Dissociation of Cellular Functions in Bacillus cereus by 5-Fluorouracil

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Received for publication 20 July 1965

ABSTRACT

REICH, MELVIN (The George Washington University School of Medicine, Washington, D.C.), AND H. GEORGE MANDEL. Dissociation of cellular functions in Bacillus cereus by 5-fluorouracil. J. Bacteriol. 91:517–523. 1966.—5-Fluorouracil (FU) produced a marked inhibition of growth and deoxyribonucleic acid (DNA) synthesis in Bacillus cereus 569H. Protein and ribonucleic acid (RNA) synthesis were not specifically inhibited, and proceeded at the rate of turbidimetric increase of the cells. Cell-wall synthesis, respiration, and penicillinase production continued in the presence of FU at essentially the control rate. The addition of equimolar concentrations of uracil and FU prevented growth inhibition but did not restore DNA synthesis. The addition of thymidine with FU did not relieve growth inhibition but did restore the DNA content to normal. Thymidine supplementation also increased the quantity of FU, but not uracil, incorporated into RNA and the acid-soluble fraction. The data indicate that inhibition of growth can be dissociated from inhibition of DNA synthesis and that more DNA is present in normal cells than is needed for growth and reproduction.

The metabolism and mechanism of action of 5-fluorouracil (FU) have been studied in bacteria. In Escherichia coli the drug was incorporated into the acid-soluble fraction and into ribonucleic acid (RNA) in place of uracil, forming the corresponding metabolic derivatives containing FU (5). The most active form of the drug appears to be 5-fluoro-2-deoxyuridine-5'-monophosphate, which inhibited the methylating enzyme, thymidylate synthetase, and prevented the conversion of deoxyuridine monophosphate to thymidine monophosphate. The result was a reduced deoxyribonucleic acid (DNA) content, unbalanced growth, and thymineless death (7).

Additional observations include alterations in ribosome metabolism (3), changes in amino acid incorporation into the soluble pool (10) and into cellular protein (8), decreased enzyme synthesis (9), formation of altered or inactive enzymes (8), and interference with cell-wall synthesis (25).

This paper deals with the effect of FU on the synthesis of protein, nucleic acid, and cell wall, and on the respiration of Bacillus cereus. The effects of uracil and thymidine upon DNA synthesis and FU incorporation is also described. A preliminary report of this work has been presented (Reich and Mandel, Federation Proc.21:176, 1962).

MATERIALS AND METHODS

Growth. B. cereus was grown at 37°C in a Gyrotory Shaker (New Brunswick Scientific Co., New Brunswick, N.J.) in media containing 2.5 × 10⁻³ M KH₂PO₄, 2.0 × 10⁻³ M MgSO₄·7H₂O, 1.2 × 10⁻³ M Fe(NO₃)₂·(SO₄)₂·6H₂O, and 1% Casamino Acids (Difco) at pH 7.0. Growth was measured turbidimetrically at 540 nm in a Bausch & Lomb Spectronic-20 colorimeter. Radioactive compounds or pyrimidines were added during the early stage of logarithmic growth at an optical density (OD) of 0.3. One flask received 20 µg/ml of FU (0.16 µmole/ml) dissolved in 0.5% Na₂CO₃, and others, serving as controls, received Na₂CO₃ only. Zero-time samples were removed for analysis immediately after addition of the drug and at absorbancy increments (ΔOD) of 0.1 up to 0.6 or at 0.6 only. At this point both the control and the inhibited culture contained 0.3 mg (dry weight) and about 2 × 10⁸ cells per ml.

Cell fractionation. After growth in the presence of FU-2-C¹⁴, the cells were fractionated by the method of Brockman et al. (5) or Mandel and Markham (14) to establish the sites of drug incorporation.

Analytical methods. Total protein was determined by the procedure of Oyama and Eagle (17), RNA by the orcinol method (27), DNA by the diphenylamine reaction (6), and RNA stability by the method of
and glycine-2-C\(^{14}\) were purchased from Tracerlab, Waltham, Mass., and uracil, thymidine, pyridoxine hydrochloride, and penicillin G from Nutritional Biochemicals Corp., Cleveland, Ohio. Tritiated di-aminopimelic acid was furnished by J. L. Strominger of the University of Wisconsin, Madison.

**RESULTS**

*Growth.* FU caused a 75% reduction in the growth rate of *B. cereus*, as measured by the time required to double the initial OD of 0.3 (Fig. 1). This growth rate was intermediate between that of the control and that of completely inhibited cells at 0.8 \(\mu\)mole of FU per ml. Microscopic examination of the inhibited cells revealed long chains as opposed to the normal independent cellular morphology. Individual cells also appeared to be elongated.

