Biochemistry VOLUME 1, NUMBER 4

July 20, 1962

The Mechanism of an Osmotic Instability Induced in *E. coli* K-12 by 5-Fluorouracil*

Alexander Tomasz† and Ernest Borek

From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York

Received February 20, 1962

The bactericidal action of 5-fluorouracil in *E. coli* K-12 can be reversed by increasing the osmotic pressure of the growth medium. The lethal action of the drug requires active growth of the organisms. The formation of osmotically sensitive "spheroplast"-like bodies during the process of the drug-induced disintegration of the cells is demonstrated. 5-Fluorouracil inhibits the incorporation of $\alpha\epsilon$-diaminopimelic acid into the cell walls of this organism; large amounts of *N*-acetyl hexosamine esters and diaminopimelic acid accumulate in the acid-soluble fraction of the cells. The mechanism of action of 5-fluorouracil in this organism seems to involve an abnormal course of cell wall synthesis.

The pyrimidine analogue 5-fluorouracil (FU), which was originally developed as a carcinostatic agent (Duschinsky *et al.*, 1957), also has profound inhibitory effect on both the growth and the reproduction (viability) of various microorganisms (Heidelberger *et al.*, 1957).

Extensive studies on the toxicity of 5-fluorouracil in *E. coli B* and *E. coli 15* have been reported, and it was suggested that the rapid loss of viability of 5-fluorouracil-treated cells was most likely the result of the inhibition of thymine synthesis by the drug (Cohen *et al.*, 1958). The cessation of DNA synthesis and a subsequent "thymine-less death" was suggested as the cause of the death of the organisms. A similar mechanism was offered for the inhibition of ascites cells (Mukharjee and Heidelberger, 1960).

The mechanism of the bactericidal action of 5-fluorouracil seems to be basically different in the K-12 strain of *E. coli*. During studies on the lethal action of 5-fluorouracil in this bacteria we have encountered a heretofore unobserved mode of action of this drug: the induction of an osmotically sensitive state in the bacteria which causes rapid loss of viability in 90–99% of the organisms (Tomasz and Borek, 1959). This effect of 5-fluorouracil could be prevented by uracil and uridine but not by thymine or thymidine. Moreover, the organisms could be restored to viability by increasing the osmotic strength of their environment. We report here evidence that the bactericidal action of 5-fluorouracil under the experimental conditions used can be fully explained by the osmotic fragility of the cells. An abnormal course of cell wall synthesis seems to be the basis for the toxicity of the drug.

* This work was supported by a grant (E-1181) from the National Institutes of Health, U. S. Public Health Service, and by a contract [AT(30-1)2358] from the U. S. Atomic Energy Commission.

† Predoctoral Fellow of the National Institutes of Health (EF-9085). This communication is part of a thesis submitted by A. Tomasz to the Graduate Faculties of Columbia University in partial fulfillment of the requirements for the Ph.D. degree. Present address: The Rockefeller Institute, New York.

Materials and Methods

Escherichia coli K-12 (wild type)[1] was used in most of the work, but in some experiments nutritional mutants of this strain as well as *E. coli B* and *E. coli 15* were also used. *E. coli 15* and *E. coli* 15_{T-U-} were gifts of Dr. S. S. Cohen of the

[1] Three independent isolates of *E. coli K-12* were used in these experiments with identical results. The sources of the organism were the stocks of Dr. N. Zinder of the Rockefeller Institute, the stocks of Prof. F. Ryan of Columbia University, and The American Type Culture Collection (Clifton strain).

University of Pennsylvania. In some experiments the double auxotrophic mutant of *E. coli* W 175-25 (Lys^-, DAP^-), requiring lysine and $\alpha\epsilon$-diaminopimelic acid (DAP) for growth, was used. This organism was kindly supplied by Dr. W. Farkas of New York University. Before each series of experiments the mutant was reisolated from a single colony.

In most of the experiments the medium of Gray and Tatum (1940) was used with glucose, lactose, or glycerol as carbon source. The nutrient broth, nutrient agar, and Bacto-agar used were Difco products.

Bacterial cultures used in the experiments were prepared in the following way: 250-ml Erlenmeyer flasks containing 100 ml defined medium were inoculated with 1 ml of a culture of $1-8 \times 10^8$ cells per ml which had been grown from a loopful of bacteria in defined medium. The culture was incubated overnight on limiting carbon source (0.3 mg glucose per ml) so that growth was limited at a cell density of $1-3 \times 10^8$ cells per ml. The incubation was done under aerobic conditions at 37°. On the morning following inoculation the cultures were supplemented with optimal amount of carbon source (3 mg/ml glucose) and growth was followed in a nephelometer. In all experiments the cultures were used in the exponential phase of growth. Bacteria were allowed to go through at least 2 or 3 divisions in the exponential phase before being used. If necessary the cultures were diluted with fresh, sterile, warm medium to convenient cell density.

Transfer of the bacteria from one medium to another was carried out by centrifugation at $5000 \times g$ for 8–15 minutes in sterile tubes at room temperature; the bacterial residue was rinsed with the new medium and then resuspended in it.

Growth was determined in a photoelectric nephelometer calibrated with viable counts.

Viability was determined by spreading 0.1 ml of the appropriately diluted bacterial culture on the surface of agar plates. The concentration of the agar used was 1.5%.

Cytologic observations were made under the phase-constrast microscope (Zeiss Model W). In the photomicrography "Panatomic X" (Kodak) film was used.

For the determination of ultraviolet absorbancy and in the colorimetric procedures the Beckman DU spectrophotometer was used.

