<td>DNA</td>
<td>71.4%</td>
</tr>
<tr>
<td>rRNA</td>
<td>99.1%</td>
</tr>
<tr>
<td>Q1-RNA</td>
<td>100.0%</td>
</tr>
<tr>
<td>Q2-RNA</td>
<td>94.6%</td>
</tr>
<tr>
<td>Total nucleic acids</td>
<td>94.4%</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>82.1%</td>
</tr>
</tbody>
</table>

- The values represent the rate of synthesis of the nucleic acid fraction as a percentage of the control rate, during the 40 min after the elapsed hours of asparagine depletion. The determinations were made by the dual-label technique and MAK chromatography as described in the text.
- L-1 RNA possibly represents 5 S RNA.
- The DNA values were calculated from the amounts of isotope incorporated into the DNA peak as it elutes from the MAK column. A small amount of the isotope (approximately 10%) is present in RNA, and this tends to reduce the magnitude of the change in DNA synthesis when DNA is the primary target of the effects under study. The effect is small as can be seen from the value in parentheses which was calculated from the alkali-resistant isotope present in the DNA peak.
- As described in "Materials and Methods" the 95% confidence interval was calculated for the samples with the least number of recorded counts. This figure defines the maximal interval of uncertainty at this level of significance for comparison of rates between different nucleic acids within an experiment.

Asparaginase inhibition of macromolecular synthesis

Asparagine from the medium. This also occurs in bacteria starved for an amino acid for which they are auxotrophic (e.g., Maaloe and Kjeldgaard (29)) and is selective for rRNA in them (33). Therefore, we next examined the rate of synthesis of the individual nucleic acid types for any signs of selectivity of the asparagine deprivation on them.

MAK Analysis of Lymphoma Nucleic Acids. The method used for analyzing the synthesis of the nucleic acids of 6C3HED cells was MAK chromatography. The elution pattern of the nucleic acids was found to be similar to those of other mammalian cells and, in particular, to that obtained by Lingrel (28) with rabbit bone marrow cells. The order of elution by a gradient from 0.3 to 1.6 M NaClO4.05 M tris-HCl, pH 6.7, was tRNA, tRNA and 5 S RNA, L1-RNA, Q1-RNA, and Q2-RNA, (e.g., Refs. 17, 39). L1-RNA has not been fully characterized but is rapidly labeled, is detected in both nucleus and in polysomes, and has a guanine + cytosine composition and sedimentation resembling 5 S RNA (39). It may represent some of the 5 S RNA of the cells. The bulk (60 to 70%) of the D-RNA was retained tenaciously by the MAK column, but it could be eluted by raising the pH of the eluting fluid, or by adding hot 1.6 M NaCl solution or 2% SDS (e.g., Ref. 17). Recently, it has been found that a concentration gradient of guanidine thiocyanate elutes the TD-RNA with some selectivity because it can fractionate the TD-RNA from different sources (19). The RNA types that eluted in the TD-RNA region were poorly resolved so that Q2-RNA was often not distinctly separated from Q1-RNA for periods of labeling longer than 30 min (18). This was especially noticeable with the lymphoma nucleic acids. For confirmation that a Q2-RNA moiety was present but obscured in the rRNA complex the selective and virtually complete inhibition of rRNA (and Q1-RNA) synthesis by low doses of actinomycin D was used (12, 18). Accordingly, aliquots of a suspension of cells were labeled in the presence or absence of actinomycin D (0.03 μg/ml) with cytidine-14C or cytidine-3H, respectively, 30 min after adding the actinomycin D. After 40 min incubation the cells were centrifuged (1000 X g, 3 min) and lysed in SDS. The 2 lysates were mixed and deproteinized; the nucleic acids were applied to a MAK column.

