In the experiments with pacinian corpuscles, the toxin was applied to single intact or decapsulated corpuscles (*4*) isolated from the cat's mesentery. The preparation was set up in a drop of Krebs's solution surrounded by mineral oil. The solution-oil interface, which served as peripheral recording electrode (another electrode was placed on the central stump of axon), was adjusted to lie at the point of axon emergence from the intact corpuscle; in the decapsulated preparation, the solution-oil interface was lying at the level of the first Ranvier node. For tetrodotoxin application, the Kreb's fluid was exchanged for one containing the compound. Generator and action potentials were elicited by mechanical pulses from a piezoelectric crystal (*4*).

Tetrodotoxin in concentration of 1 g in $10^5$ ml blocked the action potential in both the intact and decapsulated corpuscles, leaving no detectable all-or-none residue (Fig. 1*B*). The generator potential was not appreciably affected. The generator potential–stimulus strength relation and the rates of rise and fall of the potential were the same as in the untreated receptor. It is noteworthy that the generator potential is $Na^+$ dependent in both the pacinian (*11*) and stretch receptor neuron (*10*).

The above-described findings indicate that spike and generator potential in the two receptors examined are independent events being subserved by different mechanisms. If so, these are likely to reside in separate regions of membrane, although the spatial arrangement may vary in different receptors (coarsely discernible regions of the cell, separate membrane patches within a region, and submicroscopic mosaic).

Tetrodotoxin has also been shown to block the action potential in muscle fibers (*8*) and electroplates (*12*), without affecting the endplate potential or the depolarizing action of externally applied acetylcholine. These findings and the present results are taken here as lending support to Grundfest's generalization that synaptic and generator processes are analogous transducer actions (*1; 13*).

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13. The experiments reported here were designed following a suggestion by Dr. H. Grundfest to one of us (C.A.T.) to apply tetrodotoxin to the crustacean stretch receptor. Tetrodotoxin was purchased from the Sankyo Co., Tokyo, Japan.

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## Mitomycin C: Chemical and Biological Studies on Alkylation

Abstract. *The presence of an aziridine ring in mitomycin C suggests that the mechanism of action of the antibiotic is like that of the antitumor alkylating agents. However the compound is unexpectedly stable during aerobic incubation with rat liver homogenates although rapidly metabolized anaerobically. Mitomycin is not reactive with γ-(4-nitrobenzyl)pyridine and reacts only slowly at acid pH with thiosulfate. It is proposed that mitomycin is activated in vivo, possibly by a reduction which "unmasks" the potential activity of the fused aziridine ring.*

Mitomycin C is an antibiotic and also a potent inhibitor of transplantable rodent tumors (*1*). The structure of the agent (*2*) suggests possible relations between its chemical configuration and biological activity (Fig. 1). The aziridine ring is an alkylating group, similar to those present in antitumor mustards (*3*), and the quinone and carbamate moieties are like those found in some mitotic inhibitors (*4*). The carbamate and the aziridine groups, and the possibility that an *o*-aminophenol derivative may be formed by reduction of the aminoquinone, are indications that mitomycin may be a carcinogen (*5*). Biotransformation of mitomycin might occur by reduction, by O-demethylation, by deamination, or by any change which increases the reactivity of the aziridine ring.

On the basis of the known properties of the antitumor alkylating agents, which include numerous ethylenimines (*3*), it is reasonable to propose that the aziridine group is primarily responsible for the biological effects of mitomycin. This is consistent with the antitumor actions, with the toxicity which is characterized by lesions in hematopoietic tissues and intestinal epithelium and by delayed deaths (*6*), and with the observation that mouse ascites sarcoma made resistant to alkylating agents is also resistant to mitomycin (*7*). It is also consistent with mutagenesis (*8*) and phage-activation (*9*) in *Escherichia coli*, and with a primary inhibition of DNA synthesis in *E. coli* (*10*), in mammalian cells in culture (*11*), and in proliferative tissues of rats (*12*).