5-Fluorouracil, 5-fluorouridine, and 5-fluorodeoxyuridine were gifts of the Hoffman-LaRoche Company. 5-Hydroxyuridine was kindly given to us by Dr. G. B. Brown of the Sloan-Kettering Institute; tryptazan and chloramphenicol were the gifts of Dr. G. Brawerman of this Department, 6-azauracil was kindly supplied by Dr. P. Lengyel of New York University, *N*-acetylglucosamine was a gift of Dr. A. Linker of this Department, and 5-nitro-, 5-diazo, 5-bromo-, and 5-aminouracil were kindly supplied by Dr. S. Zamenhof of this Department.

5-Fluorouracil migrated as a single UV absorbing component in three chromatographic solvents:

	R_F
1 M ammonium acetate–95% ethanol (3:5.7 v/v) (Paladini and Leloire, 1952)	0.61
formic acid–ethylacetate–water (1:2:7 v/v) (upper phase)	0.47
isobutyric acid–0.5 M ammonia (5:3 v/v) (Magasanik *et al.*, 1950)	0.38

The UV spectra of 5-fluorouracil in 1 N HCl, water, and 1 N NaOH were identical to those described by Horowitz *et al.* (1958) for this compound.

Uniformly labeled glucose-C^{14} was purchased from California Foundation. All other chemicals were commercial products.

Determination of Radioactivity in C^{14}-Labeled Compounds.—One-tenth ml of the diluted samples was plated on stainless steel planchets, and to ensure even spreading 3 drops of a wetting agent ("Ultrawet") were added to each planchet. Radioactivities were determined at "infinite thinness" in a GM end-window apparatus.

The tritium-labeling of DAP was carried out by the New England Nuclear Corporation (exposure: 3 C of H^3-gas, 27°, 0.39 atm. for 2 weeks) by the Wilzbach procedure.

The tritium-labeled DAP (H^3-DAP) was purified by the following method: labile tritium was exchanged by dissolving the H^3-DAP in hot water and refluxing it for 1–2 hours; the water was then removed *in vacuo*. This procedure was repeated four or five times. The dry residue was transferred to large sheets of Whatman No. 3MM filter paper and was chromatographed in the methanol - pyridine - water - HCl (80:10:-17.5:2.5) (Rhuland *et al.*, 1955) solvent system. Spots corresponding to the location of DAP on the paper were cut out and eluted with water, and the specific activity of the DAP was determined. The whole procedure—including the refluxing—was repeated until a constant specific activity (38 μC per μmole) was reached. The DAP was stored in frozen water solution. *N*-acetyl hexosamines were determined by the procedure of Reissig *et al.* (1955); diaminopimelic acid was determined by the method of Gilvarg (1958).

Determination of Radioactivity in H^3-Labeled Compounds (DAP).—One-tenth-ml samples of the diluted extracts or other solutions were introduced into the counting vials (low K-content silica glass, Packard Corp.) and were dissolved in a mixture of 6 ml of toluene (analytical reagent) and 2.5 ml of absolute ethanol, the former solvent containing 0.3% of PPO (2,5-diphenyloxazole) and 0.01% of POPOP (1,4-bis-2,5-phenyloxazolylbenzene).

After vigorous shaking the liquid in the vials formed a single homogeneous phase. The vials were introduced into a Tricarb semiautomatic

liquid scintillation counter and were counted at 1100 v photomultiplier voltage; 10 to 50 to 100 v windows were used. The efficiency of counting under these conditions was 14%, as determined by a standard H^3 sample in toluene. The background counting rate was 80–100 cpm under the conditions used. Quenching was determined by the use of internal standards. The standard used for this purpose was H^3-labeled dihydroisoandrosterone kindly supplied by Dr. S. Lieberman of this Department.

Fractionation of the Bacterial Constituents.—The bacterial culture was poured onto 2–3 times its volume of crushed ice, stirred, and centrifuged in the cold. The fractionation procedure essentially followed that of Schneider (1945).

Protein and Cell Wall Fraction.—The bacterial residue after the extraction with hot trichloroacetic acid was washed with 50% ethanol, 95% ethanol-ether (1:1), and finally with ether. The dry powder was resuspended in 1 N NaOH, immersed in a boiling water bath for 30 minutes, and allowed to stand at room temperature overnight. The extracts were then made up to precise volumes.

Isolation of DAP for Specific Radioactivity Determination.—The ether-dried residue of the fractionation procedure was hydrolyzed in sealed tubes with 6 N HCl at 95° for 16–20 hours. After removal of the HCl *in vacuo* the residue was chromatographed on Whatman No. 3 MM filter paper in methanol-pyridine-HCl-water (80:10:-2.5:17.5) in a descending direction for 15–17 hours. Spots corresponding to the isomers of DAP and of other amino acids were cut out and eluted with water. Radioactivity was determined in a scintillation counter. The quantity of DAP was determined in triplicates by the method of Gilvarg (1958); racemic (D,L) DAP was used as standard. In experiments where H^3-DAP was used as tracer in *E. coli* W 175-25 (lys^-, DAP^-), no radioactivity could be detected in amino acid other than DAP.

Results

The Effect of 5-Fluorouracil on Growth and Viability; the Induction of an Osmotically Sensitive State in the Bacteria.—When exponentially growing *E. coli K-12* cells in liquid medium were exposed to various concentrations of 5-fluorouracil the growth rate of the culture, measured as turbidity, slowed down, but kept increasing even on prolonged incubations. On the other hand, the drug had a much more pronounced effect on the viability of the organisms: the cells rapidly lost the ability to form colonies on nutrient agar. However, if aliquots of the same culture were plated on nutrient agar into which high concentrations of salt or other solutes were incorporated, the number of viable cells in the culture remained constant for up to 60 minutes of incubation with the drug. Apparently, under these conditions 5-fluorouracil acted only as a bacteriostatic agent: it inhibited the multiplication of the cells but did not affect their viability.