Chart 1 shows the pattern of rRNA and Q2-RNA eluted by the NaCl gradient and of TD-RNA eluted by a 0 to 6 M guanidine thiocyanate gradient. The salient features were the gross inhibition of incorporation of cytidine-14C (actinomycin D-treated cells) into the ribosomal RNA area, with relative sparing of RNA which eluted in a position corresponding to Q2-RNA (12, 18). There was a small amount of residual labeling of the RNA eluting with the A250 peak of rRNA (Tubes 31 to 34) which was less than the equivalent of 25% of the amount of isotope present in the same tubes from the control cells (cytidine-3H). This is mainly contributed by D-RNA (12). Thus, after a short incubation with labeled precursor, when relatively little of the isotope in RNA has been processed from precursor RNA (Q1-RNA) to mature 18 S and 28 S rRNA, a significant amount of the isotope in the tubes containing the 18 S and 28 S RNA was present as D-RNA. The values for this contamination (Table 2) represent upper limits and possibly overestimate it depending on the presence of residual rRNA synthesis. There was no selective inhibition of labeling across the guanidine thiocyanate elution pattern of TD-RNA except for a barely significant inhibition in the first peak (Tube 66) most easily seen in the fall in the 14C/3H ratio in that tube, and this can be attributed to inhibition of the small amount of isotope in the contaminating rRNA which elutes in that peak (19). As with other cells in culture (12, 18) this dose of actinomycin D has a 10-fold difference in its effect on the transcription of rRNA and D-RNA. Thus the D-RNA of 6C3HED cells is fractionated into Q2-RNA (about 40% of the D-RNA) and TD-RNA which constitutes the remaining 60% in a fashion similar to that of other cells.

The method used for quantitation of the rates of nucleic acid synthesis was the dual-label technique recently described (18) (see "Materials and Methods"). Because the two samples being compared by cochromatography may be recognized by the different isotopes in them the influence of asparagine depletion on Q3-RNA can be partially assessed despite the absence of a discriminable Q2-peak. Because Q1-RNA is a precursor to rRNA the rate of incorporation of labeled precursor into Q1-RNA is the best measure of the transcription rate of rRNA from the RNA cistrons, while the rate of appearance of label in mature rRNA is further influenced by the intermediate steps involved in the conversion (reviewed in Ref. 14). Any differences noted between the rates for rRNA and Q1-RNA incorporation may thus
Chart 2. Kinetics of inhibition of protein, RNA, and DNA synthesis after addition of GPS (2%) to cultures of 6C3HED Asn– cells. The synthesis of protein, RNA, and DNA were measured by the incorporation of leucine-14C, uridine-5-3H, and thymidine-5-3H, respectively, as described in the text. Protein and nucleic acid synthesis were measured simultaneously in the same samples by taking advantage of the 2 different isotopes. Each point is the mean of duplicate aliquots. A, decline of RNA and protein synthesis over a 16-hr interval after the addition of GPS to a 6C3HED Asn– culture. Synthesis in the GPS-treated culture is expressed as a percentage of the control value. Labeling time was 1 hr. B and C, more detailed comparison of the decline in RNA and DNA synthesis in relation to protein synthesis in the 2 hr after GPS treatment. The control and GPS-treated cells were with the precursors at the concentrations described in the text, for 10 min at the indicated time intervals. Bars, range of duplicate aliquots. Control values are plotted as open symbols; GPS-treated values are solid symbols. o—o, protein synthesis; o—o, RNA synthesis; o—o, DNA synthesis. D, detailed kinetics of GPS inhibition of DNA and protein synthesis. Cells were labeled for 10-min periods; aliquots were taken for labeling at 5-min intervals after addition of GPS to culture. Exponential decline of DNA synthesis (o—o) and biphasic exponential decline of protein synthesis (o—o) are revealed by the semilogarithmic plot.

indicate alterations in the rate of maturation of rRNA from its precursor.

Pattern of Inhibition of Nucleic Acid Synthesis Resulting from Asparagine Deficiency. The gradual decline in RNA synthesis caused by deletion of asparagine from the medium was analyzed for evidences of selectivity.

Two appropriately sized aliquots were taken from a culture and centrifuged (200 X g for 10 min). The cells were resuspended in McCoy's medium lacking asparagine. The control sample was made 0.34 mM with asparagine while the other was left deficient in asparagine. Both samples were then incubated in spinner flasks and aliquots were removed after different intervals of time for exposure to labeled cytidine for measurement of the rates of nucleic acid
Asparaginase Inhibition of Macromolecular Synthesis

Chart 3. Decline in RNA and protein synthesis in 6C3HED Asn− cells resuspended in McCoy's medium lacking in asparagine. The incorporation was measured and expressed as in Chart 2, control cells being resuspended in McCoy's medium containing asparagine. •—•, protein synthesis; ●—●, RNA synthesis.

synthesis by the dual-label technique described in “Materials and Methods.” Each aliquot was divided in 2 and cytidine was added to 10 μg/ml and cytidine-3H (2 μCi/ml) or cytidine-14C (0.5 μCi/ml) was added to 0 of each pair. After 40 min isotopic incorporation the cells were collected by centrifugation and the 3H-labeled cell pellet of 1 pair added to the 14C-labeled sample of the other, each mixture being lysed with SDS and deproteinized with phenol. The mixed samples (each containing a treated and control lysate) were processed and fractionated on MAK. The relative rates of incorporation were calculated from the 3H/14C ratios of either pooled fractions, or from individual tubes in the rRNA-Q2-RNA area of the elution, as described in “Materials and Methods.”