However, the high potency and specificity of action of mitomycin in vivo are unexpected, for such properties are not usually found in monofunctional alkylating agents (*3*). [Mitomycin C is more potent in laboratory animals than most polyfunctional alkylating agents, with the possible exception of triethylene melamine (*13*).] Moreover, mitomycin C does not alter virus infectivity (*11*) or transforming activity (*14*), viscosity and melting-out characteristics (*14, 15*), or x-ray diffraction patterns (*16*) of isolated DNA, even though these may be directly affected by antitumor alkylating agents (*3*).

Another curious property of mitomycin is its relative stability in tissue breis under aerobic conditions. Our previous studies (*17*) showed that the antibiotic is rapidly metabolized anaerobically by rat liver homogenates. The anaerobic metabolism, measured by changes in ultraviolet absorption at 363 mμ, was proportional to loss of biological potency which was assayed with *Bacillus subtilis* spores. These earlier studies have now been extended to a comparison of the changes in biological activity with spectral changes under aerobic conditions (Table 1). The results agreed within the reliability of the bioassay (20 percent), although recoveries were slightly higher by the spectro-

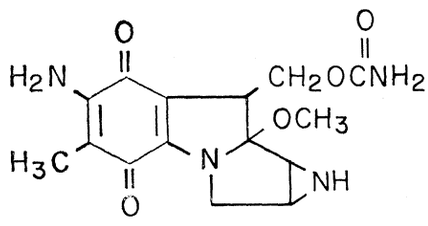

Fig. 1. The structure of mitomycin C.

photometric method. (Product absorption at 363 m$\mu$ probably accounts for this.) It is apparent that the compound was more stable under aerobic conditions. Such results were unexpected because typical antitumor ethylenimines are believed to react directly and nonenzymically with cellular constituents (*3*).

We encountered additional evidence for anomalous stability when attempting to react mitomycin with $\gamma$-(4-nitrobenzyl) pyridine (NBP) (*18*). Times of heating were varied between 5 and 60 minutes, and tests were conducted with freshly prepared and with incubated mitomycin (that is, samples taken from flasks immediately after the incubation periods in experiment 4, Table 1). Colored products, indicative of the alkylation of NBP, were not obtained.

Another test for alkylation, reaction with thiosulfate at acid *p*H, was studied in two series (Table 2). In series *A*, the method of Allen and Seaman (*19*) was followed (except that a *p*H meter, rather than indicators, was used because of blue color of mitomycin). A small net $H^+$-uptake was observed, but it was considerably less than a mole-equivalent. In series *B*, a predetermined *p*H ($\pm$0.25 units) was maintained by additions of acid until the *p*H was stable for 10 minutes or longer. At *p*H 6.0, there was no evidence of acid-uptake. At *p*H 4.0, $H^+$-uptake continued slowly for more than 3 hours, but was still incomplete. The reaction proceeded slightly faster at *p*H 3.5, but mitomycin and thiosulfate decompose independently at this hydrogen ion concentration. The sluggishness of mitomycin in its alkylation reactivity was in contrast with the vigor of typical antitumor ethylenimines (*19*).

The contrast between the high potency of mitomycin C in vivo and the relative lack of activity of the aziridine ring under the aforementioned conditions suggests that mitomycin C may require a metabolically induced structural alteration to become an effective alkylating agent in biological systems. Examples of metabolic enhancement of alkylating activity have been cited by Ross (*3*), while the studies of Webb *et al.* (*2*) demonstrate the susceptibility of mitomycin to rearrangement, cleavage, and opening of the aziridine ring under relatively mild conditions. This provides a plausible explanation for the lack of direct effect of mitomycin on isolated DNA and viruses even though these are highly susceptible in vivo.

We reported previously that mitomycin was reduced by dithionite, ascorbate, and hydrogen with pallidium-charcoal, and suggested that anaerobic conditions were necessary for reductive metabolism in liver homogenates (*17*). We now propose that enzymic reduction of mitomycin, presumably to the hydroquinone form, facilitates protonation of the aziridine nitrogen, thereby increasing its reactivity and promoting intracellular alkylation. Attack by the activated agent on a vital cellular moiety, presumably DNA, would result in cytotoxicity; conversely, reaction with noncritical moieties or with water would lead to detoxification.

