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SOME EFFECTS OF 5-BROMOURACIL ON A THYMINE-REQUIRING MUTANT OF *ESCHERICHIA COLI*

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Abstract—Studies of the biochemical effects of the thymine analog, 5-bromouracil (BU), on a thymine-requiring mutant of *E. coli*, were carried out. Partial inhibition of growth was noted within 30 min after the compound was added to an exponentially growing culture. With cultures grown to the same turbidity, the analog produced no effect on the utilization of the nucleic acid precursors, 2-^{14}C-uracil and 4-^{14}C-guanine, whereas that of 2-^{14}C-thymine was blocked completely. Correspondingly, the content of RNA was identical when cells were grown to the same turbidity in the presence or absence of BU; on the other hand, the synthesis of DNA was only 25 per cent of that of the control, and cell division stopped entirely. During this period, BU continued to be incorporated into new DNA, but it did not displace the thymine already in DNA. Although the drug decreased the rate of protein synthesis for corresponding increases in cell mass, more protein synthesis took place in the BU-inhibited cultures, and certain amino acids were utilized to a still greater extent. The possibility of disruption in the normal process of protein synthesis is discussed.

INTRODUCTION

THE growth inhibitory activity of the thymine analog, 5-bromouracil (BU), first reported by Hitchings *et al.* for *Lactobacillus casei*,[1] also is characteristic of other bacterial systems.[2–5] The incorporation of BU into the bacterial deoxyribonucleic acids (DNA) has been unequivocally demonstrated by the recovery of the analog from the DNA as the free base, the deoxyribonucleoside (5-bromo-2′-deoxyuridine) and the deoxyribonucleotide (5-bromo-2′-deoxyuridylic acid).[3, 5, 6] Inhibition of growth by BU has been shown to be associated with morphological changes[7, 8] and related to "thymineless death" due to "unbalanced growth".[9] BU produces mutations in bacterial cells which are thought to be specific effects and not mere enhancement of spontaneously occurring mutations.[10]

The major emphasis in the early work on BU concerned incorporation into the DNA and reversal of growth inhibition with natural nucleic acid components or their precursors. Although some of the over-all biochemical effects of BU have been recognized for several years, it was intended in the present work to correlate quantitatively the effect of the compound on purine, pyrimidine and amino acid incorporation, to associate the inhibition of growth with the observed dissociations of biosynthetic functions, and to determine the likelihood of replacement of preformed DNA-thymine by the analog. The highly sensitive membrane filtration method

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employed previously in this laboratory was used, and allowed the frequent sampling and analysis of cells during inhibition. Preliminary results have been presented previously.[11]

MATERIALS AND METHODS

Bacterial growth

Escherichia coli I, a thymine-requiring mutant (ATCC No. 11117), kindly furnished by Dr. S. Zamenhof, Columbia University, New York, was used throughout. Thymine-dependency was maintained by periodic streaking of the cells on nutrient agar supplemented with thymine and selecting individual colonies requiring thymine by the replica plate technique. The cells were stored in the cold and every few weeks retested for thymine-dependency.

Bacterial growth studies were carried out employing either a basal salts medium-(A) (supplemented with glucose (1 per cent) and thymine (2 mg/l.)) or medium-(S) (supplemented with sulfanilamide, xanthine and casamino acids), as previously described by Dunn and Smith.[7] The latter medium has been used to maintain thymine-dependency. The cells were incubated overnight in medium-(A) and were centrifuged, washed twice with medium-(A) and resuspended in a small volume of the same medium. The cells were then added to medium-(S) to give an initial turbidimetric reading of 0·04 at 540 mμ in a Beckman spectrophotometer, model DU (approximately 2×10^7 cells per ml of medium). The cells were grown to turbidimetric readings of 0·08, i.e. the early phases of logarithmic growth. The labeled compound under study was then added, the culture thoroughly mixed and divided into equal portions. Bacteria were grown in plastic wash bottles at 37 °C and were aerated by vigorous shaking. BU dissolved in 1 per cent sodium carbonate was then added to one subculture of cells; another, which served as control, received only the sodium carbonate. The procedure for sampling and assaying by the membrane filtration technique has been previously described.[12] In the present work, control and inhibited cells were compared at similar turbidities.