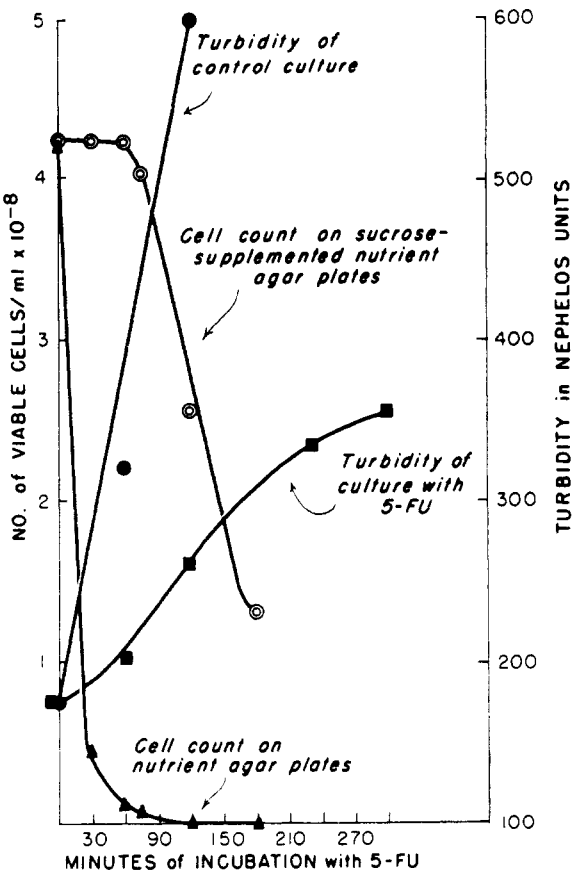

FIG. 1.—The effect of 5-fluorouracil (FU) on the viability and growth of *E. coli K-12*.

A typical experiment is summarized in Figure 1, which shows that, even after 60 minutes of exposure to 5-fluorouracil (100 μg/ml), 95% of the viable cells initially present in the culture gave rise to normal colonies if plated on nutrient agar which was supplemented with 20% sucrose. Incubation of the cells with 100 μg/ml 5-fluorouridine (FUR) or 5-fluorodeoxyuridine (FUDR) or with concentrations of 5-fluorouracil from 1 μg/ml up to 1 mg/ml gave qualitatively the same results.

The following facts suggested that the restoration of the viability of the cells was a function of some colligative property of the solutes added to the agar: K^+, Na^+, NH_4^+, chlorides, nitrates, and sulfates, as well as sucrose, glucose, galactose, mannose, maltose, mannitol, lactose, and glycerol, were found to be equally effective in enhancing the viability of the bacteria. Furthermore, isoosmotic concentrations of various electrolytes and nonelectrolyte solutes were approximately equally effective. If aliquots of 5-fluorouracil treated bacteria were plated on agar containing increasing concentrations of some solute and the

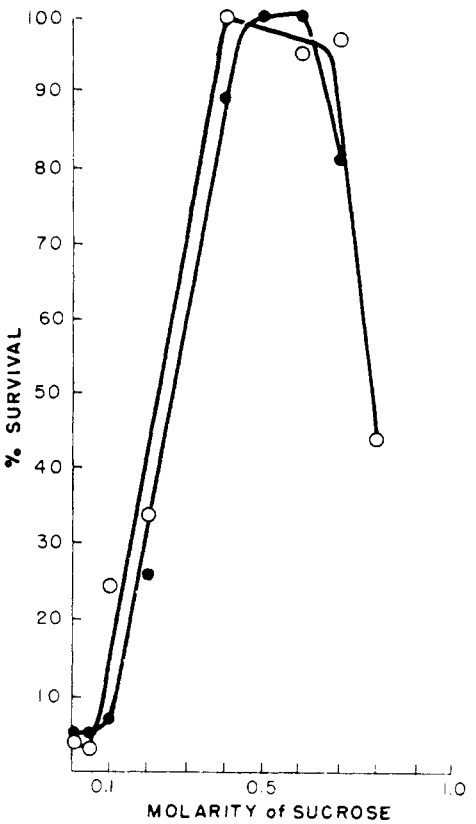

FIG. 2.—Survival of the 5-fluorouracil-treated bacteria as a function of the sucrose concentration of the nutrient agar. Open and full circles indicate two separate experiments.

viable counts were plotted against concentration of the solute, a series of curves were obtained the shape of which resembled the shape of protoplast "stability curves" (Mitchell and Moyle, 1956) (Fig. 2). These observations suggested that the 5-fluorouracil treatment induces an osmotically sensitive condition in the cells.

The Bactericidal Action of 5-Fluorouracil and the Osmotic Sensitivity.—Figure 1 shows that during the first 60 minutes of incubation in synthetic medium with 5-fluorouracil the loss of viability in over 90% of the cells was caused by the osmotic sensitivity, since the viability of the bacteria could be restored by osmotic supplementation of the agar. On more prolonged incubations with the drug, however, the survival of the cells started to decline even on the supplemented agar plates. Loss of viability under these longer exposures to 5-fluorouracil must have been caused by additional damage to the cells other than osmotic sensitivity.

Because of this fortuitous timing of the 5-fluorouracil-produced injuries our studies could be focused on the initial osmotically reversible phase of the drug action. The standard conditions used were 60 minutes of incubation at 37° in the presence of 5-fluorouracil (100 μg/ml) in synthetic salts medium under aerobic conditions and in the exponential phase of growth.

The Effect of Growth During Exposure to 5-Fluorouracil on the Development of Osmotic Sensitivity.—In these experiments nutritional mutants of the *E. coli K-12* strain as well as metabolic inhibitors were used. The auxotrophs were washed free of the growth medium, resuspended in a medium lacking some essential metabolite, and then incubated with 5-fluorouracil under the standard conditions.

In other experiments some antimetabolite was added to wild-type *E. coli K-12* cells at the beginning of the incubation with 5-fluorouracil. Appropriate controls were performed for the effect of the starvation and of the antimetabolites on the viability of the organisms. It is apparent from these experiments (Table I) that suppression of growth by any method tried during incubation with 5-fluorouracil inhibited the development of osmotic sensitivity.

The Effect of Growth After the Exposure to 5-Fluorouracil.—It is clear from Figure 1 that the viability of the 5-fluorouracil-treated cells was only lost *after* they had been transferred from the 5-fluorouracil-containing salts-medium to the nutrient agar (otherwise they could not have been rescued by the osmotic supplement). When 5-fluorouracil-treated bacteria were plated on a series of minimal agar plates "enriched" to various degrees with nutrients (casamino acids, uracil, nutrient broth), maximum loss of viability occurred on the "richest" nutrient agar plates. Since bacteria have relatively short generation times in these enriched media, it appeared likely that it was the rate of the general metabolism or growth of the cells during a period following the 5-fluorouracil treatment which determined the fate of the bacteria. Further evidence supporting such a conclusion came from experiments in which 5-fluorouracil-treated bacteria were plated on nutrient agar plates chilled to 0°; the plates were immediately returned to the low temperature and were kept there for several hours before transferral to the 37° incubator. It is apparent from the data in Table II that under these conditions the cells were able to recover their viability just as well as on sucrose-supplemented agar plates.

The Mechanism of Lethality of Osmotic Sensitivity.—The "death" of the 5-fluorouracil-treated cells during their growth in rich media may have occurred in either one of two ways: they may have disintegrated or they may have stopped dividing after a few divisions, forming invisible microcolonies.

In order to determine whether the loss of viability was due to actual disintegration of the cells the following experiment was performed. To *E. coli K-12* cells grown on uniformly labeled glucose-C^{14}, 5-fluorouracil was added in the exponential phase of growth; after incubation for 60 minutes the drug and the radioactive glucose were removed by centrifugation and the cells were resuspended in warm broth and incu-

TABLE I

THE EFFECT OF GROWTH INHIBITION DURING EXPOSURE OF THE CULTURE TO 5-FLUOROURACIL ON THE DEVELOPMENT OF OSMOTIC SENSITIVITY

Strain of Bacteria Used	Addition to Incubation Medium	% Survival[a]	
		On Nutrient Agar	Nutrient Agar + Sucrose
E. coli K-12	Glucose + 5-FU[b]	18	100
	No glucose + 5-FU	100	100
E. coli W 6 (Me)⁻	Glucose + L-methionine (25 μg/ml) + 5-FU	15	100
	Glucose, no L-methionine + 5-FU	100	100
E. coli C-600-25 (Threo, Leu)	Glucose + D,L-threonine (250 μg/ml) + D,L-leucine (120 μg/ml) + 5-FU	40	100
	Glucose, no threonine + D,L-leucine (120 μg/ml) + 5-FU	100	100
	Glucose + D,L-threonine (250 μg/ml) + no leucine + 5-FU	100	100
E. coli K-12	Glucose + chloramphenicol (100 μg/ml) + 5-FU	90	100
	Glucose + Na-azide (100 μg/ml) + 5-FU	100	100

[a] Determined after 60 minutes of incubation in the various media. [b] FU = fluorouracil; 100 μg/ml FU was used in all experiments.

bated at 37°. Viability of cells on sucrose-supplemented plates, as well as radioactivity and UV absorbance of the supernatant fluid (obtained by centrifugation of the culture), were determined at intervals. Figure 3 shows that at about the 20th minute of incubation the bacteria abruptly disintegrated, as indicated by a drop in viability and by the visible agglutination of the cells. The coincident appearance of radioactivity and of UV absorbing compounds in the supernatant fluid was confirmatory evidence of the disintegration of the cells.

It was possible to correlate these changes with cytologic events occurring at the same time. So that single fields could be observed for longer periods of time, droplets of a heavy suspension of 5-fluorouracil-treated bacteria in water were plated on microscope slides coated with a thin layer of nutrient agar. The bacteria were observed under the phase-contrast microscope. At about the time (20–25th minute) when the macroscopic changes in the broth culture occurred, a series of abnormal cytologic changes could be observed in rapid succession. The cells underwent a process resembling a plasmolysis. This was followed by the sudden disappearance of all optically dense material from the cells, leaving the empty ghosts of walls (Fig. 3). If the nutrient agar used for the coating of the slide was supplemented with 0.001–0.002 M Mg^{++}, a further "stage" in this process could be observed: the formation of spherical bodies, initially attached to the cell walls as a sort of "protrusion" of the cytoplasm. The spherical bodies soon became irregularly shaped; occasionally they grew to 10–20 times their original size before they disintegrated (Fig. 4).

TABLE II

PREVENTION OF THE DEVELOPMENT OF THE OSMOTICALLY SENSITIVE STATE BY COLD TREATMENT

Plate	Incubation After Plating	% Survival On Nutrient Agar	% Survival Nutrient Agar + Sucrose (20%)
1	16 hr. at 37°	10	100
2	60 min. at 0° followed by 16 hr. at 37°	50	—
3	120 min. at 0° followed by 16 hr. at 37°	55	—
4	8 hr. at 0° followed by 16 hr. at 37°	100	—

Experiments on the Mechanism of Osmotic Sensitivity.—The abnormal osmotic sensitivity of 5-fluorouracil-treated *E. coli K-12* cells and their disintegration via a "self-destructive" growth could be the consequence of a number of quite different mechanisms.

(*a*) Since 5-fluorouracil is a uracil as well as a thymine antagonist in many biological systems, the possibility that the effect of high osmotic environment might be indirect had to be considered. The high solute concentration in the medium might facilitate the uptake of the normal metabolites uracil or thymine from the medium, thereby helping to overcome an intracellular

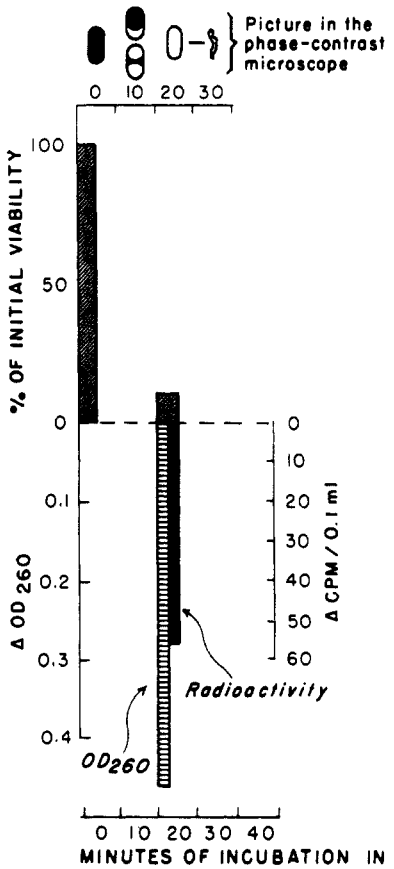

FIG. 3.—Disintegration of bacteria in broth after 5-fluorouracil treatment. For experimental details see the text. ΔOD_{260} indicates the increase in the UV absorbance of the supernatant of the culture after removal of the cells by centrifugation; Δcpm/0.1 ml indicates the increase of radioactivity in aliquots of the same supernatant.

pyrimidine deficiency induced by the 5-fluorouracil treatment. This possibility was eliminated, however, by an experiment in which bacteria were plated—in addition to plating on normal and salt-supplemented plates—on nutrient agar enriched with high concentrations of uracil and thymine with and without the addition of salt to the medium. It was found that the viability of the cells was a function *only* of the salt present and was unaffected by thymine or uracil.

(*b*) The osmotically fragile state of bacteria might be related to the "thymine-less death" (Cohen and Barner, 1955) which has been suggested to be the major mechanism of the cytolytic action of 5-fluorouracil in both bacteria and mammalian cells. For this reason we tested the strain 15_T^- of *E. coli*, in which the phenomenon of thymine-less death was originally described. Under conditions which produce thymine-less death, cells could not be saved by plating on the osmotically supplemented agar.

The conclusion that an interference with thymine metabolism is not involved in the phenomenon was also supported by the finding that supplementation of the growth medium with various concentrations of thymine or thymidine during the exposure of the cells to 5-fluorouracil did not have any effect on the development of osmotic fragility. On the other hand, addition of uracil or uridine to the medium in equimolar concentration with 5-fluorouracil completely protected the cells against the bactericidal action of the drug.

(*c*) The possibility that the phenomenon might be related to the lysogenic condition of the K-12 strain was ruled out since nonlysogenic members of the strain also showed the effect.

(*d*) An explanation for the high osmotic requirement could be the development of an abnormally high internal pressure in these bacteria for which the normal mechanical rigidity of the cell walls would be inadequate support.[2] This possibility was eliminated, however, by determining the approximate internal osmotic pressure of 5-fluorouracil-pretreated bacteria by the plasmolysis threshold method. There was no detectable difference between the internal osmotic pressure of normal and 5-fluorouracil-treated bacteria.

(*e*) A more plausible mechanism for the hyperosmotic requirement would be that the 5-fluorouracil treatment somehow destroys the normal rigidity of cell walls. The appearance of structures morphologically resembling "spheroplasts" during the process of disintegration of 5-fluorouracil-treated bacteria strongly suggests such a mechanism. Induction of a number of lethal biosyntheses, *e.g.*, autolytic systems, (for review see McQuillen, 1960) defective lysogeny (Jacob and Fuerst, 1958), or synthesis of bacteriocines (Ivanovics *et al.*, 1959) could result in such a defect in the walls of the bacteria. However, lytic "principles" once formed could be expected to act on normal bacteria too. If this assumption is valid then the cell-free supernatant fluid of disintegrated bacteria after 5-fluorouracil treatment should be able to induce the disintegration of normal bacteria—in the absence of 5-fluorouracil. Such an experiment was performed, but the results were negative. As substrates for the hypothetical lytic enzyme we used normal K-12 cells, *E. coli B* cells, *E. coli K-12* cells pretreated with versene in *p*H 8 Tris buffer for 5 minutes (*i.e.*, conditions under which these bacteria become sensitive to lysozyme). No lysis could be demonstrated in any of these bacterial preparations on incubation for various times with broth-lysates of *E. coli K-12* cells pretreated with 5-fluorouracil.

(*f*) Finally the possibility remained that 5-fluorouracil induced the osmotic sensitivity by a more direct metabolic interference with some step in the biosynthesis of the cell wall. In order to test this possibility the incorporation of H^3-

[2] Abnormally increased pool sizes might result in such a condition.

