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THE ROLES OF VARIOUS BIOCHEMICAL EFFECTS PRODUCED BY 5-FLUOROURACIL IN EARLY GROWTH INHIBITION OF *BACILLUS CEREUS**

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Abstract—By the utilization of uracil and thymidine to selectively protect against some of the inhibitory effects of 5-fluorouracil (FU) in *Bacillus cereus*, it was demonstrated that the early phases of growth inhibition produced by FU could not be correlated with inhibition of DNA synthesis, or with the quantity of FU incorporated into the whole cell or the ribosomal fraction. By contrast, cultures grew at control rates only when the quality and quantity of ribosomes synthesized appeared normal, suggesting that an effect of FU on ribosome synthesis was most closely related to the rapid onset of growth inhibition.

DRUGS USUALLY produce more than one biochemical effect on a biological system, and frequently it is difficult to discriminate amongst these simultaneously occurring actions as to which may be the principal and primary drug action. For the growth-inhibitory primidine analog, 5-fluorouracil (FU), two major drug effects have been consistently established. FU in the form of its anabolite, deoxyfluorouridylate, inhibits thymidylate synthetase, and thereby blocks the formation of DNA.[1,2] The analog also follows many of the reactions of uracil in its metabolism and is incorporated into RNA.[3] More recently, it has also been discovered that in microbial cells, FU inhibits ribosome formation.[4–6] A similar effect has been observed in mammalian cells, where FU or fluoro-orotate inhibits ribosomal RNA maturation.[7,8] The major actions of FU have been the subject of two extensive reviews.[9,10] These drug effects have also been observed to take place in the microbial system, *Bacillus cereus*.[11–13]

By the use of either uracil or thymidine when co-administered with FU, it has been possible to prevent selectively certain of the alterations produced by FU in this microbial system, and to correlate these effects with growth inhibition which occurs within the first doubling of the bacterial population. We now propose that the rapid inhibition of growth observed after the addition of FU to this microbial system is most closely related to the drug's actions in inhibiting ribosome formation, rather than

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inhibition of DNA synthesis or the incorporation of FU into total RNA or into the major ribosomal fractions.

METHODS AND MATERIALS

Cultures of *B. cereus* 569 were grown at 37° in casamino acids salts medium and growth was monitored as previously described.[13] Logarithmically growing cultures were divided, and additions of compounds were made at an absorbancy of about 0·1 at 540 nm. The final concentrations of fluorouracil (FU), uracil (U) and thymidine (TdR) were 48, 48 and 160 μM respectively. Thymidine was added every 15 min because of its instability in the presence of bacterial cells.[14] ^{14}C-guanine incorporation (20 nCi/3·3 μg/ml) into the trichloroacetic acid-insoluble cell residue was used as a measure of total RNA synthesis, whereas ^{14}C-guanine incorporation into the KOH-insoluble cell residue was found to be a measure of incorporation into DNA.[15] In FU-treated cells, the reduced incorporation of ^{14}C-guanine was shown to be linked to a lowered content of DNA of the bacterial cells.[16] ^{14}C-FU was utilized to follow the incorporation of the drug in the RNA. Incorporation of radioisotopes was measured by the membrane filtration technique,[15] and comparisons were made after similar increases in bacterial turbidity, approximating similar increases in cell mass.[12]

For preparation of ribosomes, cultures were incubated until the absorbance at 540 nm had doubled, and were then harvested and extracts prepared for analysis.[13] Suspensions of the cells in 10 mM Tris buffer, pH 7·8, containing 10 mM Mg acetate and 60 mM KCl were disintegrated ultrasonically for 5 min at 0° in an MSE model 60W ultrasonic disintegrator, followed by centrifugation for 30 min at 10,000 *g*. The supernatant solutions were dialyzed at 4° for 18 hr against 500 vol. of the same suspension medium. Samples of 0·2 ml of this supernatant containing ribosomes were then layered over 5 ml of a linear 5–20% sucrose gradient in the suspension medium and were centrifuged in a Spinco model L ultracentrifuge at 105,000 *g* for 90 min in an SW 39 rotor. The density gradients were analyzed using an ISCO automatic density gradient fractionator (Instrumentation Specialties Co., Inc., Lincoln, Neb.) equipped with an ultraviolet analyzer. Fractions were collected directly in counting vials and 10 ml of Bray's solution was added. The radioactivity of each fraction was determined in a Beckman CPM-100 liquid scintillation counter. For the distribution calculations, ribosomes were considered present in tubes 1–19, whereas tubes 20–28 represented the soluble fraction.