Chemicals

2-^{14}C-Uracil and 4-^{14}C-guanine have been prepared previously.[13, 14] 5-Bromouracil-2-^{14}C (0·19 μc/mg) was synthesized from 2-^{14}C-uracil and bromine, as described by Weygand and Wacker,[4] and was isolated by ion-exchange chromatography on Dowex 1–X8 (200–400 mesh)-acetate. Elution was effected by progressively increasing concentrations of acetic acid from 0·01 N to 0·1 N. 2-^{14}C-Thymine, ^{14}C-formate, 1-^{14}C-alanine, 3-^{14}C-serine and 1-^{14}C-leucine were obtained from the Isotopes Specialties Co., Burbank, California. ^{35}S-Methionine and ^{35}S-cystine were purchased from Schwarz Laboratories, Mount Vernon, New York. ^{3}H-Diaminopimelic acid was kindly furnished by Dr. J. L. Strominger, Washington University, St. Louis, Missouri.

Paper chromatography

Solvent systems included: (a) *n*-butanol saturated with water to separate uracil from BU; (b) *iso*propanol-HCl, as previously described by Wyatt[15] for the resolution of nucleic acid bases, and (c) 70 per cent *iso*propanol–water with ammonia in the vapor phase to distinguish BU from thymine in nucleic acid hydrolysates.

Compounds which absorbed in the ultraviolet range, located photographically,[16] were cut out, measured directly for radioactivity and extracted with suitable solvents for estimations of purity and concentration in a Beckman spectrophotometer, model

DU. Areas identical in size were cut out from adjacent strips for use as blanks. Radioassay was performed in a proportional gas-flow counter.

Fractionation procedures

Nucleic acids. Ribonucleic acid (RNA) was separated from DNA by the Schmidt and Thannhauser[17] procedure. Total RNA and total DNA were estimated by the methods described by Ceriotti for RNA[18] and DNA[19]. Mononucleotides were separated by paper electrophoresis at pH 3·5.[16] Treatment with 5 per cent trichloroacetic acid (TCA) at 100 °C for 30 min led to partial hydrolysis of total nucleic acids.[20] Nucleic acids were hydrolyzed to the free purines and pyrimidines with 12 N perchloric acid.[21] The cooled hydrolysates were neutralized with N KOH to precipitate $KClO_4$ and the supernatant solution of nucleic acid bases was chromatographed.

Protein. The insoluble protein fraction after extraction with boiling 5 per cent TCA was hydrolyzed in a pressure cooker with 6 N HCl at 12 lb/in^2 for 3 hr. After washing with water to remove HCl, aliquots of the hydrolysate were chromatographed on Whatman no. 1 filter paper. The two-dimensional procedure was essentially that described by Roberts *et al.*[22] Total protein was estimated by the method of Lowry *et al.*[23] Blue-sensitive Kodak X-ray film was used for the preparation of radioautograms which were exposed for a period of from 1 to 2 weeks to locate labeled amino acids.

Isotope distribution

The uptake of radioactivity from a radiolabeled precursor into the unfractionated cell (growth-medium-insoluble) and the cold acid-insoluble and hot acid-insoluble fractions of the cell was measured readily by the membrane filtration technique, as described for *Bacillus cereus* by Roodyn and Mandel.[12] With the proper precursors this method gave a rapid estimation of radioactivity in the nucleic acid, protein and acid-soluble fractions of the cell.

RESULTS

Effects of 5-bromouracil on the growth of E. coli

A concentration of BU of 200 μg per ml (approx. 1 mM) was partially inhibitory to the exponential growth of *E. coli I* in medium-(S), as measured turbidimetrically, and was used throughout. This slowing resulted within 30 min after the addition of BU. By using this thymine-requiring mutant in a medium containing sufficient thymine to allow near-maximal growth, precise and reproducible antagonism was observed in the presence of varying concentrations of BU.

The profound morphological changes induced by the analog, producing some cells up to forty times the normal length, necessitated a clarification of the application of turbidity as a measure of bacterial growth. In control cultures, cell numbers increased logarithmically and optical density was directly related to cell numbers. After exposure to BU for 30 min, the increase in cell numbers, measured by direct count in the inhibited culture, ceased abruptly, while optical density continued to increase and the cells continued to increase in size (Fig. 1). A comparison of the dry weights for both the inhibited and control cultures showed insignificant differences between the two at the same turbidity. It was concluded, therefore, that the application of turbidity measurements to assessment of bacterial growth was a good index of cell mass rather than of cell number.

In the present work, comparisons between cultures exposed to BU and control cultures have been made after corresponding increases in cell mass. In this manner it

was possible to focus greater emphasis on those analog-induced biochemical reactions affected to a different extent than growth. It should be emphasized, however, that an increased response, as measured in this manner, does not necessarily imply an absolute increase in the *rate* of this response over that of a control culture, but refers to the relative composition of the newly formed cellular mass.

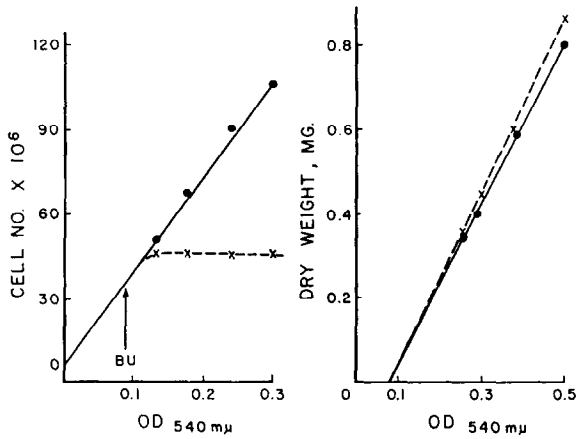

FIG. 1. Effect of 5-bromouracil, 200 μg/ml, on cell number (left) and dry weight of cells (right) per ml of suspension of growing *E. coli*, strain I. Growth was measured turbidimetrically, and the analog was added at OD_{540} of 0·08. Control culture (●), BU-treated culture (×).

Uptake of 5-*bromouracil*-2-^{14}C

To determine the pattern of incorporation of BU into cells, bacteria were grown in the usual manner and, to each ml, 200 μg of 5-bromouracil-2-^{14}C were added. Thereafter, as growth continued at a greatly diminished rate, samples of the suspension were removed, precipitated with TCA, filtered to separate the cells, washed and the radioactivity was determined, as described.[12] The results are shown in Fig. 2. It is to be noted that the analog was incorporated immediately, and that this process continued throughout the period of growth inhibition, even though cell division no longer took place (cf. Fig. 1).

All of the radiocarbon from the analog was solubilized by treatment of the cells with hot 5 per cent TCA, a procedure which extracts nucleic acids, but not proteins. The lack of radioactivity in the RNA extracted by the Schmidt and Thannhauser procedure, the recovery of radiolabeled 5-bromo-2′-deoxyuridine after hydrolysis, and electrophoretic and chromatographic behavior, confirmed the observations of Dunn and Smith[3] that BU was incorporated into the DNA.

Using the present procedure, it was not possible to estimate whether the quantity of BU incorporated was the molar equivalent of the thymine which it replaced in the newly formed DNA. The replacement of thymine by BU has been determined previously by base-ratio calculations.[7]

5-*Bromouracil effect on components of* E. coli I

Colorimetric determination of the protein, RNA and DNA components of cells grown to the same turbidity in the presence and absence of BU revealed that, while

total RNA was relatively unaffected by the analog, the synthesis of DNA was depressed markedly (Table 1). Since the inhibitor was added at OD 0·10 and the cells were harvested at OD 0·30, and since the DNA made before the addition of the analog was still present, it can be calculated that the DNA content of the bacterial mass formed during inhibition was only 25 per cent that of control cells. Likewise, the bacterial mass formed during inhibition by BU contained from 8 to 28 per cent more new protein than did the control mass.

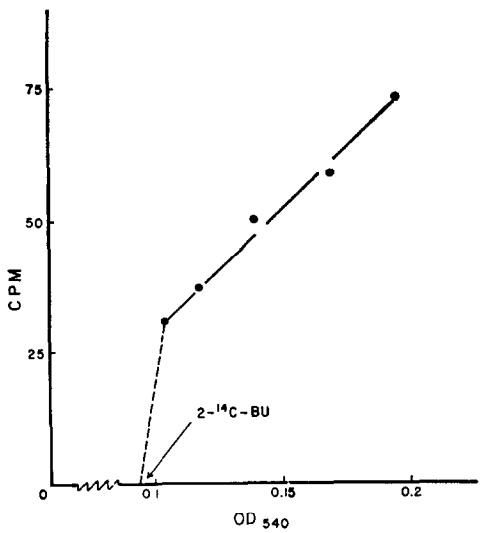

FIG. 2. Uptake of 5-bromouracil into bacterial cells. The culture was grown with BU-2-^{14}C, 200 μg/ml. One-millilitre samples of the suspension were treated with cold trichloroacetic acid, filtered through membranes, and the cells were assayed for radioactivity. Radiocarbon was recovered in the DNA of cells.

TABLE 1. EFFECT OF 5-BROMOURACIL ON THE CONTENT OF PROTEIN, RNA AND DNA OF *E. coli*

Chemical component	Number of experiments	Percentage of control
Protein	4	103
		105
		105
		117
RNA	4	109
		86
		91
		96
DNA	4	50
		50
		65
		50

Bacteria at OD_{540} of 0·1 were grown to OD_{540} of 0·3, in the presence or absence of 5-bromouracil, and bacterial contents per ml of suspension were assayed colorimetrically.

Effect of 5-bromouracil on the utilization of uracil and guanine

The nucleic acid precursors, 2-^{14}C-uracil and 4-^{14}C-guanine, were added to bacterial cultures during the early stages of logarithmic growth, and uptake and distribution of the radiolabeled compounds were measured by membrane filtration. The incorporation of isotope into the nucleic acid fraction (insoluble in cold 5 per cent TCA) is presented in Fig. 3. In the case of either precursor, the uptake of radioactivity was essentially equal for the inhibited and control cultures. More than 99 per cent of the radioactivity was present in the nucleic acid fraction.

Effect of 5-bromouracil on the utilization of thymine

Cells grown in medium-(A) containing non-labeled thymine were transferred for growth in medium-(S). Cells were then resuspended in medium-(S) supplemented with

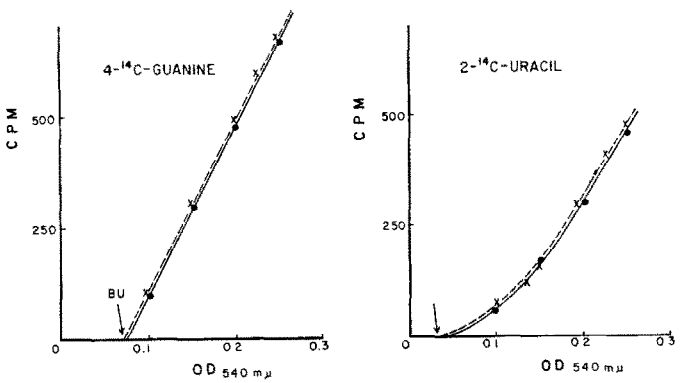

FIG. 3. Uptake of radioactive nucleic acid precursors into bacterial cells. The cultures were grown with labeled guanine (20 μg/ml) (left) and uracil (20 μg/ml) (right) in presence of (×) or absence of (●) 200 μg of 5-bromouracil per ml. Additions were made as indicated by arrow. The radioactivity was found exclusively in the nucleic acids. One-millilitre samples of suspensions were treated with cold trichloroacetic acid, filtered and the cells were assayed for radioactivity.

0·75 μg of thymine-2-^{14}C per ml, and the radioactivity and optical density of aliquots were measured. After some growth, BU, 200 μg/ml, was added to one portion of the cells, and both cultures were allowed to continue growth. Fig. 4 represents the radioactivity in the nucleic acid fraction of cells.

It is evident that the uptake of thymine-2-^{14}C was blocked completely by the addition of BU. The level of radioactivity, after the addition of the analog, remained constant, while the turbidity increased. Thus, there was no indication of displacement by the analog of the labeled thymine already present in the DNA. Such results differ from those of Weygand *et al.*[24] and Zamenhof *et al.*[8, 25] who, using resting cells. have reported that exchange takes place between BU and thymine in *S. faecalis* and *E. coli.*

Effect of 5-bromouracil on the utilization of ^{14}C-formate for nucleic acid synthesis

Experiments involving the utilization of ^{14}C-formate were conducted with cells in the usual way. At OD_{540} of 0·1 the isotope was introduced, and, at OD_{540} of 0·3, cells from the cultures exposed to the analog and from control cultures were harvested and fractionated as described.[17, 21] Determination of uptake, carried out by the membrane

filtration technique, indicated that the incorporation of radioactivity from ^{14}C-formate was reduced by treatment with BU.

In these experiments, it was important that sodium carbonate was added to the control culture in an amount equivalent to that used to dissolve the analog, in order to prevent the conversion of formate to CO_2, with resultant extensive labeling of the

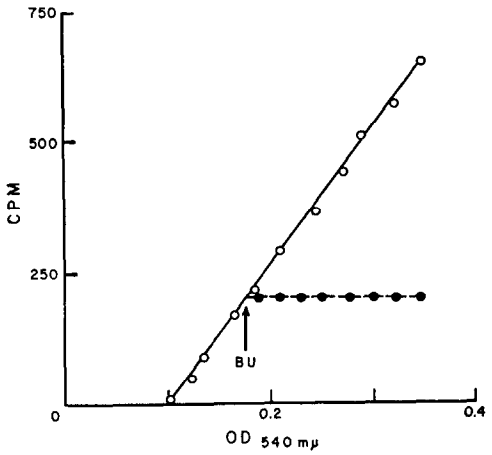

FIG. 4. Radioactivity in DNA of an aliquot of cells grown with 2-^{14}C-thymine (0·75 μg/ml) added at OD_{540} of 0·1. 5-Bromouracil (200 μg/ml) was added at arrow to one portion of the culture. Control culture (○), BU-treated culture (●). Procedure as in Fig. 3.

pyrimidines by CO_2 rather than formate. Under the present conditions, pyrimidines were still radioactive, but treatment with BU exerted little effect on the specific activity values of cytosine and uracil, in contrast to the decreased specific activity of adenine (Table 2). It was concluded, therefore, that the utilization of formate was affected by the analog, rather than the conversion to and uptake of CO_2.

TABLE 2. SPECIFIC ACTIVITIES OF RNA BASES ISOLATED FROM CULTURES INHIBITED BY 5-BROMOURACIL AND CONTROL CULTURES GROWN TO THE SAME TURBIDITY IN THE PRESENCE OF ^{14}C-FORMATE

BU	Folic acid	Molar activity		
		Adenine	Cytosine	Uracil
—	—	(100)	8·9	10·3
+	—	70·0	10·1	11·0
+	+	67·5	11·2	11·8

Relative specific activities of RNA-adenine set at (100).

The relative specific activity of guanine was considerably lower than that of adenine, undoubtedly because the presence of xanthine in the medium reduced the *de novo*-formation of guanine by dilution or feed-back inhibition. The effects on DNA bases were identical to those on the bases of RNA. DNA-thymine contained only 2 per cent of the molar activity of adenine in the control culture, a finding which indicated that this mutant lacks the ability to methylate deoxyuridylic acid.

The uptake of formate was not influenced by folic acid, even at a concentration equimolar with that of BU, and no effect on the growth inhibition was apparent. Thus, the effect on formate utilization apparently did not involve interference at the cofactor level of the one-carbon transfer mechanism.

Effect of 5-bromouracil on the utilization of amino acids

Radioactive amino acids were added directly to medium-(S), which already contained casamino acids, and incorporation of isotope into the cell fraction insoluble in hot TCA, consisting largely of bacterial proteins, was measured. With most of the amino acids used, more radioactivity was taken up by the cells exposed to BU than by the cells of the control culture, grown to the same turbidity. For example, this ratio, in several such isotope experiments, was, respectively, 1·29, 1·35 and 1·30 for 1-^{14}C-alanine; 1·28, 1·37 and 1·23 for 1-^{14}C-leucine; 1·32 and 1·20 for ^{35}S-methionine; 1·39 for ^{35}S-cystine; but only 0·87 and 0·99 for 3-^{14}C-serine. The probable cleavage of this latter amino acid to labeled one-carbon fragments which were utilized less in the BU-treated culture (cf. the formate-^{14}C experiment above) may explain this deviation.

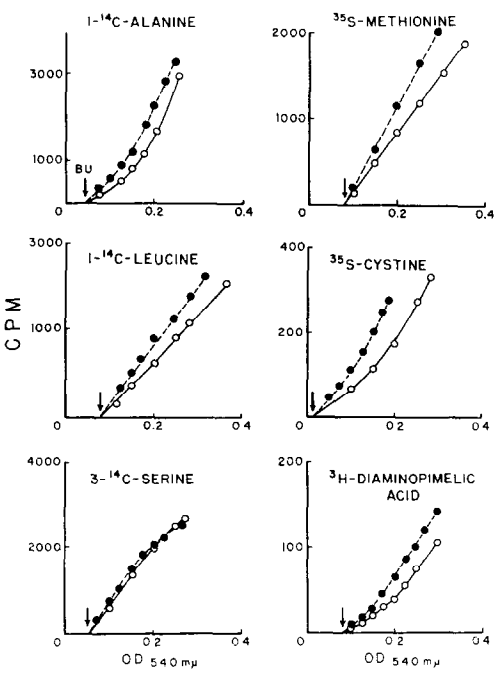

FIG. 5. Radioactivity in protein fraction of an aliquot of cells grown with labeled amino acids in presence of (●) and absence of (○) 200 μg of bromouracil per ml. BU and isotopes were added at arrow. Procedure as in Fig. 3.

