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Effect of Mitomycin C on Nucleic Acid Biosynthesis in Ehrlich Ascites Tumor Cells

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SUMMARY

The effect of mitomycin C on nucleic acid biosynthesis in Ehrlich ascites tumor cells was investigated *in vivo* as well as *in vitro* with relation to the mitotic cycle.

When the cells were exposed *in vivo* to 50 μg of mitomycin C per mouse for 3 or 6 hours, the incorporation of H^3-thymidine into DNA was markedly inhibited, while the H^3-cytidine incorporation into RNA was less suppressed and the S^{35}-methionine incorporation into protein was scarcely affected.

Such an inhibition of DNA synthesis does not seem to be induced by an interruption of the mitotic cycle but the inhibition rather precedes the disturbance of mitosis.

The mode of inhibition of DNA synthesis by mitomycin C could not be clarified by experiments *in vitro* using a cell-free DNA synthesizing system.

INTRODUCTION

Mitomycin C (MC), an antibiotic, has a marked inhibitory effect on various tumors of experimental animals (Shiba *et al.*, 1957; Usubuchi *et al.*, 1958; Teranaka, 1958; Sugiura, 1959). This antibiotic also possesses antibacterial activity, and a characteristic evidence was shown by Shiba *et al.* (1958) that DNA formation in *Escherichia coli* strain B is selectively inhibited by MC. It is uncertain whether the antitumor action of MC is analogous to the antibacterial action, and it is also of interest to study the action of MC as a radiomimetic substance in comparison with the effect of radiation.

For such experiments ascites tumor cells seem to be good material. Ehrlich ascites tumor of mice is one of the susceptible tumors to MC; by treatment with MC, animals bearing this tumor survive longer than untreated animals, ascitic fluid is reduced and the tumor cells, especially their nuclei, appear to be damaged.

The present studies are on the effects of MC *in vivo* and *in vitro* on nucleic acid metabolism in Ehrlich ascites tumor cells with relation to the mitotic cycle.

The following abbreviations are used: MC for mitomycin C, dAMP, dGMP, and dCMP for the 5'-monophosphates of deoxyadenosine, deoxyguanosine, and deoxycytidine respectively, and Tris for tris (hydroxymethyl) aminomethane.

MATERIALS AND METHODS

1. *Cells and animals*

Ehrlich ascites tumor (tetraploid), kindly given by Dr. Kawamata of this Institute, was maintained in mice by serial weekly intraperitoneal transplantation, and in all the experiments cells were used 4 - 5 days after implantation of 10^7 cells into the peritoneal cavity of mice.

Male albino mice (ddO), 6 - 7 weeks of age, supplied from the Central Breeding Station of Experimental Animals of Osaka University were used in this study. The mice were fed with mouse chow MF (Oriental Yeast Co., Ltd.) and given water *ad libitum*.

2. *Reagents*

Tritium labeled thymidine (2.5 c/m mole) and tritium labeled cytidine (0.16 c/m mole) were purchased from the Radiochemical Centre (England), and were diluted with sterile physiological saline to the desired concentration for *in vivo* experiments. For *in vitro* experiments H^3-thymidine was diluted with non-radioactive thymidine solution to give a specific activity of 250 mc/m mole.

Salmon sperm DNA, d MP,dGMP, and dCMP were obtained from the California Corporation (U. S. A.), and disodium ATP and DPN from the Sigma Chemical Company (U. S. A.).

Mitomycin C was given by Kyowa Hakko Kogyo Co., Ltd. (Japan).

3. *Assay of radioactivity incorporated into nucleic acid in cells*

After the incorporation period, the ascitic fluid was withdrawn from the peritoneal cavity of the mice. The tumor cells were separated from the ascitic plasma, washed repeatedly with saline by low speed centrifugation to eliminate erythrocytes, and then suspended in 1 ml of distilled water. They were disrupted by the freezing-thawing technique using a solid CO_2-acetone mixture. The resulting suspension was acidified by addition of 1 ml of 6 % perchloric acid in the cold. After centrifugation, the precipitate was washed twice with 2 % cold perchloric acid, and then with ethanol and ethanol-ether. From the cold acid insoluble precipitate hot acid soluble materials were obtained by two extractions, each with 1 ml of 3 % perchloric acid at 90°C for 15 minutes.

