Properties of 5-Fluorouracil-containing Ribonucleic Acid and Ribosomes from Bacillus subtilis

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Growth of a strain of Bacillus subtilis that requires uracil, thymine, adenine, and tryptophan in the presence of 5-fluorouracil (FU) results in the synthesis of ribonucleic acid (RNA) and ribosomes in which 55 to 65% of the RNA uracil has been replaced by the fluorine derivative. Examination of analogue-containing ribosomes by sucrose density gradient centrifugation and thermal denaturation studies suggests that, as far as the size, shape, and packing structure are concerned, extensive FU substitution has little or no effect. FU appears to replace uracil in RNA without selectivity for one RNA class over another, as determined by methylated albumin-kieselguhr column chromatography and sucrose density gradient centrifugation. The total amino acid content of the cells is markedly affected by growth in the presence of FU. The possibility of an FU effect on genetic translation is discussed.

In view of previous investigations indicating production of inactive or altered enzymes in the presence of 5-fluorouracil (FU) (4, 8) it is probable that functional differences exist between FU-containing and natural ribonucleic acids (RNA) (9). Several investigations on the biological and physicochemical properties of FU polyribonucleotides have been reported. Experiments on amino acid incorporation demonstrate that FU polymers behave exactly like uracil (U) polymers in coding for protein synthesis. In physical studies, the FU polymers form less stable complexes with polyadenylic acid (poly A) than do their U counterparts (16). Gros et al. (7) presented evidence suggesting that natural FU messenger RNA can be defective. The possibility of FU replacing cytosine (C) in RNA messages was studied in detail by Bujard and Heidelberger (3) with the deoxyribonucleic acid (DNA)-dependent RNA polymerase reaction using a DNA primer of defined base sequence. They found the frequency of replacement to be less than one uracil or FU per 3,000 cytosines. They pointed out, however, that this level of replacement could be explained by DNA contamination in the RNA polymerase preparation.

A companion paper (18) describes an abnormal pattern of growth in the presence of FU exhibited by a strain of Bacillus subtilis that required uracil and thymine. The cells continued to synthesize DNA, RNA, and protein in the presence of the drug but were unable to undergo cell division. This paper will describe the properties of the FU-containing RNA formed by this organism and attempt to elucidate the direct cause of death.

MATERIALS AND METHODS

The methods employed are generally as described in the previous paper (18) with the exception of those detailed below.

Measurement of growth and isotope incorporation. A minimal medium (18) containing 20 μg each of adenine and thymine per ml, 2 μg of uracil per ml, and, when desired, 8.0 μg of FU per ml was inoculated with a suspension of cells obtained from a Trypticase-agar plate that had been incubated overnight at 37°C.

Growth studies were carried out in 250-ml Delong flasks containing 50 ml of medium with vigorous shaking at 37°C in a model G-77 water-bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.). At various time intervals, 1-ml samples were removed, and their absorbancy at 420 mμ was measured in a Zeiss PMQ II spectrophotometer. Additional 1-ml samples were removed at appropriate intervals and transferred to tubes containing 1 ml of cold 20% trichloroacetic acid. The trichloroacetic acid precipitates were collected on 25-mm type B6 (Schleicher & Schuell Co., Keene, N.H.) membrane filters, washed with six successive 5-ml portions of cold 10% trichloroacetic acid, dried, and counted for 10 min in a Packard Tri-Carb liquid scintillation spectrometer. The counting solution employed contained 0.01% 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene and 0.4% 2,5-diphenyloxazole in toluene.

Amino acid analysis. Cells grown as described above were harvested when the absorbancy at 420 mμ
reached 0.60. The cells were washed twice with 0.2 m
NH₄Cl, dialyzed against deionized water, and lyo-
philized. Approximately 2 mg of protein, estimated by
the method of Lowry et al. (14), were hydrolyzed for
24 hr in 1.0 ml of glass-distilled constant-boiling HCl
at 110°C under vacuum. The amino acid composition
of the hydrolysates was determined on a Spinco
(model 120B) automatic amino acid analyzer as de-
scribed by Spackman, Stein, and Moore (20). The
analyses were corrected for a 10% decomposition of
threonine and serine. The standard deviations (SD)
of duplicate analyses were less than ±0.03 mole per cent
for all amino acids except the following from the FU
cells: aspartic acid, SD = ±0.045; threonine, SD = ±
0.034; glutamic acid, SD = ±0.23; and alanine, SD =
±0.30.