Fluorouracil (NSC-19893) was kindly donated by the Cancer Chemotherapy National Service Center, Bethesda, Md. 2-^{14}C-FU (0·32 mCi/m-mole) was purchased from CalBiochem, Bethesda, Md. 8-^{14}C-guanine (0·9 mCi/m-mole) was obtained from New England Nuclear Corp., Boston, Mass. Sucrose for density gradients was RNase-free and was obtained from Schwartz BioResearch, Inc., Orangeburg, N.Y. All other chemicals were purchased from Fisher Scientific, Philadelphia, Pa.

RESULTS

The effect of FU and various combinations of U and TdR on growth, isotopic incorporation into DNA and RNA and the synthesis of ribosomes are presented in Table 1. The addition of TdR or U to control cultures had no effect on any of the parameters measured in this report. Treatment of *B. cereus* with FU produced a

TABLE 1. EFFECT OF FU AND PYRIMIDINES ON GROWTH AND ISOTOPIC INCORPORATION INTO MACROMOLECULES

Treatment*	Doubling time† (min)	^{14}C-guanine incorporation into‡ DNA (%)	RNA (%)	^{14}C-FU incorporation‡ (%)	RNA base replacement FU/(FU + U)§ (%)	Total radioactivity in ribosomes after ^{14}C-FU administration‖ (%)	Total FU in ribosomes¶ (μg/mg dry wt)	Total radioactivity in ribosomes after ^{14}C-guanine administration‖ (%)
Control	50	(100)	(100)	0	0	0	0	75·1 ± 4·0
FU/U/TdR	50	99 ± 3	101 ± 11	28 ± 4	5·5	61·9 ± 4·6	0·26	74·1 ± 1·8
FU/U	50	26 ± 2	99 ± 10	14 ± 3	2·7	56·0 ± 2·4**	0·08	71·9 ± 2·1**
FU	120	26 ± 2	106 ± 15	(100)	19·5	25·1 ± 2·6††	0·38	35·2 ± 3·0††
FU/TdR	120	102 ± 3	108 ± 9	236 ± 60	46·0	18·3 ± 2·1	0·65	19·5 ± 3·3

* Results presented are the average of three experiments and where listed are ± standard error of the mean. Drug concentrations are specified in the legend of Fig. 1.

† Doubling time is defined as the time required for a doubling of turbidity (or dry weight) at 540 nm

‡ Incorporation of ^{14}C-isotopically labeled compounds into DNA and RNA was measured by the membrane filtration technique.[15] About seven serial samples were measured during the growth of each culture. The results for ^{14}C-guanine are expressed as per cent of the control at identical cell mass. For ^{14}C-FU, the value for incorporation of radioactivity in the FU culture was taken as 100 per cent. Average incorporation of ^{14}C-guanine into DNA and RNA was 1,000 and 15,000 cpm respectively, and for ^{14}C-FU, 1,000 cpm into cells/ml of bacterial suspension after one doubling of bacterial cell mass.

§ Values calculated from incorporation data for newly formed RNA only.

‖ Ribosomes prepared as described in text. For the distribution calculations, ribosomes were considered present in tubes 1–19, whereas the soluble region was represented in tubes 20–28.[13]

¶ Calculated from cellular FU content and data on distribution of FU in ribosomes per mg dry weight of bacteria.

** Statistically significant $P < 0.01$.

†† Statistically significant $P < 0.05$.

slowing of the rate of growth, decreased the incorporation of labeled purine into DNA without any corresponding effect into total RNA, and resulted in the incorporation of FU into total RNA.[12] From studies on the incorporation of ^{14}C-FU, the replacement of U by FU was then calculated for the newly synthesized RNA.