Growth inhibition was almost completely prevented by the simultaneous addition of an equimolar concentration of uracil. An equimolar concentration of thymidine, added at zero-time and at 15-min intervals, to compensate for its rapid cleavage to thymine (2), did not reverse growth inhibition. The growth rate of the control culture was not affected by the addition of either of the natural pyrimidines alone.

It has been reported that pyridoxine acted as a noncompetitive antagonist of FU in *Candida albicans* (11). No such effect was observed on FU-treated *B. cereus* with up to 4.9 \(\mu\)moles of pyridoxine per ml.

*Protein synthesis.* In most of the present experiments, biosynthesis was measured in cultures at similar bacterial turbidities. In this manner, an allowance was made for the slower rate of growth of the FU-treated cells, and the differences due to effects beyond that due to growth inhibition become more apparent. Turbidometric increases are related to increases in cell dry weight. Table 1 compared the incorporation of several amino acids into the hot trichloroacetic acid-insoluble fraction of cells grown with and without FU to similar turbidities. This fraction has been shown to contain all the bacterial protein as well as cell-wall constituents (21). FU did not inhibit the uptake of those amino acids which act solely as protein precursors [i.e., tyrosine, proline, arginine (22)], but those amino acids which contribute carbon for the formation of cell wall (i.e., glycine, serine, aspartic acid) were all used more extensively. It should be clear, however, that, since FU inhibited growth, the rate of protein synthesis, in accord with that of growth, was similarly inhibited. Colorimetric analysis of the total protein content revealed no specific inhibition of protein synthesis in the FU-treated culture (Table 2).
TABLE 1. Incorporation of radioactive amino acids into the hot trichloroacetic acid-insoluble (protein plus cell wall) fraction of Bacillus cereus during growth inhibition by FU

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Percentage of control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine-2-\textsuperscript{14}C</td>
<td>187</td>
</tr>
<tr>
<td>Serine-3-\textsuperscript{14}C</td>
<td>165</td>
</tr>
<tr>
<td>Aspartic acid-4-\textsuperscript{14}C</td>
<td>148</td>
</tr>
<tr>
<td>Valine-1-\textsuperscript{14}C</td>
<td>112</td>
</tr>
<tr>
<td>Tyrosine-2-\textsuperscript{14}C</td>
<td>103</td>
</tr>
<tr>
<td>Proline-carboxy-\textsuperscript{14}C</td>
<td>102</td>
</tr>
<tr>
<td>Arginine-guanido-\textsuperscript{14}C</td>
<td>100</td>
</tr>
<tr>
<td>Methionine-2-\textsuperscript{14}C</td>
<td>97</td>
</tr>
<tr>
<td>Phenylalanine-3-\textsuperscript{14}C</td>
<td>94</td>
</tr>
</tbody>
</table>

* Radioactive amino acids were added to portions of an exponentially growing culture at OD 0.3 with or without FU (0.16 \textsuperscript{µ}mol/ml). At OD 0.6, samples were removed and analyzed by the membrane filtration technique as described in Materials and Methods. Control values (counts per minute) were arbitrarily assigned values of 100.

TABLE 2. Protein, RNA, and DNA contents of Bacillus cereus during growth inhibition by FU in the presence and absence of added pyrimidines

<table>
<thead>
<tr>
<th>Addition</th>
<th>δOD</th>
<th>Protein</th>
<th>RNA</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FU</td>
<td>0.0</td>
<td>94</td>
<td>105</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>103</td>
<td>94</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>91</td>
<td>105</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>96</td>
<td>99</td>
<td>55</td>
</tr>
<tr>
<td>FU plus uracil</td>
<td>0.0</td>
<td>102</td>
<td>104</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>104</td>
<td>102</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>96</td>
<td>103</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>102</td>
<td>106</td>
<td>49</td>
</tr>
<tr>
<td>FU plus thymidine</td>
<td>0.0</td>
<td>100</td>
<td>102</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100</td>
<td>106</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>91</td>
<td>107</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>100</td>
<td>92</td>
<td>103</td>
</tr>
</tbody>
</table>

* Compounds were added as in Fig. 1. Results are expressed as a percentage of the control value (obtained from a control culture without added pyrimidines) at each OD increment of 0.1 from 0.3 to 0.6. Actual initial values for the control culture were (per milliliter): 30.0 μg of protein, 17.0 μg of RNA, 2.7 μg of DNA.

Cell-wall synthesis. The effect of FU on cell-wall synthesis was determined by measurement of the incorporation of tritiated diaminopimelic acid (DAP-H\textsuperscript{3}) into the cold trichloroacetic acid-insoluble residue. As shown in Fig. 2 (bottom), the drug caused an increased uptake of this cell-wall precursor when compared with control cells at the same OD. This result corresponds to the increased incorporation of these amino acids which are also cell-wall precursors. The rate of cell-wall synthesis, however, was not affected (Fig. 2, top).