Isolation of ribosomes. Cells were grown as de-
scribed above, harvested in the cold, and washed once
with a standard buffer (SB) containing 10⁻² m tris-
(hydroxymethyl)aminomethane (Tris), 1.2 × 10⁻³ m
magnesium acetate, 6 × 10⁻⁴ m KCl, and 6 × 10⁻³ m
mercaptoethanol (pH 7.8). All subsequent isolation
steps were carried out at 4°C. The cells were broken by
grinding with 100-5005 Superbrite glass beads (Minne-
sota Mining and Manufacturing Co., St. Paul, Minn.).
The cell debris was removed by one 20-min centrifuga-
tion at 20,000 × g and two 30-min centrifugations
at 35,000 × g in a Sorvall refrigerated centrifuge (rotor
SS34). Ribosomes were pelleted by centrifugation at
105,000 × g for 2 hr (40 rotor) in a Spinco (model L)
preparative ultracentrifuge. The pellet was suspended
in SB, pelleted again at 105,000 × g, and dialyzed
overnight at 4°C against the desired buffer (SB).
The FU cells did not break as easily as the normal cells
when ground with glass beads, so it is possible that our
ribosome preparations derive from only the most
fragile part of the FU cell population. Physical studies
were conducted only on ribosome batches that had
been shown to have 55 to 65% FU substitution. The
purity of the ribosome preparations was determined by
sucrose density gradient centrifugation (Fig. 3) and
absorption spectra (legend, Fig. 4).

²³P labeling was accomplished by growth of cells on
minimal medium without FU plus 10 μC of carrier-free
²³P phosphoric acid per ml (International Chemical
and Nuclear, City of Industry, Calif.). After growth
had reached mid-log phase, the cells were harvested
in the cold and washed twice with SB. The pellet
was frozen and mixed with 2 g of frozen B. subtilis
cells (Miles Laboratories, Inc., Elkhart, Ind.) to give a
sufficient cell mass for breakage by grinding with
glass beads. The ribosomes were isolated and purified
as described. The specific activity of the preparation
shown in Fig. 2 was 1,900 counts per min per μg of
ribosomes. Therefore, the amount of carrier ribosomes
in the ²³P preparation, which was layered on the
gradient, would not be detected by absorbancy
measurements.

Extraction and purification of RNA. Lysozyme (400
μg/ml) and 50 μg of electropheretically purified de-
oxyribonuclease I per ml (Worthington Biochemical
Corp., Freehold, N.J.) were added to washed cells in
3 × 10⁻² m Tris-chloride buffer (pH 7.7). The mixture
was incubated at 37°C for 5 min, and then MgCl₂ was
added to give a final concentration of 10⁻³ m. The
mixture was incubated at 37°C for 3 min, sodium
dodecyl sulfate (to a final concentration of 0.4%) was
added, and the mixture was shaken for 5 to 10 sec. An
equal volume of phenol buffered with 0.01 m KH₂PO₄
(pH 6.7) was added immediately to minimize activity
of the cellular ribonuclease.

RNA was purified according to the method of Gierer
and Schramm (6), and was precipitated from the
aqueous phase, after the removal of ether, with 3
volumes of cold 95% ethanol alcohol; the precipitate
was then dissolved in 0.1 × SSC (0.015 m NaCl-
0.0015 m trisodium citrate (pH 7)). The RNA solution
was dialyzed against 2,000 volumes of 2 × SSC in the
cold for 18 hr.

Base composition analysis of RNA. The RNA solu-
tion was hydrolyzed by adding an equal volume of 0.6
N KOH and incubating the mixture at 37°C for 18 hr.
The resulting 2'- and 3'-nucleotides were separated on
a Dowex-1-8X formate column with a resin bed 9 mm
in diameter by 50 mm high. The method of elution
was described by Key (12). Unlabeled Escherichia coli
carrier RNA of known base composition was added
prior to hydrolysis to give an absorbancy marker in
the nucleotide regions and to provide internal controls
for the preferential losses of individual nucleotides.
The eluate was collected in 5-mI fractions, and the absorb-
cy at 260 mλ was determined for each fraction. The
radioactivity of each fraction was determined by liquid
scintillation counting in a Packard Tri-Carb spectrom-
eter as previously described (18).

RNA fractionation on methylated albumin-kies-
selghur (MAK) columns. MAK columns were prepared
as described by Sueoka and Chang (21). Cells were
grown as described (18) with the addition of 1 μC/ml
of FU-³H₃. A column containing 25 ml of MAK was
charged with 50 ml of an RNA solution containing 1.5
mg of U-RNA and 90 μg of ³H₃FU-RNA (1,690
counts per min per μg of RNA) in 0.1 m NaCl-0.05 m
potassium phosphate buffer. RNA was eluted with a
linear gradient of NaCl, and 5-mI fractions were
collected. U-RNA was identified by absorbancy at
260 mλ. For the detection of tritium, 0.5-mI portions
of each fraction were diluted with 0.1 ml of carrier
salmon sperm DNA (1 mg/ml in water) and 5 ml of
12% trichloroacetic acid, and incubated in an ice bath
for 30 min. Precipitates were collected on 25-mm type
B6 membrane filters, washed with 20 ml of cold 10%
trichloroacetic acid, dried under a heat lamp, and
counted as described.

Melting temperatures. Thermal denaturation of
RNA and ribosomes was followed in a Zeiss PQM II
spectrophotometer equipped with a heated cell com-
partment and a temperature readout assembly. All
samples had initial absorbancies of 0.50 to 0.70 at 260
mλ.

RESULTS

Incorporation of ³H₃FU and ³H-thymidine into
trichloroacetic acid-insoluble material. Figure 1
shows an initial experiment designed to verify the
assumption that FU is incorporated into the
macromolecules of strain 568(b). The incorpo-
ration of ³H₃FU into trichloroacetic acid-
insoluble material continued throughout the growth period as did the incorporation of $^3$H-thymidine.

**Extent of FU substitution.** A crucial point in studies regarding the effect of base analogues on RNA and ribosome structure is the extent of substitution of FU for U in the RNA in question. Nucleotide base substitution was monitored by two independent methods. The first involved radioactive labeling and elution from an ion-exchange column of the nucleotide bases derived from purified ribosomes (Fig. 2). This resolved the uridylate fraction into two peaks consisting of FU (65%) and U (35%). The presence of tritium in the cytosine peak indicates that a significant fraction of the input $^3$H-uracil was converted to cytosine. The level of crossover of $^3$H into the $^{14}$C channel in our radioactivity measurements was about 15%, which accounts for the small peak in the cytosine region and does not indicate any conversion of $^{14}$C-FU to either U or cytosine. The second method, paper chromatography, which involves formic acid hydrolysis of ribosomes, separation in ethylacetate-water-formic acid (60:35:5), base identification with the appropriate standards, and elution of the bases with 0.1 M HCl, routinely gave approximately 55% FU and 45% U. Therefore, the level of substitution was in the range of 55 to 65%, sufficient to warrant a search for structural alterations in the cellular RNA and ribosomes.

**Properties of FU ribosomes.** To determine the effect of U replacement by FU on the physical stability of ribosomes, a series of experiments designed to assess the effect of alterations in the nucleoprotein packing structure of ribosomes were carried out.

Co-sedimentation of the $^{32}$P-U and unlabeled FU ribosomes in a sucrose density gradient (Fig. 3) revealed no marked differences between the two types of ribosomes. In both cases, the ribosomes were primarily 70S. Thus, one can conclude that ribosome size and shape are not altered significantly by extensive FU substitution. This is in contrast to the work on E. coli ribosomes, such as that of Iwabuchi et al. (11) and Andoh and Changaff (1), who found an accumulation of non-functional FU-containing particles at essentially the same degree of substitution.

The stability of the nucleoprotein packing structure can be monitored by thermal-induced absorbancy changes. Figure 4 shows that the FU and U ribosomes have essentially the same $T_m$ (about 70 C). However, the shapes of the melting curves differ significantly. The normal U ribosomes are stable up to 60 C and then begin to dissociate over a 10 C temperature range. The drop in absorbancy above 70 C is due to protein precipitation and frequently occurs in these analyses. It has no effect on the midpoint of the absorbance transition.

**FU-RNA characterization.** From the degree of analogue substitution observed, one would expect some changes in physicochemical properties to be induced by FU in RNA. The finding that FU polynucleotides form less stable structures of either the complementary hydrogen bonded type (i.e., the poly FU-poly A complex) or the intramolecular base stacked type [reviewed by Michel-son et al. (17)] supports the notion that one could detect structural changes if a sufficient quantity of FU stretches exist in the RNA. We have used two independent methods to monitor changes in RNA secondary structure. The first method (Fig. 5), sucrose density gradient centrifugation, shows that the size and shape of FU- and U-RNA are essentially the same since there is no reason to suspect an FU effect on RNA chain length.

The fractionation of RNA on MAK columns is a useful method for showing changes in secondary structure (5). Once again, the FU- and U-RNA species chromatograph together (Fig. 6).
Although the tritium background was somewhat high, the ratio of \(^3\text{H}\) to \(A_{260}\) was essentially constant in the major RNA peaks, indicating no dramatic selectivity of incorporation of the natural base over the halogenated form into a particular class of RNA. This is unlike certain other base analogues whose distribution in RNA has revealed marked selectivity [i.e., azaguanine incorporation into soluble RNA in \(B.\) \textit{ceres}\ (13)].

**Amino acid composition of FU cells.** Under these conditions of continued growth (and presumed protein synthesis), and the high degree of incorporation of FU into RNA, it is conceivable that the observed aberrant growth could be reflected in the amino acid analyses of 568(b) cells grown in the presence and absence of FU. One can see that there were distinct differences in the relative amounts of several amino acids. The most pronounced were the increased relative ratios of glutamic acid, glycine, and alanine. All the other amino acids decreased with the exception of arginine which remained essentially constant. The possible significance of these findings will be considered in the Discussion. An extensive study of the changes in amino acid composition in several cellular components at various FU to U ratios is currently in progress.

**DISCUSSION**

In view of the finding that FU polymers form less stable complexes with poly A than do uracil
polymers (16), one might expect a greater probability of incomplete translation with FU messenger RNA molecules. If this were true, it is possible that the regulation of RNA synthesis might be impaired, resulting in the increased production of certain messages and their corresponding proteins or peptides and a decreased production of others. A rough approximation of such a translational effect would conceivably be evident from a total cellular amino acid analysis. A study of this type was carried out in E. coli by Aronson (2), who reported that FU did not affect the amino acid composition of the cells. Gros and Naono (8), on the other hand, reported that FU inhibits the incorporation of proline and tyrosine, and stimulates incorporation of arginine in E. coli.

The total cellular protein of B. subtilis strain 568(b) grown in the presence of FU shows an increased relative ratio of glutamic acid, glycine,
TABLE 1. Amino acid composition (mole percentage) of 568(b) cells grown on U and FU

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>U cells</th>
<th>FU cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>7.38</td>
<td>7.20</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.54</td>
<td>1.38</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.78</td>
<td>3.81</td>
</tr>
<tr>
<td>Aspartic</td>
<td>9.63</td>
<td>7.63</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.20</td>
<td>4.69</td>
</tr>
<tr>
<td>Serine</td>
<td>4.79</td>
<td>4.19</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>13.67</td>
<td>14.61</td>
</tr>
<tr>
<td>Proline</td>
<td>3.38</td>
<td>3.05</td>
</tr>
<tr>
<td>Glycine</td>
<td>8.40</td>
<td>10.01</td>
</tr>
<tr>
<td>Alanine</td>
<td>13.61</td>
<td>19.40</td>
</tr>
<tr>
<td>Valine</td>
<td>6.61</td>
<td>5.94</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.91</td>
<td>1.43</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.75</td>
<td>4.93</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.45</td>
<td>6.14</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.08</td>
<td>2.92</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.78</td>
<td>3.28</td>
</tr>
<tr>
<td>Total</td>
<td>99.96</td>
<td>100.61</td>
</tr>
</tbody>
</table>

* Corrected for 10% decomposition.