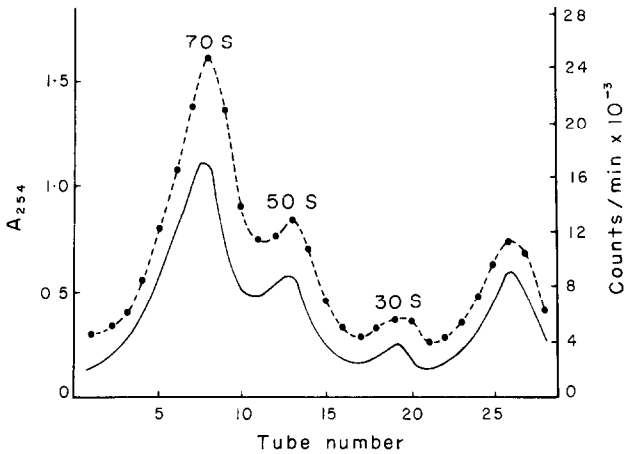

FIG. 1. Distribution of absorbancy and radioactivity after sucrose gradient analysis of *B. cereus* extracts from control cells. Cultures were incubated in the presence of 8-^{14}C-guanine (20 nCi/3·3 μg/ml). After one doubling of turbidity, cells were harvested and extracts prepared in 10 mM Mg^{2+} buffer as described in the text. These extracts were layered over 5–20% sucrose and centrifuged for 90 min at 105,000 *g* in a Spinco SW 39 rotor. Fractions were collected as described in Methods. Absorbancy at 254 nm, —; cpm, ---.

The distribution pattern of radioactivity and ultraviolet absorption after sucrose gradient centrifugation of an extract prepared from untreated cells grown in the presence of ^{14}C-guanine is shown in Fig. 1. The profile was characterized by a large peak corresponding to the 70S ribosomal fraction and smaller peaks corresponding to the 50S and 30S ribosomal subunits; the area corresponding to soluble RNA (4S) was smaller than that of the 70S ribosomal fraction. The distribution of radioactivity parallels that of absorbancy. The distribution pattern of an extract from a culture treated with FU (Fig. 2) was characterized by a reduced 70S ribosomal fraction and the appearance of increased quantities of 50S and 30S ribosomal subunits. Furthermore, isotopic incorporation into the 4S fraction was markedly increased (Fig. 2). Table 1 also describes the percentage of total radioactivity, following growth in the presence of ^{14}C-guanine or ^{14}C-FU, which was incorporated into the ribosomal portion of the cell extracts. The total incorporation of FU into ribosomes expressed per mg of dry weight of the cells was then calculated for each culture.[13]

When uracil was co-administered with FU(FU/U), the slowing of the rate of proliferation characteristic of FU alone was prevented for about 50 min, although thereafter growth inhibition became noticeable.[16] During this early period when growth of the FU/U culture proceeded at a normal rate, DNA synthesis still remained sharply curtailed, but the presence of U reduced the incorporation of FU (Table 1) into RNA and thus the replacement of U by FU. Figure 3 demonstrates that, in the presence of U, the FU-related inhibition of ribosome formation was prevented, and

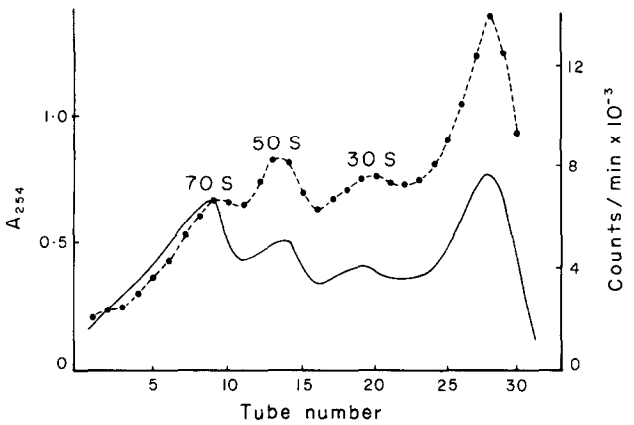

FIG. 2. Analysis as in Fig. 1, but cells were grown with 48 μM FU.

the elution pattern after sucrose density gradient centrifugation was indistinguishable from that of control cells.

In the presence of thymidine and FU(FU/TdR), which prevents the FU-related inhibition of DNA formation without significantly modifying the FU-produced slowing of growth,[17] the incorporation of FU into total RNA and the replacement of U by FU were greatly enhanced (Table 1). However, ribosomal formation still remained grossly abnormal (Fig. 4).