Nucleic acid synthesis. The RNA and DNA contents, measured colorimetrically, of control and inhibited cultures grown to identical turbidities are presented in Table 2. At the end of one generation time, the RNA content of the inhibited cells was normal, whereas the DNA content was reduced to a value 55% that of the control. By subtracting the DNA content present at zero-time, it was calculated that the DNA content of the newly formed cells was only 25% of the control in the FU culture and 12% in the FU plus uracil culture. Subcultures of cells grown with or without the drug and with adenine-8-\textsuperscript{14}C, when regrown in drug-free media containing added nonlabeled...
adenine, did not lose any radioactivity from the KOH-insoluble (DNA) residue. Thus, the observed reduction in DNA content was not due to its instability or turnover after synthesis. Likewise, the RNA synthesized in the presence of FU was no less stable than that of the control cells.

As reported previously (19), the addition of uracil did not produce an increase in the DNA content, although it acted to restore the growth rate to normal. The present experiments show (Table 2) that thymidine, which did not relieve growth inhibition, was effective in preventing the decrease in DNA. These results were confirmed by a study of the incorporation of radioactive nucleic acid precursors (Fig. 3). FU did not inhibit the utilization of adenine-2-C14 or orotic acid-2-C14 for RNA synthesis but markedly reduced their incorporation into DNA. This reduction was prevented by thymidine or thymidine plus uracil. Thus, uracil and thymidine must be added together for the cells to both grow and synthesize DNA at a normal rate. Wachsmann et al. (28) reported that one partially FU-resistant strain of B. megaterium required only uridine and another required only thymidine to reverse FU-induced inhibition.

**Incorporation of FU-2-C14.** After growth in the presence of FU-2-C14, radioactivity was isolated from RNA as fluorouridylic acid. No activity was recovered in the area corresponding to fluorocytidylic acid or in the DNA hydrolysate.

### Table 3. Effect of thymidine on the incorporation of FU-2-C14 and uracil-2-C14 into cell fractions of Bacillus cereus

<table>
<thead>
<tr>
<th></th>
<th>FU-2-C14</th>
<th>Uracil-2-C14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cold trichloroacetic acid-insoluble</td>
<td>Cold trichloroacetic acid-soluble</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>Plus thymidine</td>
<td>Control</td>
</tr>
<tr>
<td>0.1</td>
<td>1921</td>
<td>405</td>
</tr>
<tr>
<td>0.2</td>
<td>453</td>
<td>1,228</td>
</tr>
<tr>
<td>0.3</td>
<td>695</td>
<td>2,127</td>
</tr>
</tbody>
</table>

*FU-2-C14 or uracil-2-C14 (0.16 μmole/ml) in the presence or absence of thymidine (0.16 μmole/ml) was added at an OD of 0.3, and thymidine, every 15 min thereafter. At OD increments of 0.1, samples were removed, treated with cold trichloroacetic acid, and filtered as described in Materials and Methods.

† Results expressed as counts per minute.

The acid-soluble fraction of the cells contained the fluorinated analogues of the corresponding uracil-containing cofactors.

The addition of an equimolar amount of uracil simultaneously with FU almost completely prevented the incorporation of FU-2-C14 into *B. cereus* (19).

Since Sells (23) reported that thymidine can reduce the incorporation of uracil into *B. cereus*, it was thought that it might also antagonize FU incorporation, thereby permitting the observed normal rate of DNA synthesis. No such reduction was observed. On the contrary, it was found (Table 3) that thymidine stimulated the incorporation of FU into RNA and the acid-soluble fraction by 206 and 176%, respectively, after one generation time when compared with a control culture. The incorporation of uracil-2-C14 remained unaffected by thymidine addition. The addition of thymine (0.16 μmole/ml) every 15 min stimulated the incorporation of FU-2-C14 into RNA by only 9%.

Thymidine continued to stimulate FU incorporation into RNA for at least 90 min after supplementation was stopped (Table 4) and for at least 160 min (time required to reach OD 0.6 upon subculturing) after its removal by centrifugation (Table 4). In both experiments, the continued addition of thymidine stimulated drug uptake still further. Again, the incorporation of uracil-2-C14 remained unaffected by thymidine.

**Respiration and enzyme synthesis.** FU produced up to a 100% increase in both O2 and CO2 production when comparisons were made between growing cultures over equal OD increments. The
rate of respiration, however, was slightly reduced by FU.

The penicillinase activity assayed in the cell-free media of the inhibited cells was essentially equal to that of the control.

**DISCUSSION**

The results presented here suggest that FU exerts at least two effects on *B. cereus*. One effect, the inhibition of DNA synthesis, is prevented by thymidine but not by uracil. Thus, the addition of thymidine permits the synthesis of normal amounts of DNA despite the increased incorporation of FU, without relieving the inhibition of growth. Gros and Naono (8) also reported that thymidine could restore DNA synthesis in *E. coli* but did not report its effect on growth.