The values in Table 1 represent the percentage rate of synthesis of the individual nucleic acids of Asn−cells relative to the control cell rate. It can be seen that 3 hr after deletion of asparagine from the cells there was no significant depression of RNA synthesis in any of the RNA types. However, most significantly, there was already detectable an inhibition of precursor incorporation into DNA. By 6 hr (Column 2) the inhibition of DNA synthesis was much more marked (53%) and every RNA species now showed depressed incorporation. The inhibition of RNA synthesis was not uniform but was selectively marked on ribosomal RNA. The extent of inhibition of the transcription of ribosomal precursor Q1-RNA was 51%. The low-molecular-weight RNA’s (tRNA, 5 S RNA, and L1-RNA) were inhibited to the same extent as the DNA-like RNA of the cell, all about 35%.

With increase of the time of incubation of the cells in the absence of asparagine, the inhibition of incorporation of isotope into DNA became greater than 90% of the control rate (by 12 hr) while the synthesis of ribosomal and DNA-like RNA were also severely inhibited. The incorporation into tRNA and 5 S RNA was less inhibited.

The values calculated for rRNA synthesis were different when rates based on the isotope chromatographing in the 18 S to 28 S RNA area were compared with those of the Q1-RNA peak. Therefore, a tube-by-tube comparison of the rates of rRNA synthesis was made across the zone of elution of the rRNA complex to obtain an estimate of the maturation of rRNA, as explained in “Materials and Methods.” Table 2 embodies these results. The upper set of figures which have not been corrected for D-RNA contamination show a tendency, in the more severely inhibited cultures, for the matured RNA (18 S and 28 S rRNA) to be more inhibited than the precursor rRNA (Q1-RNA). The lower set of figures has been corrected for D-RNA contamination on the basis previously described and these show that the maturation process was more markedly inhibited than the transcription of the ribosomal precursor RNA (Q1) by a factor of 1/3 to 1/4 at the later time periods.

Pattern of Inhibition of Nucleic Acid Synthesis Induced by GPS. The rates of synthesis of the individual nucleic acids were determined in the 6C3HED cells after GPS had been added to the medium, relative to the same cells in normal medium. Two aliquots were taken from the same culture 4 hr after refedding to stabilize the stimulatory effect of fresh medium (K. A. O. Ellem, A. M. Fabrizio, and L. Jackson, in preparation), so that the cells would be in a similar nutritional status to those used in the preceding experiments on asparagine deletion. To one of them GPS was added to a final concentration of 2%, the other was left as a control. After incubation for different time intervals after adding the GPS, aliquots were taken, labeled with cytidine-3H or -14C as described in the previous section and analyzed by the dual-label MAK method. The results in Table 3 (Columns 1 and 2) show a similar sequence of inhibition of nucleic acid synthesis to that resulting from asparagine deletion tabulated in Table 2. The speed of the shutdown is, however, very much faster. Thus, in the interval 10 to 50 min after the GPS was added DNA synthesis fell by 60% and rRNA synthesis fell by 24%. After 2 hr of GPS action the pattern of inhibition of the nucleic acids resembled that of cells which had been deprived of asparagine for 17 hr (Table 2, Column 4). The same selectivity of inhibition, viz., DNA > rRNA > D-RNA > tRNA, was observed in both sequences.

Pattern of Inhibition of Nucleic Acid Synthesis after Inhibition of Protein Synthesis by Cycloheximide. The fall in nucleic acid synthesis always followed a decline in protein synthesis induced by the asparaginase state. For the examination of the hypothesis that the nucleic acid events could be simply secondary to the interruption of protein synthesis, the pattern of nucleic acid inhibition after drug-mediated termination of protein synthesis was explored. Aliquots of cells from a culture refed 4 hr previously were handled in a similar fashion to the previous experiments except that instead of adding GPS, cycloheximide was added to the "treated" group in a final concentration of 10 μg/ml. Columns 3 and 4 of Table 3 record the relative rates of synthesis of the individual nucleic acids in the intervals 10 to 50 and 60 to 100 min after the addition of the cyclo-
Table 2