With o e portion of this extract the amount of DNA or RNA was determined colorimetrically by the method of Burto · (1956) and of Mejbaum (1939) respectively.

Another portion of this extract was neutralized with 6 N KOH, and put in a freezer to precipitate $KClO_4$, which was then removed by centrifugation. After suitable dilution with distilled water, 0.2 ml aliquots of the supernatant solution were pipetted onto planchets and their radioactivity was measured in a windowless gas-flow counter (Nuclear Chicago, U.S.A.). Corrections for self-absorption were not made. All the samples were triplicated.

4. *Autoradiography*

Smears of the ascites tumor cells were stained by the Feulgen reaction and were autoradiographed using a stripping film (London Kodak AR-10).*

As an index of DNA synthesis in individual cells, the numbers of silver grains in the nuclei were calculated from counts on 50 cells.

5. *Mitotic index*

Ascitic fluid was withdrawn from the peritoneal cavity of mice at definite intervals after MC-injection, and the numbers of cells in mitotic phases were calculated from counts on 2000 cells on the smear preparations stained with hematoxylin solution.

6. *Standard assay of enzymic activities*

The assay of enzymic DNA synthesis by cell-free extracts of Ehrlich ascites tumor cells was carried out according to the method of Smellie *et al.* (1959) by measuring the incorporation of H^3-thymidine into an acid insoluble product.

Cell extracts: Ascites tumor cells were collected from several mice, washed repeatedly with buffered (Tris, pH 7.9) 0.25 M sucrose solution to separate the cells from the ascitic plasma and ery-

* The autoradiography was carried out by courtesy of Dr. Kato of this Institute.

throcytes, and then resuspended in 8 - 9 volumes of distilled water. The resulting cells, which had osmotically swollen, were disrupted in a Potter glass homogenizer, and the homogenate was then centrifuged in a Spinco centrifuge at 105,000 $\times g$ for 60 minutes. The protein content of the supernatant was determined by the method of Gornall *et al.* (1949).

Reaction: The reaction mixture was 0.05 M with respect to Tris buffer, pH 7.9, and contained 2 μc/8 mμ mole/ml of H^3-thymidine, 5 mμ mole/ml of dAMP, dGMP, and dCMP respectively, 5 μ mole/ml of ATP, 0.25 μ mole/ml of DPN, 5 μ mole/ml of $MgCl_2$, 50 μg/ml of DNA, and 3 - 4 mg protein/ml of cell extracts. The volume of the mixture in each tube was 2 ml. Only DNA was added to the reaction mixture after 10 minutes' preincubation. The incubations were carried out at 37°C with shaking for 40 or 60 minutes.

Analytical procedure: To terminate the reaction, the mixture was quickly chilled and acidified by addition of 2 ml of 6 % perchloric acid. Then DNA was extracted and determinations of the amount of DNA and radioactivity incorporated were made as described above. In later experiments radioactivity was measured in a liquid scintillation spectrometer (Packard, Tri-carb, U.S.A.). In these cases aliquots of the extract without removal of perchloric acid or dilution were mixed with ethanol and toluene containing scintillators (2,5-diphenyloxazole and 1,4-di-[2-(5-phenyloxazole)]-benzene). Corrections were made for quenching using a H^3-thymidine solution of known radioactivity as a standard.

7. *Isolation of DNA from the tumor cells*

The DNA of the tumor cells was isolated using sodium dodecyl sulfate according to the method of Marmur (1961).

Thermal denaturation was carried out by heating the DNA solution at 100°C for 10 minutes and then cooling it rapidly in an ice bath.

RESULTS

1. *Effect of MC on the incorporation of H^3-thymidine into DNA in tumor cells*

Mice bearing Ehrlich ascites tumor cells were intraperitoneally injected with 50 μg of MC per mouse (in 0.5 ml of physiological saline). Then at various times after the injection they were given 10 μc of H^3-thymidine per mouse intraperitoneally. After an incorporation period of 20 minutes the mice were sacrificed by cervical dislocation and cooled by rapid intraperitoneal infusion of cold saline. Then the ascitic fluid was withdrawn and the incorporation of H^3-thymidine into DNA in the tumor cells was determined.