and alanine, while arginine remained at the same ratio (Table 1). Although it has been well established that the genetic code is degenerate and multiple codons exist for all the amino acids, Marshall et al. (15) have shown species-dependent differences in the response of amino acyl-transfer RNA to trinucleotide codons among bacteria, amphibia, and mammalia. Using E. coli as a representative of the bacteria, they find that the codons that give the greatest response (at least twice that of other recognizable codons) for the amino acids relevant here are: glutamic-GAG, GAA; glycine-GGC; alanine-GCG, GCA; arginine-CGA, CGU. It is interesting to note that only one of these codons contains uracil. In the present study, all of the other amino acids analyzed decreased in ratios of 10% or more.

The FU ribosomes display one mode of denaturation not found in the normal ribosomes. This effect is observed as an increase in absorbancy between 45 and 60°C and may reflect a high degree of structural heterogeneity which does not alter the sedimentation pattern (Fig. 3). It is quite probable that alterations in ribosomal RNA base stacking do not result in changes in the ribosome sedimentation coefficients. Although FU may destabilize the ribosome, the effect is not as marked as one might expect from the extent of analogue substitution in the ribosomes. This suggests that the effect is quite subtle and could involve RNA loops where base pairing and base stacking are not essential to maintain the ribosome packing structure. A more sensitive assay for RNA loops would involve in vitro protein synthesis experiments or the messenger RNA-catalyzed binding of amino acyl-soluble RNA to ribosomes. These determinations are currently under investigation.

The influence of FU on ribosome synthesis has been shown by Aronson (2) and Iwabuchi et al. (11) in E. coli, and by Hignett (10) in Staphylococcus aureus, to result in the accumulation of small inactive FU-containing particles. The experiments
described here with strain 568(b) of B. subtilis are in apparent contrast to these situations.

It is clear from this and the companion paper (18) that, in the presence of an exogenously supplied source of thymine, protein synthesis in strain 568(b) occurs in spite of a high degree of substitution of FU for U in all classes of RNA. The physical studies with FU ribosomes from 568(b) indicate that, as far as the size, shape, and packing structures are concerned, FU substitution (55 to 65%) has little, if any, effect. This discrepancy may conceivably reflect the different methods of FU exposure employed. Ribosomes were obtained from 568(b) after growth in the presence of FU (8 µg per ml of FU, 2 µg per ml of uracil). On the other hand, the ribosomes of E. coli and S. aureus were isolated from cells that had been exposed to a high level of FU (20 µg/ml) in the absence of U for a relatively short period of time (40 to 60 min). Since the RNA of these particles contained 60 to 70% substitution of FU for U, they, as well as all RNA species synthesized during the interval, probably contain numerous FU stretches. If one assumes that the distribution of FU along an RNA molecule is similar to that of U, and that both bases are incorporated into RNA at a rate which is proportional to the relative concentration of each base in the precursor pool, then the number or chain length, or both, of pure FU clusters in RNA would be reduced under conditions of prolonged analogue exposure at FU/U = 4. As mentioned in the Results section, evidence obtained from physical studies on synthetic homopolyribonucleotides suggests that the presence of FU stretches should result in less stable RNA base-pairing reactions of either the inter molecular or intramolecular types.

The fact that only slight changes were observed in the 568(b) FU ribosome melting curve suggests either that very few FU stretches existed in the ribosomes or that uridylic stretches are rare in B. subtilis ribosomal RNA. The recent work of Slapikoff and Berg (19) supports the former idea. They investigated the effect of different nearest neighbors on the specificity with which an adenine residue in the polydeoxyxynucleotide template directs the incorporation (by RNA polymerase) of either uridine 5’-monophosphate or one of its analogues. It was found that U is incorporated more efficiently than FU next to adenine or cytosine. Little or no preference exists between uridine 5’-triphosphate and 5-fluorouridine 5’-triphosphate for incorporation next to guanine residues. When U is the nearest neighbor, U is preferentially incorporated compared to pseudouridine. Unfortunately, the case of FU with a U nearest neighbor was not examined. These data suggest that there will be fewer FU than U stretches found in the RNA of a cell that was grown in the presence of both nucleotide bases.

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LITERATURE CITED


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