Effects on the maturation of rRNA in 6C3HED Asn—cells of growth in the absence of asparagine

<table>
<thead>
<tr>
<th>RNA marker(^a)</th>
<th>A(_{260}) of 3 hr(^b)</th>
<th>Hours elapsed after deletion of asparagine from the medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Set I</td>
<td>Set II</td>
</tr>
<tr>
<td>18 S + 28 S RNA</td>
<td>0.140</td>
<td>0.140</td>
</tr>
<tr>
<td>(A_{260}) Peak</td>
<td>0.220</td>
<td>0.185</td>
</tr>
<tr>
<td>Q(_1)-RNA</td>
<td>0.160</td>
<td>0.120</td>
</tr>
<tr>
<td></td>
<td>0.095</td>
<td>0.100</td>
</tr>
<tr>
<td></td>
<td>0.070</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>0.045</td>
<td>0.040</td>
</tr>
<tr>
<td></td>
<td>0.035</td>
<td>0.035</td>
</tr>
<tr>
<td>TD-RNA = (R_{TD})(^d)</td>
<td>94.6 ± 1.7</td>
<td>63.3 ± 1.2</td>
</tr>
</tbody>
</table>

\(95\%\) confidence interval

Values corrected for D-RNA contamination \(R_f\)\(^e\)

<table>
<thead>
<tr>
<th>pf</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 S and 28 S RNA</td>
</tr>
<tr>
<td>(A_{260}) Peak</td>
</tr>
<tr>
<td>Q(_1)-RNA</td>
</tr>
</tbody>
</table>

\(R_{18S + 28S}/R_{Q_1}\) 1.07 0.54 0.25 0.36

\(a\)Marker indicates the predominant form of ribosomal RNA present in the tube. The predominant species present in the \(A_{260}\) peak is 28 S rRNA with much 18 S rRNA also present.

\(b\)For illustration of the method of tube matching in the ribosomal RNA elution zone the \(A_{260}\) readings for the 2 sets of tubes from the ribosomal RNA area of the elution from 2 dual-labeled analyses are given for the first time period. Where any difficulties in matching arose from phase differences between sets values were calculated for each of the 2 fits and the geometric means of these values were used.

\(c\)\(R_f\) is the relative rate of synthesis of RNA in each tube of the rRNA complex calculated by using the total isotope incorporated into each area.

\(d\)\(R_{TD}\) is the relative rate of synthesis of TD-RNA.

\(e\)\(R_f\) is the corrected value for rRNA using the formula which allows for D-RNA contamination: \(R_f = (R_f - p \times R_{TD})/(1 - p)\).

\(p_f\) is the fraction of total RNA of control cells present as D-RNA in each tube. The values were calculated as described in the text.

\(\delta\)These values do not take into account the possible effects which decrease in the maturation rate may have on the apparent rate of transcription due to delay in processing the precursors.

heximide. The patterns of shutdown in RNA synthesis were similar to those after the addition of GPS, and again DNA synthesis was most dramatically affected. The major difference was in the immediacy of the inhibition with the cycloheximide, which can be correlated with the almost instantaneous effectiveness with which it shuts off protein synthesis (13, 20, 56).

The rate of the maturation of rRNA from its precursor to the mature 18 S and 28 S RNA forms was again assessed by a tube-by-tube analysis of the rRNA complex eluted from MAK. Table 4 compares the results for GPS and cycloheximide. In both cases the processing of the precursor Q\(_1\)-RNA to stable 18 S and 28 S RNA components was rapidly and almost completely inhibited when the transcription process had been reduced only by \(1/2\) and \(1/3\). There was thus clear evidence of a marked dependence of the successful maturation of rRNA on concomitant protein synthesis.

Inhibition of protein synthesis by asparagine depletion or cycloheximide was not accompanied by any localized changes in the guanidine thiocyanate elution pattern of the TD-RNA. The inhibition of D-RNA synthesis was thus general.

Effects of GPS on S-Phase. Because the culture consists of unsynchronized cells it is possible that the effect of GPS on DNA synthesis was accomplished either by preventing new cells from entering S-phase or by decreasing the rate of DNA synthesis in all cells in S-phase or possibly both. In order to resolve this question the phenomenon was studied with a two-emulsion autoradiograph technique (2). A culture was exposed to thymidine-\(^{14}\)C for 60 min 14 hr after
Table 3

Inhibition of nucleic acid synthesis after exposure of 6C3HED cells to guinea pig serum and cycloheximide

The times listed are the periods for which the agent (guinea pig serum or cycloheximide) was present in the cell suspension before the isotope labeling began. The period of precursor incorporation was 40 min. All values and symbols are the same as those used in Table 1.