The simultaneous presence of FU, uracil and thymidine (FU/U/TdR) resulted in essentially control values for growth, DNA synthesis and the pattern of ribosome formation, but permitted extensive incorporation of FU into RNA (Table 1).

DISCUSSION

It appears from an examination of Table 1 that there was no correlation between the mass doubling time and the rate of DNA synthesis of the bacterial cultures grown

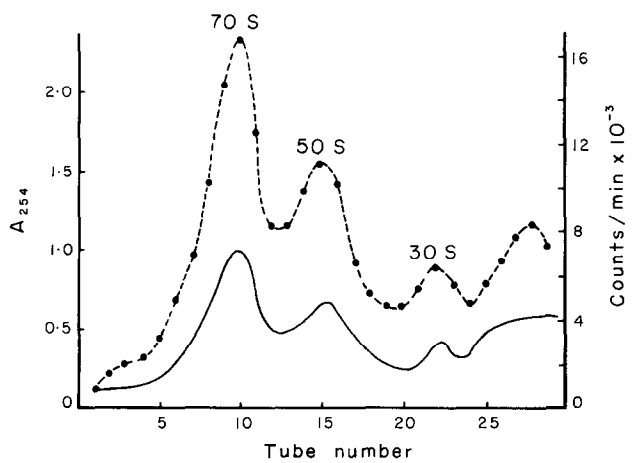

FIG. 3. Analysis as in Fig. 1, but cells were grown with 48 μM FU and 48 μM U.

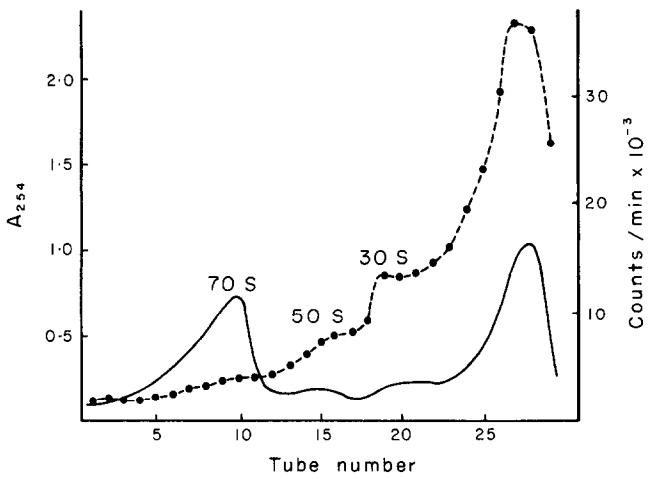

FIG. 4. Analysis as in Fig. 1, but cells were grown with 48 μM FU and 160 μM TdR.

under the various conditions of drug treatment. For the same increase in bacterial mass, total RNA synthesis was identical for all cultures regardless of treatment. Furthermore, there was no simple and quantitative relation between the rate of increase in cell mass and the total quantity of FU in the cells. For instance, the addition of TdR to either the FU or the FU/U cultures had no effect on the rate of increase in cell mass, although the incorporation of FU into the cells doubled.

Similarly, the quantity of ^{14}C-FU incorporated specifically into the ribosomal fraction of the total cell mass was not directly related to the inhibition of growth, since the FU and FU/U/TdR cultures incorporated approximately equal quantities of ^{14}C-FU into their ribosomal fractions (0·38 and 0·26 μg FU/mg dry weight of cells, respectively), although their growth rates differed widely. Therefore, we conclude that growth inhibition probably was not a result of the incorporation of FU into the whole cell or the ribosome fraction.

On the other hand, those cultures which grew at control rates maintained normal ribosomal synthesis (FU/U, FU/U/TdR), whereas those in which growth was delayed had a characteristically altered pattern of ribosomal synthesis (FU, FU/TdR). Thus, a clear-cut correlation exists between these sets of measurements.

It has been suggested by DeKloet and Strijkert[18] that interference with DNA formation may be a cause of altered ribosomal RNA. In this study, however, it has been demonstrated that the addition of TdR to FU-treated cultures returned DNA synthesis to normal levels but did not reverse the inhibition of ribosome synthesis produced by FU. In addition, FU/U-treated cultures produced ribosomes indistinguishable from those of untreated cells, even though DNA synthesis was inhibited. Therefore, interference with DNA synthesis as a cause of altered ribosomal synthesis by FU was excluded in the *B. cereus* system.