A second effect, the incorporation of FU into RNA and the soluble uracil-containing cofactors, is prevented by uracil but not by thymidine. With the addition of uracil, the growth rate remains normal while DNA synthesis is reduced to a value only 12% of the control. In several instances, the added uracil, although preventing FU incorporation, actually reduced the DNA content below that of the FU-inhibited culture. Although this effect was small (6% in Table 2, see also 19), it was verified by isotope incorporation (Fig. 3, columns B and C) and seems to be real. The ability of uracil to reverse FU-induced growth inhibition in *E. coli* has been demonstrated by Cohen et al. (7) but not by Gros and Naono (8).

The ability of *B. cereus* to grow at a control rate in cultures containing FU plus uracil and to synthesize protein and RNA in normal amounts while forming only 12% of the normal quantity of DNA suggests that the inhibition of DNA synthesis precedes the resulting inhibition of growth. Indeed, similar experiments have shown that growth was not inhibited until after three doublings of the initial OD despite a marked reduction in the DNA content (19).

It should be stressed, however, that the RNA which is formed, although present in normal amounts, as well as the acid-soluble components, is atypical in that it contains FU. The synthesis of messenger RNA containing FU may explain the report of Gros and Naono (8) regarding the formation of bacterial protein with a reduced content of tyrosine and proline and an elevated arginine level. In the present experiments, no changes in the pattern of amino acid incorporation were observed, nor was the enzymatic activity of penicillinase affected.

In the time interval between the inhibition of DNA synthesis and the inhibition of growth, the small amount of newly synthesized DNA might be sufficient to permit normal growth, protein, and RNA synthesis provided, as is true only in the uracil-supplemented culture, that little or no FU is incorporated into RNA or the soluble cofactors. This would seem to indicate that most of the cellular complement of DNA normally formed during growth is not needed for growth. If true, this situation could be analogous to the finding by Allfrey, Littau, and Mirsky (1) that up to 80% of the DNA in thymus nuclei could be removed without affecting protein or RNA synthesis. They postulated that most of the DNA was inactive or repressed since it was not synthesizing messenger RNA. In a multinucleate cell such as *B. cereus*, this excess DNA may be associated with the four chromatin bodies containing DNA (16).

An alternative explanation might be the utilization of pre-existing DNA formed prior to
the addition of FU and pyrimidines. This does not appear likely, because the thymidine-supplemented culture has the same quantity of pre-existing DNA, as well as a normal content of newly synthesized DNA, yet its growth rate remains inhibited. It is possible, however, that the DNA made during growth inhibition by FU, in the presence or absence of thymidine, is not functional.

The selective stimulation by thymidine (or a derivative) of FU incorporation into RNA and the acid-soluble fraction without influencing uracil incorporation is surprising. Thymidine may act by affecting the anabolism or catabolism of FU, its ribosides and ribotides. Maley and Maley (12) have shown that added thymidine (acting as thymidine triphosphate) stimulated the incorporation of deoxythymidine into chick-embryo DNA by blocking the conversion of cytidine to uridine and uridine monophosphate. On the other hand, Mukerjee and Heidelberger (15) found that thymine reduced the catabolism and increased the toxicity of FU in mice by competing for the pyrimidine catabolizing enzymes.

If thymidine did act by increased anabolism or decreased catabolism of FU in B. cereus, one might expect an increase in intracellular concentration of 5-fluoro-2-deoxyuridine-5'-monophosphate, with a resultant increase in growth inhibition in those cultures receiving thymidine. This was not observed. Further, to explain the selective action of thymidine by this mechanism would require the existence of separate sets of anabolic and catabolic enzymes for FU and uracil, yet both pyrimidines are apparently acted upon by identical enzymes (15, 24).

The inability of thymine to stimulate FU incorporation (9% increase in RNA versus 206% for thymidine in Table 3) indicates that it is not the active derivative. If FU inhibits thymidine phosphorylase in B. cereus, as has been shown in B. subtilis (4), then even less thymine (or deoxyribose-1-phosphate) would be formed. Indeed, the presence of an intracellular pool of thymidine would explain the continuing action of thymidine long after its removal.

The continued synthesis of cell wall by cells grown in the presence of FU suggests that the fluorouridine analogue of the cell-wall precursor, uridine-diphosphate-N-acetylglucosamine, may be readily utilized and may function similarly to the normal metabolite. This is not the case in Staphylococcus aureus, where FU causes the accumulation of cell-wall precursors (20).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Grant CA-02978 from the National Cancer Institute and by Public Health Service grant AI-04264 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED