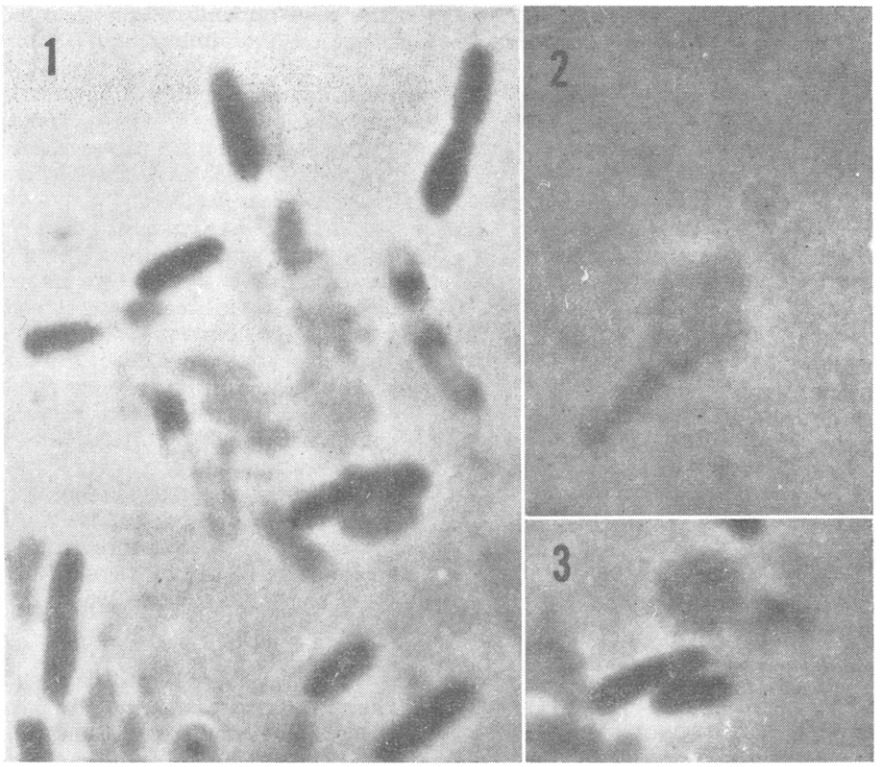

FIG. 4.—Formation of spheroplast-like bodies from *E. coli K-12* after treatment with 5-fluorouracil (phase contrast; magnification, ×2000).

labeled DAP into the cell walls of normal and 5-fluorouracil-treated bacteria was studied.

A culture of *E. coli* W 175-25 (DAP^-, Lys^-) in a salts medium supplemented with DAP (7.5 μg/ml) and lysine (30 μg/ml) with glucose (3 mg/ml) as major source of carbon was allowed to grow to a density of about 5×10^8 cells per ml. The cells were washed free of the medium and resuspended in fresh salts medium supplemented with lysine but no DAP. The cells were incubated for 15 minutes at 37° in the absence of DAP in order to deplete intracellular pools of this amino acid and then H^3-DAP (0.3 μC per ml, 1.9 μg per ml) was added to the medium. The culture was divided and to one half of it 5-fluorouracil (100 μg/ml) was added and both cultures were incubated with shaking at 37° for 60 minutes. At intervals samples were removed, quickly chilled, and centrifuged. The pellet was washed free of adsorbed extracellular radioactivity and fractionated as described in Materials and Methods.

By the end of 60 minutes of incubation 80% of the 5-fluorouracil-treated cells developed "osmotic sensitivity" as revealed by the differential counts of viable bacteria on sucrose-supplemented and normal agar plates. Figure 5 and Table III show the kinetics of DAP uptake and the distribution of DAP into various cellular fractions.

It can be seen that while the total uptake of H^3-DAP by 5-fluorouracil-treated cells was almost identical to that of the control (within about 10%), the 5-fluorouracil-treated cells differed greatly in the distribution of H^3-DAP between the acid-soluble (precursor) fraction and the cell wall. In normal cells, by the end of the incubation about 90% of the total DAP taken up was in the cell wall, about 10% in the acid-soluble fraction. At the same time, in the 5-fluorouracil-treated cells 65% of the total radioactivity remained in the acid-soluble fraction and only 35% was incorporated into the cell wall. A diminution of the incorporation of DAP into the cell wall of 5-fluorouracil-treated cells became evident about 15 minutes after the addition of the drug.

Table III also includes results obtained when 100 μg/ml uracil was added to the culture together with 5-fluorouracil (100 μg/ml) in an experiment otherwise identical to the one just described. It can be seen that in the presence of uracil the inhibition of incorporation of the tracer into the cell walls was reversed.

The substantial inhibition of DAP-incorporation into the cell walls by 5-fluorouracil was confirmed in an experiment[3] in which DAP was isolated from the acid hydrolysates of the cell wall fraction of normal and 5-fluorouracil-treated bacteria and the specific radioactivities were determined as described under Materials and Methods. The results are shown in Table IV. Since DAP is a unique component of the mucopeptide

[3] The experimental conditions were identical to the ones used in the previous experiment.

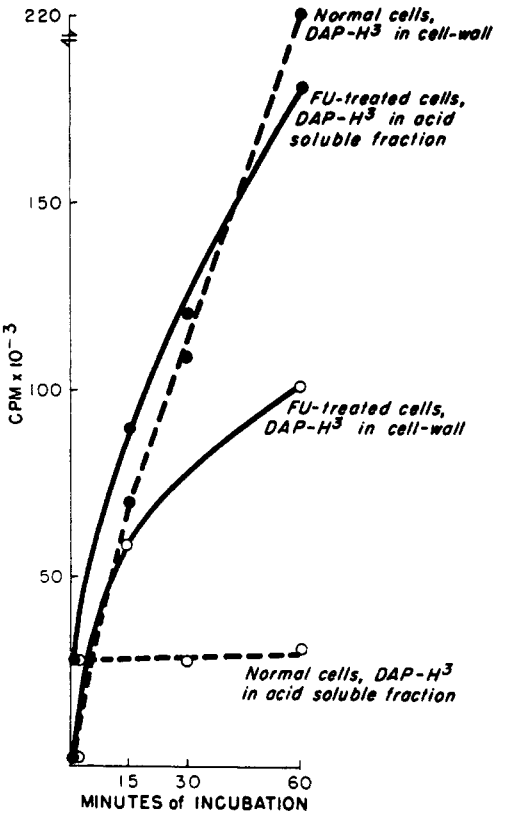

FIG. 5.—Incorporation of H^3-DAP into *E. coli K-12* in the presence (100 μg/ml) and in the absence of 5-fluorouracil. Experimental details are given in the text. Owing to technical difficulties no 0 minute sample was taken; the earliest sample represents 3rd minute of incubation.

polymer of the bacterial cell wall, and since 5-fluorouracil treated bacteria were found to accumulate *N*-acetyl hexosamine–containing cell wall precursors also (Tomasz and Borek, 1960), this inhibition of DAP incorporation was assumed to reflect an inhibition of the formation of the whole mucopeptide polymer.