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Guinea pig serum (2%)</th>
<th>Cycloheximide (10 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min</td>
<td>2 hr</td>
</tr>
<tr>
<td>tRNA</td>
<td>93.7</td>
<td>38.8</td>
</tr>
<tr>
<td>tRNA + 3 S RNA</td>
<td>88.5</td>
<td>36.1</td>
</tr>
<tr>
<td>L-1 RNA</td>
<td>90.0</td>
<td>38.3</td>
</tr>
<tr>
<td>DNA</td>
<td>41.9</td>
<td>16.0</td>
</tr>
<tr>
<td>rRNA</td>
<td>60.7</td>
<td>22.0</td>
</tr>
<tr>
<td>Q1-RNA</td>
<td>76.0</td>
<td>28.2</td>
</tr>
<tr>
<td>TD-RNA</td>
<td>80.3</td>
<td>35.9</td>
</tr>
<tr>
<td>Total nucleic acids</td>
<td>72.0</td>
<td>29.4</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>± 2.5</td>
<td>± 3.3</td>
</tr>
</tbody>
</table>

Table 4

Analysis of the maturation of rRNA after inhibition of RNA synthesis by inhibition of protein synthesis with guinea pig serum and cycloheximide

<table>
<thead>
<tr>
<th>Elution marker</th>
<th>Guinea pig serum (2%)</th>
<th>Cycloheximide 10 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min</td>
<td>120 min</td>
</tr>
<tr>
<td>Values calculated from total ³H/¹⁴C ratios in each tube (Rf)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 S + 28 S RNA</td>
<td>60.7</td>
<td>22.0</td>
</tr>
<tr>
<td>A₂₆₀ peak</td>
<td>61.4</td>
<td>26.3</td>
</tr>
<tr>
<td>Q₁-RNA</td>
<td>76.0</td>
<td>28.2</td>
</tr>
<tr>
<td></td>
<td>76.6</td>
<td>27.9</td>
</tr>
<tr>
<td></td>
<td>77.7</td>
<td>30.7</td>
</tr>
<tr>
<td></td>
<td>79.3</td>
<td>32.2</td>
</tr>
<tr>
<td></td>
<td>81.4</td>
<td>33.1</td>
</tr>
<tr>
<td></td>
<td>79.6</td>
<td>34.8</td>
</tr>
<tr>
<td>TD-RNA = R_TD</td>
<td>80.3 ± 1.0</td>
<td>35.9 ± 2.0</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Values corrected for D-RNA contamination (Rf)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 S + 28 S RNA</td>
<td>24.2</td>
<td>3.9</td>
</tr>
<tr>
<td>A₂₆₀ peak</td>
<td>53.8</td>
<td>22.4</td>
</tr>
<tr>
<td>Q₁-RNA</td>
<td>75.5</td>
<td>27.3</td>
</tr>
<tr>
<td>Factor of maturation inhibition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 18 S + 28 S/RQ₁</td>
<td>0.32</td>
<td>0.14</td>
</tr>
</tbody>
</table>

*Values and symbols as for Table 2.

Asparaginase Inhibition of Macromolecular Synthesis

replicating with the serum. Control cells were similarly labeled but were not treated with GPS. Autoradiographs were prepared and scored as described previously. As can be seen in Tables 5 A and B the percentage of cells synthesizing DNA before and after GPS treatment, as well as the percentage of treated as compared to control cells in S-phase, remained essentially unaltered. On the other hand grain counts of the ³H layer show that the rate of incorporation of thymidine decreased with GPS treatment, as compared with the control cells, so that in this experiment the inhibition of DNA synthesis was 65% in the interval from 30 to 120 min. This indicates that the rate of DNA synthesis rather than the percentage of cells in S-period was affected by the asparaginase in the serum. The cells used in this experiment were taken from a culture which had been refed 14 hr previously, and were then resuspended in fresh medium. The replenished supply of nutrients caused an increase in the total number of grains over the nuclei of controls and GPS-treated cells from 30 to 90 min as had been found previously (K. A. O. Ellem, A. M. Fabrizio, and L. Jackson in preparation). The experiment was repeated with just the thymidine-³H to label the cells at defined times after adding GPS to 1 of a pair of aliquots from a culture which had not been refed in the previous 24 hr. The grain counts shown in Table 6 demonstrate the progressive decline in DNA synthesis after GPS and the slight decline in the control cells which can be attributed to the effects of nutrient depletion.

