*THE MECHANISM OF BACTERIAL FRAGILITY PRODUCED BY 5-FLUOROURACIL: THE ACCUMULATION OF CELL WALL PRECURSORS**

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In a previous communication we have reported that there is an early phase in the bacterial action of 5-fluorouracil (FU) on *E. coli* K_{12} which produces an "osmotic imbalance" in the cells.[1]

Further study of this phenomenon revealed that FU exerts its lethal action only on growing bacteria.[2] Inhibition of growth during the time of FU treatment by metabolic inhibitors or by omission of growth factors from the media of auxotrophs prevented the development of osmotic imbalance.

When exponentially growing bacteria were treated with FU and incubated on nutrient agar plates for various times, 90–95 per cent of the cells did not give rise to colonies. However, it was possible to recover these cells by a transfer from the nutrient agar plates to sucrose-supplemented agar provided the replica plating was done *before* a critical 25 minutes of incubation on the nutrient agar. After 25 minutes of incubation, 90 per cent of the cells could no longer be recovered by this method, possibly since they had already disintegrated on the nutrient plates.

When the FU-treated bacteria were plated on a thin layer of agar, observation under a phase contrast microscope revealed a series of anomalous cytological changes which culminated in the sudden disintegration of the cells.[3]

The disintegration of FU-treated bacteria could also be demonstrated in fluid medium: after 25–30 minutes of incubation in broth, about 90 per cent of the cells abruptly disintegrated and sedimented out of the medium. The timing of these events suggests that a great part of the population must have been synchronized, possibly by the FU treatment itself (thymine starvation). The disintegration of the cells at the end of a normal generation time suggests a possible connection between the division process and the events of disintegration.

The fate of the bacteria treated with FU depends fully upon the conditions of growth during a critical period *following* the exposure to the drug. Maximum rate of growth during this time inevitably leads to cellular disintegration unless the osmotic pressure of the medium is raised before the cells reach some irreversible stage in the process of disintegration. Suppression of the metabolic rate during this critical period also "rescues" the cells from FU-induced death. This can be accomplished by keeping the nutrient plates at low temperatures for prolonged periods or by plating on minimal medium. Slow rate of metabolism apparently enables the cells to "heal" the lesion introduced by FU and thus to restore full viability. The length of the critical period during which the cells need the protection of high osmotic environment was found to be 60 minutes. There was no cell division during this time.

The prevention of cellular disintegration by high osmotic milieu suggested the involvement of some cell wall abnormality. Our recent findings that FU-treated

cells also exhibit an increased sensitivity toward heat and mechanical stress could also be interpreted as consequences of diminished rigidity of the cell wall. (FU-treated cells also show high UV sensitivity.[4])

In order to test this hypothesis we have searched for the accumulation of cell wall precursors,[5] and found a significant increase of N-acetylhexosamine esters[6] in the FU-treated cells. Table 1 summarizes the results of experiments designed to establish a possible correlation between the accumulation of N-acetylhexosamine esters and the presence of osmotic imbalance in bacteria. The data clearly establish such a correlation.

TABLE 1

Incubation medium*	Supplement added	μM-s of N-acetylhexosamine esters accumulated per 10^{12} cells	Osmotic effect‡
Complete	None	3.8	1
Complete	FU(100 μg/ml)	33.6	21
Complete	FU (5 μg/ml)†	15.0	1.5–2
No glucose	FU(100 μg/ml)	1.0	1
No glucose	None	5.3	1
Complete	FU(100 μg/ml+ uracil (100 μg/ml)	2.0	1
Complete	FU(100 μg/ml+ thymine (100 μg/ml)	28.0	18
Complete	2-thioracil (100 μg/ml)	2.5	1
E. coli, *B*, complete	FU(100 μg/ml)	1	1

* Bacteria at half-maximum growth in exponential growth phase in synthetic medium (Gray and Tatum[7]) were incubated with FU for 60 minutes at 37°C.