It may be argued that growth inhibition results from a combination of the various effects measured, and certainly this is true when growth and viability are measured over an extended period of time.[16,17] Furthermore, it is still quite possible that the incorporation of FU into a select site of m-RNA, for example, is responsible for the inhibition of growth, but any specific incorporation of FU is obscured by the relatively

gross measurements made in the present investigation. The incorporation of FU into ribosomes does not appear to be such a site, however, and the incorporation of FU into m-RNA[19] does not necessarily lead to loss of biological activity.[10]

Comparisons of the relative distribution of radioactivity between the ribosome and soluble RNA fractions show statistically significant differences depending on whether ^{14}C-guanine or ^{14}C-FU is used as the label, at least for the FU and FU/U cultures. Previously, we reported that FU was incorporated somewhat selectively into s-RNA during growth inhibition,[13] but a similar pattern now has been shown to exist for the FU/U culture, which grew at the normal rate. Thus, there is no evidence from these experiments of any correlation between a specific localization of FU in the RNA and growth inhibition. It is known that major changes in content of minor base constituents in t-RNA occur in association with replacement of U by FU, leading to alteration in normal functions in polypeptide synthesis *in vitro*.[20–22]

It therefore appears by elimination that the effect of FU on ribosome formation, or an action closely associated with it, may have major significance with respect to the early phase of growth inhibition of *B. cereus* produced by FU.

REFERENCES

1. S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb and J. Lichtenstein, *Proc. natn. Acad. Sci. U.S.A.* **44,** 1004 (1958).
2. L. Bosch, E. Harbers and C. Heidelberger, *Cancer Res.* **18,** 335 (1958).
3. N. K. Chaudhuri, B. J. Montag and C. Heidelberger, *Cancer Res.* **18,** 318 (1958).
4. A. I. Aronson, *Biochim. biophys. Acta* **49,** 98 (1961).
5. P. P. Saunders, R. E. Bass and G. F. Saunders, *J. Bact.* **96,** 525 (1968).
6. D. C. Hills and J. Horowitz, *Biochemistry, N.Y.* **5,** 1625 (1966).
7. U. Stenram, A. Bengtsson and R. Willén, *Cytobios* **5,** 125 (1972).
8. D. S. Wilkinson, A. Cihak and H. C. Pitot, *J. biol. Chem.* **246,** 6418 (1971).
9. C. Heidelberger, *Prog. nucleic Acid Res. molec. Biol.* **4,** 1 (1965).
10. H. G. Mandel, *Prog. molec. subcell. Biol.* **1,** 82 (1969).
11. M. Reich and H. G. Mandel, *Science, N.Y.* **145,** 276 (1964).
12. M. Reich and H. G. Mandel, *J. Bact.* **91,** 517 (1966).
13. G. A. Hahn and H. G. Mandel, *Biochem. Pharmac.* **20,** 1973 (1971).
14. E. L. Alpen and H. G. Mandel, *Biochim. biophys. Acta* **43,** 317 (1960).
15. D. B. Roodyn and H. G. Mandel, *Biochim. biophys. Acta* **41,** 80 (1960).
16. C. H. Doolittle, H. G. Mandel and G. A. Hahn, *J. Bact.* **113,** 1311 (1973).
17. C. H. Doolittle, H. G. Mandel and G. A. Hahn, *Chemotherapy* **19,** 22 (1973).
18. S. R. DeKloet and P. J. Strijkert, *Biochem. biophys. Res. Commun.* **23,** 49 (1966).
19. F. Gros, W. Gilbert, H. H. Hiatt, G. Attardi, P. F. Spahr and J. D. Watson, *Cold Spring Harb. Symp. Quant. Biol.* **26,** 111 (1961).
20. R. J. Lowrie and P. L. Bergquist, *Biochemistry, N.Y.* **7,** 1761 (1968).
21. I. I. Kaiser, *J. molec. Biol.* **71,** 339 (1972).
22. J. Horowitz and E. S. Ramberg, *Fedn Proc.* **31,** 867 (1972).