The results in Tables 5 A and B show that the decrease of DNA synthesis in the GPS-treated cultures was not due to large shifts in the population of cells in S-phase. Although the time interval of the experiment was small (90 min) an examination of the distribution of the 2 isotopes among the small population of cells which were labeled with only 1 isotope was determined because it would reflect the effects of GPS on the initiation of DNA synthesis (³H label only) and on the termination of S-phase (¹⁴C label only). The lower set of values represents the distribution of cells between initiation and termination. In a steady state condition a slight preponderance of ¹⁴C-labeled cells would be expected because the cells were labeled longer with ³H alone. This may imply either a reduction in the number of cells initiating DNA synthesis or possibly undue sensitivity of the DNA synthesis occurring at the end of S-phase which would make it difficult to discriminate between the light ³H- and ¹⁴C-labeled cells and the cells labeled with ¹⁴C alone. Further, more detailed autoradiographic study will be needed to determine which explanation is correct.

DISCUSSION

Production of the asparaginase-deaf state in cultures of 6C3HED Asn- lymphosarcoma cells is followed by a definite sequence of inhibitions of macromolecular synthesis. Protein synthesis declines first. This is closely followed by a fall in the rate of DNA synthesis. RNA synthesis is then depressed and follows a reproducible sequence temporally
Table 5A

**Effects of GPS on thymidine incorporation into 6C3HED Asn− Cells**

<table>
<thead>
<tr>
<th>Time after adding GPS (min)</th>
<th>³H grains/10 cells</th>
<th>Distribution of isotopes in 100 cells</th>
<th>Unlabeled</th>
<th>³H alone</th>
<th>¹⁴C alone</th>
<th>Both labels</th>
</tr>
</thead>
<tbody>
<tr>
<td>30  Control</td>
<td>416</td>
<td>20</td>
<td>9</td>
<td>8</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>154</td>
<td>21</td>
<td>4</td>
<td>4</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>60  Control</td>
<td>740</td>
<td>25</td>
<td>6</td>
<td>8</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>252</td>
<td>29</td>
<td>6</td>
<td>1</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>90  Control</td>
<td>840</td>
<td>22</td>
<td>3</td>
<td>5</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>308</td>
<td>28</td>
<td>4</td>
<td>2</td>
<td>66</td>
<td></td>
</tr>
</tbody>
</table>

*The grains in the first emulsion (³H and ¹⁴C) were counted over 20 cells by two observers. They were then corrected for the grains caused by ¹⁴C by counting and subtracting the grains in the ³H layer caused by ¹⁴C in 20 cells in parallel preparations, which had only been labeled with thymidine-³H. The increase in number of grains with time of incubation after the thymidine-¹⁴C labeling was due to the refeeding effects mentioned in the text.*

Table 5B

**Distribution of cells with only one isotope present (100 cells counted)**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>³H</th>
<th>¹⁴C</th>
<th>³H/¹⁴C</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 Control</td>
<td>30</td>
<td>70</td>
<td>0.43</td>
</tr>
<tr>
<td>Serum</td>
<td>20</td>
<td>80</td>
<td>0.25</td>
</tr>
<tr>
<td>60 Control</td>
<td>45</td>
<td>55</td>
<td>0.82</td>
</tr>
<tr>
<td>Serum</td>
<td>22</td>
<td>78</td>
<td>0.28</td>
</tr>
<tr>
<td>90 Control</td>
<td>41</td>
<td>54</td>
<td>0.76</td>
</tr>
<tr>
<td>Serum</td>
<td>11</td>
<td>89</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*One hundred cells were counted which were labeled with either ³H or ¹⁴C, but not both, according to the method outlined in the text. Four separate counts of 100 cells each on the same slide gave a standard deviation of ± 2 cells for the apportioned values for ³H or ¹⁴C, which was assumed to be the inherent error for the other counts, all done by the same observer.*

Table 6

**Inhibition of DNA synthesis by guinea pig serum in cells in nutrient depleted medium**

<table>
<thead>
<tr>
<th>Time after adding GPS (min)</th>
<th>No. of grains/10 cells</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30  Control</td>
<td>194</td>
<td>29.9</td>
</tr>
<tr>
<td>60  Control</td>
<td>184</td>
<td>34.2</td>
</tr>
<tr>
<td>90  Control</td>
<td>178</td>
<td>49.4</td>
</tr>
<tr>
<td>120 Control</td>
<td>168</td>
<td>53.6</td>
</tr>
</tbody>
</table>

*Cells were labeled for 30 min with thymidine-³H (1 μCi/ml) after the indicated time of exposure of the treated aliquot to guinea pig serum. Autoradiograms were made and the number of grains in 50 cells were counted and averaged to a 10-cell unit.*

and quantitatively of rRNA > D-RNA > low-molecular-weight RNA's > tRNA.