The Effect of Pyrimidine Analogues on the Accumulation of N-Acetyl Hexosamine Esters.—A number of pyrimidine analogues other than 5-fluorouracil were tested for their possible effect on *N*-acetyl hexosamine accumulation under conditions identical to the ones used in the 5-fluorouracil experiments. Of all the compounds tested only 6-azauracil showed an effect (Table V).

Correlation Between the Osmotically Sensitive State and the Inhibition of Cell Wall Synthesis.—The correlation between the physiologic effect of 5-fluorouracil (*i.e.*, osmotic sensitivity) and the biochemical effects (*i.e.*, inhibition of DAP incorporation into the cell walls, accumulation of acid-soluble *N*-acetyl hexosamine esters [Tomasz and Borek, 1960]) and DAP was studied under a number of conditions.

(*a*) The kinetics of accumulation of *N*-acetyl hexosamine esters was compared with the kinetics of the development of osmotic sensitivity (plotted as decrease in viability) in a 5-fluorouracil-treated culture (Fig. 6). It is apparent that the decrease in viability of the culture is paralleled by a rapid accumulation of *N*-acetyl hexosamine esters. Comparison of the kinetics of the accumulation of acid-soluble DAP and the decrease in viability gave similar results.

(*b*) The lowest concentration of 5-fluorouracil (2–5 μg/ml) which still had observable effect on the viability of the organism also caused accumulation of *N*-acetyl hexosamine esters (see Table I in Tomasz and Borek, 1960).

(*c*) Close to equimolar quantities of uracil which can completely reverse the bactericidal action of 5-fluorouracil have also significantly reversed the inhibition of DAP incorporation into the cell wall (Table III).

(*d*) It was reported before (see Table I in Tomasz and Borek, 1960) that a number of conditions which have appreciable effects on the development of osmotic sensitivity have parallel effects on the accumulation of *N*-acetyl hexosamine esters

The conclusion drawn from these experiments was that the inhibition of cell wall synthesis by 5-fluorouracil shows a positive correlation with the physiological effect of the drug.

DISCUSSION

The biosynthesis of the cell wall mucopeptide appears to be the target of the action of a number of antibiotics of diverse chemical structure (for review see Salton, 1960). It was only recently, however, that pyrimidine analogues have been added to the list of these inhibitors. Otsuji and Takagaki (1959) have suggested that the bacteriostatic action of 6-azauracil could be explained by a selective inhibition of cell wall synthesis in *E. coli K-12*. These authors found that 6-azauracil induced the accumulation of *N*-acetyl hexosamine esters. Unfortunately, inhibition of cell wall synthesis was not measured directly in these experiments. No morphologic abnormalities in the cells were reported.

Rogers and Perkins (1960) found that 5-fluorouracil inhibited the synthesis of the cell wall mucopeptide in *Staphylococcus aureus*.

From our previous studies (Tomasz and Borek, 1959) on the effect of 5-fluorouracil in *E. coli K-12* we have concluded that the drug induces some defect in the cell walls of this organism. Results of experiments described in this paper suggest more specifically that this defect may reside in an inhibition of the formation of the normal mucopeptide components of the cell wall. The alternative possibility, namely, that cell walls with abnormal composition (*i.e.*, very low in DAP and *N*-acetyl hexosamine content) may be formed in 5-fluorouracil-inhibited cells, seems less likely but cannot be excluded at present.

A prominent feature of the bactericidal effect

TABLE III

DISTRIBUTION OF DAP TAKEN UP BETWEEN THE COLD TRICHLOROACETIC ACID (TCA)-SOLUBLE FRACTION AND THE PROTEIN + CELL WALL FRACTION OF NORMAL AND 5-FLUOROURACIL (FU)-TREATED BACTERIA

	DAP (cpm) in Cold TCA-Soluble Fraction (A)	DAP (cpm) in Cell Wall + Protein Fraction (B)	Total DAP (cpm) in Fractions (A + B)	Distribution of DAP Between A and B in % of (A + B)	
				A	B
Control cells	30,000	220,000	250,000	12%	88%
FU-treated cells	180,000	100,000	280,000	64.3%	35.7%
(Cells treated with FU in presence of uracil)[a]	(38,000)	(195,000)	(233,000)	(16.3%)	(83.7%)

[a] For experimental details see text.

of 5-fluorouracil on *E. coli K-12* is the requirement for growth of the organisms. Growth of the cells is required during the exposure of the culture to the drug as well as during a post-treatment period in order to cause loss of viability of the bacteria. Part of this growth—or metabolic activity—during the incubation with the drug is probably needed to achieve the uptake and intracellular transformation of 5-fluorouracil into the active "antimetabolite," but it seems that production of cytoplasmic mass is also a major requirement. In this respect 5-fluorouracil-inhibited *E. coli K-12* cells show characteristics of "unbalanced systems" where a more or less selectively inhibited cell wall synthesis is accompanied by relatively unaffected production of cytoplasmic material (for review see McQuillen, 1960).

The fact that penicillin, oxamycin, bacitracin, novobiocin, and a number of other antibiotics are lethal only to growing organisms has been interpreted this way (for review see McQuillen, 1958). In addition, several instances have been described where bacterial mutants requiring some of the unusual cell wall components for their growth undergo lysis on the exhaustion of these growth factors from the medium, provided that cytoplasmic growth of the cells continues (for review see McQuillen, 1960). While the above-mentioned antibiotics are known to induce abnormal morphology and lysis of bacteria under conditions resembling those described in this paper, the relation between the structure and activity of these compounds is far from clear. In the case of the 5-fluorouracil-inhibited *E. coli K-12* cells the

TABLE IV

THE INHIBITION OF DAP INCORPORATION INTO THE CELL WALLS BY 5-FLUOROURACIL (FU)

	DAP Isolated[a] (μg)	DAP (cpm/μg)	Inhibition of DAP Incorporation in % of Control
Control cells	28	690	—
FU-treated cells	55	250	64

[a] See Materials and Methods.