The kinetics of the uptake results are shown in Fig. 5. The uptake curves reveal changes in the initial slopes for the various amino acids as compared to the controls. These differences were probably related to variations in the pool sizes of amino acids. Once the different dilution rates were equilibrated, the slopes after exposure to BU usually became more similar.

The hydrolyzed protein residues of cells grown with either 1-^{14}C-leucine or 1-^{14}C-alanine were resolved by two-dimensional chromatography and radio-autography, as described.[22, 28] The patterns of radioactivity on the chromatograms were unaltered by exposure of the cells to BU, and since no new compounds were labeled in the presence of the analog, it was assumed that the increased utilization of the amino acids represented an enhancement of the normal pattern of incorporation. It was observed, however, that in most experiments the relative increase in formation of total protein in the presence of the analog was considerably less than the increase in the incorporation of alanine, leucine, methionine and cystine. An altered ratio of amino acids in the newly synthesized protein might explain this discrepancy.

In order to determine whether this effect of BU was related to the synthesis of cell walls, rather than to that of protoplasmic protein, experiments were carried out with labeled diaminopimelic acid, since this compound, when present in bacteria, is a component characteristic of the cell wall.[26] Practically all radioactivity in the mutant was present as lysine, however, in agreement with earlier work on the wild strain of *E. coli*.[27]

DISCUSSION

This investigation has revealed additional quantitative evidence for dissociation of the various synthetic processes of the cell by BU. For the same turbidimetric increase, inhibited cells formed less DNA and incorporated more of certain amino acids than did control cells, whereas the contents of RNA were approximately equal. On the same basis of comparison, the incorporation of 8-azaguanine into nucleic acids of *B. cereus* has been shown to lead to the almost complete suppression of protoplasmic protein synthesis, whereas RNA and DNA (and cell wall) are increased.[28, 29] Similar results also have been obtained with chloramphenicol in this system.[30] On the other hand, 6-mercaptopurine decreased the amounts of RNA and DNA in *E. coli*, while leaving the protein content essentially unchanged.[31, 32]

The inability of the mutant to form thymine *de novo* from one-carbon units, and the complete inhibition by BU of the incorporation of thymine, exclude the common mechanisms by which DNA-thymine can be supplied. Although some DNA is still being synthesized by the cells, it must be "thymine-less", although BU may substitute for much if not all of the thymine. Such micro-organisms have lost the ability to divide, although they still retain the capacity to synthesize new cellular material containing the analog. The earlier work by Jeener and Jeener,[33] which showed that thymine-less elongated cells have an unchanged content of RNA, but a depressed amount of DNA, would also be in agreement with the present findings.

The lack of effect of BU on the synthesis of nucleic acids from the preformed bases is in contrast to the effect of the analog on the synthesis of purines from labeled formate. This discrepancy, which therefore is apparently unrelated to the effect of the analog on the synthesis of DNA, probably involves the fact that, to reach the desired turbidity, the inhibited cultures were grown for a longer period of time than were the control cells. In other experiments, similar dissociations between the formation of purines *de novo* and the utilization of formate have been observed and have been ascribed to degradation reactions of formate unrelated to bacterial growth.[30]

The increased utilization of amino acids for protein synthesis by analog-treated cells grown to the same turbidity as control cells does not imply an actual stimulation in

the rate of protein biosynthesis, but rather a change in the relative composition of the bacterial cells. No evidence is available to localize this protein effect in the cell. Kit *et al.*[34] have reported a slight stimulation by 5-bromo-2′-deoxyuridine of the incorporation of either formate or formaldehyde into the protein of neoplastic tissue.