The potent effects of mitomycin against experimental tumors seem understandable in terms of the proposed anaerobic, reductive activation. Compared with other antitumor ethylenimines which react nonspecifically in blood, mitomycin may be stable and reactive only after it has entered the cells; this could account, at least in part, for its relatively high potency. Its ability to inhibit the growth of a variety of transplanted rodent tumors may be due to the relative anoxia under which such cells grow after being implanted (*20*).

After this paper was accepted for publication, Iyer and Szybalski (*21*) presented evidence that complementary strands of DNA are in covalent linkage

Table 1. Inactivation and colorimetric changes of mitomycin (Mc) after incubation with rat liver homogenates. Recoveries were calculated as the percentage of unincubated standards. Standard and experimental mixtures were in 5.0-ml volumes; incubation was at 37°C. Proteins were precipitated by immersion in a boiling water bath for 5 minutes. Supernatants were diluted 1 : 5 with water. For descriptions of colorimetric assay at 363 m$\mu$, bioassay with *B. subtilis* spores on minimal media, preparation of fresh, aged, and dialyzed homogenates, and other details, see reference (*17*).

| Conditions | Recovery of mitomycin (%) | | | |
|---|---|---|---|---|
| | Colorimetric | Bioassay | Colorimetric | Bioassay |
| *Fresh tissue (100 mg) + Mc (125 $\mu$g)* | | | | |
| | Experiment 1 | | Experiment 2 | |
| 0 min | 98 | 88 | 94 | 87 |
| 10 min (air) | 92 | 87 | 87 | 78 |
| 30 min (air) | 84 | 73 | 82 | 68 |
| 30 min ($N_2$) | 46 | 40 | 33 | 24 |
| *Tissue (50 mg) + NADPH (0.4mM)* + Mc (125 $\mu$g)* | | | | |
| | Experiment 3 | | Experiment 4 | |
| Aged tissue, 0 min | 95 | 91 | 103 | 80 |
| Dialyzed tissue, 0 min | 95 | 90 | 105 | 87 |
| Aged tissue, 30 min (air) | 90 | 81 | 97 | 73 |
| Dialyzed tissue, 30 min (air) | 95 | 91 | 92 | 89 |
| Aged tissue, 30 min ($N_2$) | 7 | 13 | 18 | 11 |
| Dialyzed tissue, 30 min ($N_2$) | 29 | 25 | 59 | 54 |

*Nicotinamide adenine dinucleotide phosphate, reduced form.

Table 2. Acid uptake by mitomycin in the presence of thiosulfate. (Series *A*) Titrations were carried out with 50 ml 10 percent thiosulfate at 22° to 23°C; HCl (0.1*M*) was added until the *p*H (Beckman model 76) reached 4.0. Solutions were back-titrated, after 30 minutes, with standardized NaOH (0.082*N*) to *p*H 8.5. (Series *B*) Titrations were carried out with 25 ml 0.2 percent thiosulfate. The indicated *p*H was maintained by the addition of 0.10*M* HCL for the times shown and back-titrated as above. Initial and final amounts of mitomycin were calculated from absorbance at 363 m$\mu$ at *p*H 7.0 (molar extinction coefficient 2.22 $\times$ $10^4$).

| *p*H | Time (min) | Mitomycin | | Net $H^+$-uptake ($\mu$moles) | Net uptake (mole-equivalents) |
|---|---|---|---|---|---|
| | | Initial ($\mu$moles) | Final ($\mu$moles) | | |
| | | *Series A* | | | |
| 4.0 | 30 | 298 | | 60 | 0.20 |
| 4.0 | 30 | 301 | | 83 | 0.28 |
| | | *Series B* | | | |
| 6.0 | 55 | 83 | 0 | 0 | 0 |
| 4.0 | 254 | 119 | 61 | 56 | 0.47 |
| 3.5 | 191 | 59 | 42 | 52 | 0.88 |
| 3.5 | 180 | 83 | 54 | 54 | 0.65 |
| 3.5* | 180 | 83 | 14 | 0 | 0 |

* Reaction tested in the absence of thiosulfate.

after isolation from bacterial cells which have been treated with mitomycin C. The authors had no evidence of a direct effect by the agent on purified DNA. Although their results support the argument that mitomycin C requires a metabolic trigger, evidence is still lacking that mitomycin C itself forms the bridge between strands, or even reacts to form a covalent bond with any cellular component.