**Mechanism of Inhibition of Protein Synthesis.** The interruption of protein synthesis by removal of a supply of asparagine from 6C3HED Asn− cells is well documented (44, 45). It is caused by the inability of this tumor to synthesize the amino acid in adequate amounts owing to a deficiency in asparagine synthetase, an enzyme which is present and inducible in strains of the lymphoma which are independent of asparagine (Asn+)(34, 37). Removal of the external supply is followed by rapid depletion of the cell pool of asparagine (9), and the consequent low level of asparaginyl-tRNA presumably becomes rate limiting for peptide synthesis when an asparagine group is needed to continue the obligatory sequence of amino acid insertion into the polypeptides being synthesized (41). The asparagine limitation inhibits the assembly of asparagine rich proteins more than those with little of this amino acid (41).

The present observation that the inhibition of amino acid incorporation into protein is resolved into an initial rapid exponential decline followed by a more gradual one is unexplained. The initial phase could perhaps be caused by a pool expansion of leucine in the cell, although Broome (9) has found evidence of a large increase in the pool size of only aspartic acid in a similar system.

The decline in nucleic acid synthesis was found to be similar in sequence differing only in rapidity, whether the interruption of protein synthesis was caused by deletion of asparagine or inhibition by cycloheximide. The differences in speed can be correlated with the fact that cycloheximide causes almost instantaneous cessation of protein synthesis in mammalian cells at the concentration used here (13, 20, 56), whereas nutritional deprivation leads to a very slow decline (half-life 9 hr) (possibly due to a small contribution of asparagine from the fetal calf serum), while the fall of protein synthesis caused by GPS is intermediate between these (half-life of the first 60% loss was 11.1 min; subsequent decay had a half-life of 84 min).

The gross anabolic derangements which result from GPS asparaginase seem to stem from the inhibition of protein synthesis. They do not specify the mode of cell death of Asn− cells, but an explanation of this could perhaps be sought in any of several metabolic systems. It is useful, however, to consider the possible mechanisms of the effects...
which seem to be secondary to the inhibition of protein synthesis.

**Mechanism of Inhibition of DNA Synthesis.** The inhibition of DNA synthesis by asparagine depletion like that caused by cycloheximide is most probably secondary to the inhibition of protein synthesis, and this phenomenon has been the subject of considerable investigation. Studies with cells and organisms other than the lymphoma cells used in this investigation also have shown that antibiotics such as puromycin (32, 36, 42, 52) and cycloheximide (3, 25, 56), which act on protein synthesis will cause a reduction in the rate of DNA synthesis in the individual cell. In addition more physiological means of retarding protein synthesis, such as depletion of 2 essential amino acids in *Tetrahymena pyriformis* (47), will also retard DNA synthesis.

The simultaneous replication of histones and DNA in eukaryotic organisms was noted cytologically many years ago (1, 4, 5, 38). The concurrence and reciprocal dependence of histone and DNA synthesis has recently been demonstrated most elegantly by biochemical methods in physiologically synchronized cultures of HeLa cells (6, 40), and in regenerating liver (50). Also Shah (42) used 5-methyltryptophan to inhibit gross protein synthesis while selectively preserving histone synthesis. Despite a 75% fall in general protein synthesis after 18 hr of the 5-methyltryptophan action DNA synthesis was unaffected. Histone synthesis was only detectably affected after 24 hr exposure to the analog when a 37% depression in histone synthesis was accompanied by a dramatic fall (80% inhibition) in DNA synthesis. This points to histone synthesis as being a necessary and perhaps unique concomitant of DNA synthesis.