† 5 μg/ml FU is just above the threshold concentration which induces "osmotic imbalance" under the standard conditions of incubation.

‡ Osmotic effect was quantitatively expressed as the ratio of numbers of colonies recovered on sucrose supplemented plates to the number of colonies recovered on nutrient agar plates: S/N; in normal bacteria S/N = 1.

The accumulation showed linear increase with incubation time: 4μM-s at 0 minutes, 8.5 μM-s at 30 minutes and 16 μM-s at 60 minutes of incubation (expressed per 10^{12} cell.). Preliminary experiments with an amino acid auxotroph of *E. coli* K_{12} (C-600-25, Leu$^-$, Threo$^-$) showed that resting populations of these bacteria (produced by amino acid starvation) are also able to accumulate these compounds in the presence of glucose and FU.

In attempts to isolate the accumulated compounds, large batches of FU-treated bacteria were extracted and fractionated on Dowex-1-Cl columns according to the method of Strominger.[6] 90 per cent of the N-acetylhexosamine esters adsorbed were recovered in two peaks, a major and a smaller one, both eluted with 0.05 *M* NaCl–0.01 *M* HCl (Fig. 1). The peaks were located on the basis of UV absorbance and the quantitative Elson-Morgan reaction. No N-acetylhexosamine containing component came off the column with any of the successive eluents.

The two peaks were not homogeneous. Paper chromatography resolved each peak to at least 5 components all having typical nucleotide spectra in the UV. Two of the components of the major peak on strong acid hydrolysis yielded diaminopimelic acid, glutamic acid, alanine, and UV-absorbing spots but no hexosamine. Another component of the same peak on strong acid hydrolysis yielded a UV-absorbing component which had UV spectra and chromatographic mobility identical with those of an authentic sample of FU. The fourth—and largest—component of the major peak contained N-acetylhexosamine, uracil but no amino acids.

It will be of interest to determine at what site FU interferes with the assembly of the cell wall mucocomplex. It appears likely that small quantities of FU become

metabolized to some cell wall precursor and this product may inhibit the whole enzyme complex of cell wall synthesis. This may lead to the accumulation of relatively large amounts of the normal precursor(s). The restoration of cells to viability would then involve the slow removal, by some means, of this interfering compound from the synthetic pathway.

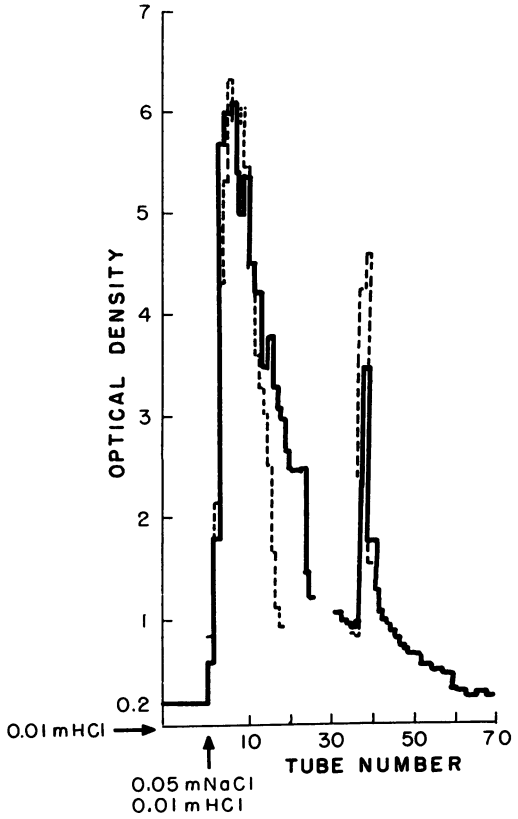

FIG. 1.—Isolation of the nucleotide linked N-acetylhexosamine esters on Dowex-1-Cl. 15 liters of bacteria at half maximum growth in synthetic medium were treated with FU(100 μg/ml) for one hour and then harvested, extracted, and fractionated on an ion-exchange column.[7] The figure only shows the part of elution diagram where the N-acetylhexosamine esters were eluted. Solid lines indicate UV absorbance at 260 mμ, dotted lines show the quantitative Elson-Morgan reaction.