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## Continuous Recording of Cell Number in Logarithmic and Synchronized Cultures

Abstract. *An instrument for the continuous recording of cell number has been developed and is being used to record changes in populations of logarithmic and synchronized cultures of protozoan flagellates. A Coulter cell counter is used in conjunction with a counting chamber that is fitted with a flexible polyethylene aperture (75 μ in diameter). This aperture rarely becomes blocked and appears to be self-clearing. The unit consists of a proportioning pump, a cell counter, a counting chamber, a rate meter, and a recorder.*

There are two common methods of detecting unequivocally cell division in a population of cells. One is by direct microscopic observation of cell division and the other is by counting the cells as a function of time. All other methods, such as those that use optical density, turbidity, mass, or DNA content, are less satisfactory since they rely on variables that may change in the absence of cell multiplication. Consequently, to detect and measure synchronous cell division, one must rely on counting the cells. Fortunately, electronic methods (*1, 2*) have been developed which are reliable, but to measure growth rates or division synchrony, one must use a batch method of counting. This is because the aperture or window in the Coulter counter through which the cells must pass is small, between 30 and 200 μ in diameter, depending on the cell being counted, and it is subject to plugging. The window surface can be cleaned between batch counts when plugging occurs. The plugging usually results from debris that is present in even the most carefully prepared culture medium. Thus, counting nonsterile aliquots is readily accomplished, but counting a sterile system directly becomes a difficult operation.

We have now developed an instrument for counting continuously the cells in a sterile, growing culture of *Astasia longa*. By means of a proportioning pump (*3*), a small part of the stirred parent culture is continuously removed, diluted, mixed, and pumped into a counting chamber (Fig. 1) whose leads are attached to a Coulter counter, model B. The electrical output of this instrument is fed into a rate meter and then to a recorder. Since the rate meter (*4*) requires a negative signal pulse, an auxiliary circuit is used to reverse the polarity of the signal. The recorder (*5*) is set at 0.66 in./hr (1.67 cm/hr). With this recorder, other signals, such as culture temperature, can be monitored along with cell number.

Figure 1 shows a schematic diagram of the apparatus and includes details of the counting chamber. The window or aperture of this unit is a perforation in a flexible polyethylene or Teflon membrane. It is mounted in an "O" ring ball joint (*6*) and held in position with a Teflon or Neoprene "O" ring. The aperture is made in the membrane with a tapered glass needle drawn to cover the range of diameters needed to count specific cells. The membrane is perforated to the appropriate diameter under a dissecting microscope. When continuous counting was attempted with a solid aperture, such as the one supplied with the counter, plugging occurred frequently and the system could not be used. The best apertures could be made in polyethylene membranes, 25 to 35 μ thick. We have used several of these for 2 weeks of continuous counting without their showing signs of deterioration. Teflon membranes of the same thickness appear to change their electric characteristics with time.

Our design is based on several factors that we consider important for the continued use of such a unit. With an aperture of about 75 μ, the flow rate must not exceed 3 ml/min or high pressure will break the membrane. To reduce coincidence error to a minimum, the output from the parent culture is diluted with 0.3 percent sodium chloride as the electrolyte. To obtain a dilution ratio of one part of the culture to 8.3 parts of the diluent, and to maintain the total flow rate at 2.8 ml/min, tubes of 0.030 inch (0.08 cm) and 0.081 inch (0.21 cm) diameter are used with the Autotechnicon proportioning pump. Since the linear flow rate must be such that settling of the cells in the tubing is reduced to a minimum, the culture input line is injected into the vertically directed diluent stream in the counting chamber. The diluent flow rate is eight times greater than the culture input flow rate, consequently the cells are washed upward, settling does not occur, and mixing is optimal in this region of the