The biosynthesis of DNA containing BU presupposes a soluble nucleotide of the analog, and this compound may interfere with the synthesis or function of thymine-containing coenzymes. Evidence for the existence of such compounds has been accumulating.[35] Nevertheless, until their function is known better, it will be difficult to ascribe the inhibition of growth by the analog to either interference at the cofactor level or to incorporation into DNA. There is considerable evidence, however, that the incorporation of BU into the DNA alone is not necessarily associated with inhibition of specific functions.[36]

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REFERENCES

1. G. H. HITCHINGS, E. A. FALCO and M. B. SHERWOOD, *Science* **102**, 251 (1945).
2. F. WEYGAND, A. WACKER and H. GRISEBACH, *Z. Naturforsch.* **6b**, 177 (1951).
3. D. B. DUNN and J. D. SMITH, *Nature, Lond.* **174**, 305 (1954).
4. F. WEYGAND and A. WACKER, *Z. Naturforsch.* **56**, 46 (1950).
5. S. ZAMENHOF and G. GRIBOFF, *Nature, Lond.* **174**, 307 (1954).
6. S. ZAMENHOF and G. GRIBOFF, *Nature, Lond.* **174**, 306 (1954).
7. D. B. DUNN and J. D. SMITH, *Biochem. J.* **67**, 494 (1957).
8. S. ZAMENHOF, R. DEGIOVANNI and K. RICH, *J. Bact.* **71**, 60 (1956).
9. S. S. COHEN and H. D. BARNER, *J. Bact.* **71**, 588 (1956).
10. S. BENZER and E. FREESE, *Proc. Nat. Acad. Sci., Wash.* **44**, 112 (1958).
11. I. A. MICHAELSON, H. G. MANDEL and P. K. SMITH, *Proc. Amer. Ass. Cancer Res.* **3**, 42 (1959).
12. D. B. RODYN and H. G. MANDEL, *Biochim. et Biophys. Acta* **41**, 80 (1960).
13. H. G. MANDEL and C. L. BROWN, *J. Amer. Chem. Soc.* **74**, 2439 (1952).
14. H. G. MANDEL and P. CARLO, *J. Biol. Chem.* **201**, 335 (1953).
15. G. R. WYATT, *Biochem. J.* **48**, 584 (1951).
16. R. MARKHAM and J. D. SMITH, *Biochem. J.* **49**, 407 (1951).
17. G. SCHMIDT and S. J. THANNHAUSER, *J. Biol. Chem.* **161**, 83 (1945).
18. G. CERIOTTI, *J. Biol. Chem.* **214**, 59 (1955).
19. G. CERIOTTI, *J. Biol. Chem.* **198**, 297 (1952).
20. W. C. SCHNEIDER, *J. Biol. Chem.* **161**, 293 (1945).
21. A. MARSHAK and H. J. VOGEL, *J. Biol. Chem.* **189**, 597 (1951).
22. R. B. ROBERTS, P. H. ABELSON, D. B. COWIE, E. T. BOLTON and R. J. BRITTEN, *Publ. Carnegie Instn*, No. 607 (1955).
23. O. H. LOWRY, N. J. ROSENBOUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
24. F. WEYGAND, A. WACKER and H. DELLWEG, *Z. Naturforsch.* **76**, 19 (1952).
25. S. ZAMENHOF, B. REINER, R. DEGIOVANNI and K. RICH, *J. Biol. Chem.* **219**, 165 (1956).
26. E. WORK, *Nature, Lond.* **179**, 841 (1957).
27. P. MEADOW and E. WORK, *Biochem. J.* **71**, 2P (1959).
28. D. B. RODYN and H. G. MANDEL, *J. Biol. Chem.* **235**, 2036 (1960).
29. H. G. MANDEL, *J. Pharmacol.* **133**, 141 (1961).
30. H. G. MANDEL and R. L. ALTMAN, *J. Pharmacol.* **133**, 151 (1961).
31. E. T. BOLTON and H. G. MANDEL, *J. Biol. Chem.* **227**, 833 (1957).
32. H. G. MANDEL, J. K. INSCOE, H. M. MALING and P. K. SMITH, *J. Pharmacol.* **120**, 195 (1957).
33. H. JEENER and R. JEENER, *Exp. Cell Res.* **3**, 675 (1952).
34. S. KIT, C. BECK, O. L. GRAHAM and A. GROSS, *Cancer Res.* **18**, 598 (1958).
35. R. OKAZAKI, *Biochem. Biophys. Res. Comm.* **1 (1)**, 34 (1959).
36. R. E. F. MATTHEWS, *Pharmacol. Rev.* **10**, 359 (1958).