These findings seem to confirm our earlier suggestion[1] that the mechanism of FU-induced "osmotic imbalance" involves a metabolic imbalance between cytoplasmic and cell wall syntheses. This condition is initiated by an early injury of the cell wall synthesizing mechanism by FU and is accompanied by relatively uninhibited cytoplasmic growth. As a result, after the removal of the FU from the medium the bacteria may resume a sort of unbalanced growth which soon leads to the disintegration of the cells.[8, 9]

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[1] Tomasz, A., and E. Borek, these PROCEEDINGS, **44**, 929 (1959).

[2] The action of FU resembles in this respect the action of penicillin and of a number of other antibiotics.

[3] Tomasz, A., and E. Borek, *Federation Proc.* (1960).

[4] (to be published).

[5] Recently, Otsuji and Takagaki have reported the accumulation of these compounds in 6-azauracil treated bacteria, *Journal of Biochem. (Japan)*, **46**, 791 (1959).

[6] Strominger, J. L., *J. Biol. Chem.*, **224**, 509 (1957).

[7] Gray, C. H., and E. L. Tatum, these PROCEEDINGS, **30**, 404 (1944).

[8] Resumption of the DNA synthesis may have a decisive role in making the unbalanced growth of the organisms irreversible.

[9] The 60 minutes' incubation time with FU—a standard condition used in our experiments—may represent the critical time up to which the above mechanism of action of the drug predominates. On prolonged incubations inhibitions of other phases of pyrimidine metabolism may become decisive.

ACTION SPECTRA OF CHROMATIC TRANSIENTS AND THE EMERSON EFFECT IN MARINE ALGAE

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Chromatic transients are the changes in oxygen evolution rate recorded on altering the color of light incident on a tissue, even though the intensities are adjusted to give equal steady rates of photosynthesis at both wavelengths. These were first observed[1] on exposing a red alga (*Porphyra*) alternately to red light (675 mμ) and green light (560 mμ). They have been reported[2] in a green alga (*Ulva*) on alternating illumination between (a) 490 and 540 mμ; and (b) 640 and 688 mμ. The location of these regions coincided fairly closely to the absorption *in vivo* of several pigments: chlorophyll *a* at 675–688 mμ; mixed chlorophylls at 540 mμ; chlorophyll *b* at 640–650 mμ; phycoerythrin at 560 mμ, and carotenoids at 490 mμ. (Alterations of wavelength within the absorption region of a single pigment produce little or no transient.)

The earlier studies were made by rapid change of setting on a monochromator, and the regions were limited by output energy and action spectra of the tissue to a few pairs of wavelengths. In order to obtain a more accurate delineation of the entire action spectrum for the transients, two sources of light have now been employed, one a fixed or reference wavelength, derived from a separate lamp with interference filter, the other the high-energy monochromator previously described.[3] The images of the two lamp filaments were focused carefully upon the tissue, usually an algal thallus one cell (or a few cells) thick, tightly held against a bright platinum electrode by means of a cellophane strip.[3] *Anabaena*, a blue green alga, was studied by lifting mats of filaments which had grown out very uniformly at the surface of sea water, and spreading them as smoothly as possible over the electrode.

Oxygen arriving at the electrode was reduced to H_2O_2 at an applied potential of 0.5 volt, the current being recorded by a Speedomax potentiometer connected across a fixed resistance (usually 1,000 ohms) in the circuit. Flowing or recirculated sea water, usually equilibrated against 5 per cent CO_2 in air, quickly established a base line which remained very steady in the dark, frequently at around