TABLE V

EFFECT OF 5-SUBSTITUTED URACIL ANALOGUES ON THE *N*-ACETYL HEXOSAMINE CONTENT OF BACTERIA

Supplement Added to Incubation Medium[a]	μM of N-Acetyl Hexosamine Esters Accumulated per 7×10^{12} Cells
5-Fluorouracil, 120 μg/ml	40
None	4
5-Bromouracil, 120 μg/ml	5.6
5-Diazouracil, 120 μg/ml	Max.[b] 9
5-Nitrouracil, 120 μg/ml	Max.[b] 8
5-Aminouracil, 120 μg/ml	Max.[b] 7
6-Azauracil, 100 μg/ml	15

[a] Minimal medium plus glucose was used as the incubation medium throughout. [b] Calculated from O.D. at 550 mμ in the Morgan-Elson reaction in borate. However, the absorption spectrum of the chromophore(s) was different from that described for *N*-acetyl hexosamines (Reissig *et al.*, 1955).

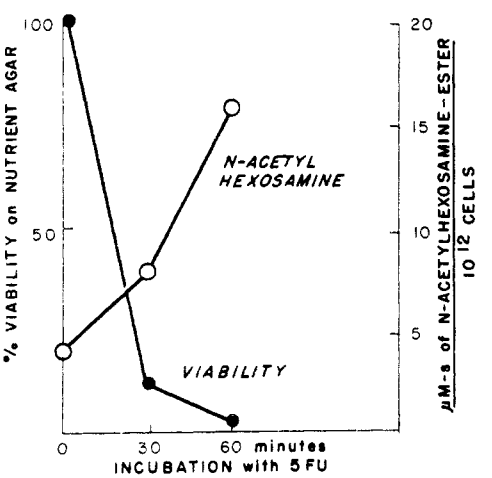

FIG. 6.—Accumulation of *N*-acetyl hexosamine esters and decrease of viability of the culture during incubation with 5-fluorouracil (100 μg/ml).

5-fluoro substituted pyrimidine structure and the reversibility of the effect by uracil suggests an interference on the level of the UDP-linked cell wall precursors (Strominger, 1960).

The effect of 5-fluorouracil described in this paper appears to be the first example of a pyrimidine analogue causing abnormal "spheroplast-like" forms and osmotic lysis in bacteria.

Acknowledgments

We wish to thank Dr. R. Duschinsky of Hoffman-LaRoche Company for the generous supply of 5-fluoro-pyrimidines. We are also grateful to Professor F. Ryan and Dr. J. Horowitz of this University and to Professor C. Gilvarg and Dr. P. Lengyel of New York University for helpful discussions of various aspects of this work.

References

Cohen, S. S., and Barner, H. D. (1955), *J. Bacteriol. 69*, 59.

Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R., and Lichtenstein, J. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 1004.

Duschinsky, R., Pleven, E., and Heidelberger, C. (1957), *J. Am. Chem. Soc. 79*, 4559.

Gilvarg, C. (1958), *J. Biol. Chem. 233*, 1502.

Gray, C. H., and Tatum, E. L. (1940), *Proc. Nat. Acad. Sci. U. S. 30*, 404.

Heidelberger, C., Chaudari, N. K., Dannenberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R. J., Pleven, E., and Scheiner, J. (1957), *Nature 179*, 663.

Horowitz, J., Saukonnen, J. J., and Chargaff, E. (1958), *Biochim. et Biophys. Acta 29*, 222.

Ivanovics, G., Alfoldi, L., and Nagy, E. (1959), *J. Gen. Microbiol. 21*, 51.

Jacob, F., and Fuerst, C. R. (1958), *J. Gen. Microbiol. 18*, 518.

Magasanik, B., Vischer, E., Doniger, R., Elson, D., and Chargaff, E. (1950), *J. Biol. Chem. 186*, 37.

McQuillen, K. (1958), *J. Gen. Microbiol. 18*, 498.

McQuillen, K. (1960), *in* The Bacteria, Vol. I, Gunsalus, I. C., and Stanier, R. Y., editors, New York, Academic Press, Inc., p. 249.

Mitchell, P., and Moyle, T. (1956), *in* Bacterial Anatomy, *Symp. Soc. Gen. Microbiol. VI*, 150.

Mukharjee, K. L., and Heidelberger, C. (1960), *J. Biol. Chem. 235*, 433.

Otsuji, N., and Takagaki, Y. (1959), *J. Biochem. Japan 46*, 791.

Paladini, A. C., and Leloir, L. F. (1952), *Biochem. J. 51*, 426.

Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955), *J. Biol. Chem. 217*, 959.

Rhuland, L. E., Work, E., Denman, R. F., and Hoare, D. S. (1955), *J. Am. Chem. Soc. 77*, 4844.

Rogers, H. J., and Perkins, H. R. (1960), *Biochem. J. 77*, 448.

Salton, M. R. J. (1960), Microbial Cell Walls, Ciba Lectures in Microbial Biochemistry, New York, John Wiley and Sons, Inc.

Schneider, W. C. (1945), *J. Biol. Chem. 161*, 293.

Strominger, J. L. (1960), *Physiol. Rev. 40*, 55.

Tomasz, A., and Borek, E. (1959), *Proc. Nat. Acad. Soc. U. S. 45*, 929.

Tomasz, A., and Borek, E. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 324.