Owing to the instability of the amide-carboxylic bond to acid hydrolysis, most amino acid analyses of proteins do not quote the content of asparagine (or glutamine) in them. In particular we have been unable to find any published value for the asparagine content of any histones (also e.g., 23, 35). Published figures for the amide content of histones vary from 2.8 to 8.1% as moles/100 moles of all amino acids (35). Recently Phillips (27) has been quoted as finding that 40% of the second carboxyl groups of aspartic and glutamic acid residues, which are present in the various histone fractions of calf thymus nuclei to the extent of 8 to 16%, are present in the amide form. It is thus probable that histones contain significant amounts of asparagine, so that asparagine deficiency in Aas− cells could lead to rapid inhibition of histone synthesis and thereby cause the inhibition of DNA replication. It would seem significant that the rate of decay of DNA synthesis is the same as the rate of decay of protein synthesis after the latter has been stabilized after the precipitous initial inhibition of leucine-14C incorporation. It emphasizes the quantitative nature of the dependence of DNA synthesis on protein synthesis.

**Mechanism of Inhibition of RNA Synthesis.** Either the specific depletion of an essential amino acid or the addition of a specific inhibitor of protein synthesis (21, 43) leads first to a selective inhibition of rRNA transcription followed by depression of D-RNA and then tRNA synthesis. Furthermore, the evidence also indicated that the maturation process, which involves the conversion of ribosomal pre-cursor RNA into the mature species, is also inhibited. In all these situations protein synthesis is depressed and the degree and rapidity of onset of inhibition of RNA synthesis is roughly correlated with that of the protein synthesis although preceded by the latter. The amino acid dependence of RNA synthesis in the 6C3HED Asn− cells as well as the few other eukaryotic cells in which it has been studied (11, 53) resembles that of stringent bacteria (16).

That the present observations of the inhibition of precursor incorporation into RNA solely reflect an increased degradation of the newly synthesized RNA by activation of, e.g., RNase seems improbable from the rapidity of the effects, in the case of cycloheximide and GPS. However, an increase in the alkaline RNase activity of the postmitochondrial fraction of 6C3HED Asn− cells from animals treated by injection 2 hr previously with guinea pig asparaginase has been reported (30). If the enzyme is only cytoplasmic (the location which was examined in that study) it would have no relevance to the inhibition of transcription of rRNA (an intranuclear event) nor would it explain the similar, selective effects of the 3 different initiating situations studied here on the various species of RNA. It could, however, be responsible for abortive maturation of rRNA, for example, by leading to rapid degradation of newly synthesized 18 S RNA-containing subunits as they emerge from the nuclei (e.g., Refs. 20, 46).

Studies of the effects of cycloheximide on RNA synthesis in L-cells (20) in regenerating rat liver (22) and in HeLa cells (46, 54) have shown the necessity for protein synthesis in the maintenance of a normal rate of rRNA transcription although this dependence is not absolute (54). In these and other studies (24, 51) with inhibitors of protein synthesis RNA synthesis was selectively inhibited. Further, Summers et al. (49) have shown that inhibition of protein synthesis results in the rapid disappearance of about 50% of the assayable RNA polymerase activity of nuclei from exponentially growing HeLa cells but not of the residual RNA polymerase of cells in stationary phase. We have observed (to be published) that there is a selective depression of rRNA synthesis in cells in stationary phase. There are different RNA polymerase activities for rRNA and DNA-like RNA in mammalian cells (31, 55) which may be due to different enzymes (31). Thus inhibition of protein synthesis could lead to differential loss of the RNA polymerase if this enzyme had a much shorter half-life than the extranucleolar enzyme.

This would explain differential inhibition of rRNA transcription but would not explain the added decrease of maturation of rRNA. Alternatively, specific proteins constituting a small pool (54) may be needed for the maturation process and transport of rRNA. Inhibition of protein synthesis may lead to a critical shortage of these, which would lead to inhibition of the maturation process, and either by mass action or by failure to remove precursor RNA from its template lead to the inhibition of rRNA transcription, which we have observed and was observed by RNA polymerase assay (49). Both of these alternative mechanisms could be contributing to the selective inhibition of rRNA synthesis. Also, in a preliminary communication it was stated (53) that valine starvation of HeLa cells leads not

ASA
only to a reduction in the rate of transcription of rRNA precursor but also in a slowing of its maturation to 18 S and 28 S RNA. Study of isotope incorporation and elimination from the various intermediates and products of rRNA synthesis by using methods with greater resolving power in these systems should throw further light on the regulatory mechanisms of rRNA production and the reason for its dependence on protein synthesis.

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The Dependence of DNA and RNA Synthesis on Protein Synthesis in Asparaginase-treated Lymphoma Cells

K. A. O. Ellem, Angelina M. Fabrizio and L. Jackson


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