Results are presented in Fig. 1. In the cells after exposure to MC for 3 hours the incorporation of H^3-thymidine into DNA was 38% of that of the controls, and after 6 hours it was more suppressed to 13% of the controls, but the cells exposed to MC for 1 hour showed an almost unimpaired ability to incorporate the labeled precursor into DNA.

The effects of MC on RNA and protein synthesis, which were deduced from the incorporation of H^3-cytidine into RNA and S^{35}-methionine into protein respectively, were compared with the effect of MC on DNA synthesis. Though H^3-cytidine was partially incorporated into DNA under the present conditions, an experiment analyzed by the method of Schmidt and Thanhauser (1945) showed that the H^3-cytidine incorporation into the alkali stable fraction was less than 8% of the total radioactivity incorporated.

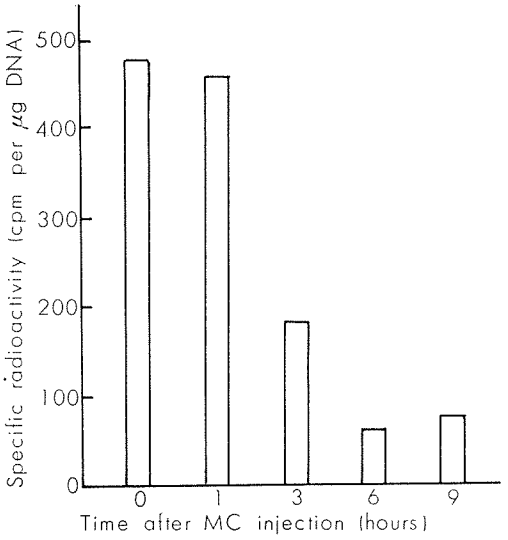

Fig. 1. Effect of MC on H^3-thymidine incorporation into DNA in Ehrlich ascites tumor cells *in vivo*.

The tumor cells were treated intraperitoneally with 50 μg of MC per mouse for the indicated periods, and then were labeled by intraperitoneal injection of 10 μc of H^3-thymidine per mouse for 20 minutes.

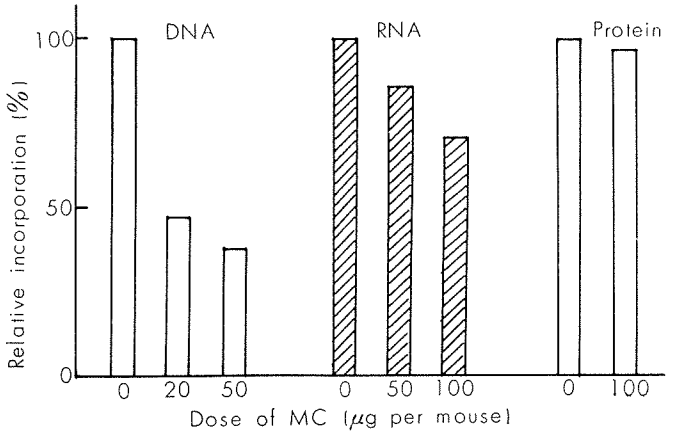

Fig. 2. Effects of MC on the incorporation of H^3-thymidine, H^3-cytidine, and S^{35}-methionine into DNA, RNA, and protein respectively in Ehrlich ascites tumor cells *in vivo*.

The tumor cells were treated intraperitoneally with the indicated doses of MC for 3 hours, and then were labeled by intraperitoneal injection of 10 μc of H^3-thymidine, 10 μc of H^3-cytidine, or 2.5 μc of S^{35}-methionine per mouse respectively for 20 minutes. The height of the bars indicates the specific radioactivity relative to that of untreated cells.

As shown in Fig. 2, when the cells were subjected to MC-treatment for 3 hours the incorporation of H^3-thymidine into DNA was markedly inhibited by 20 μg of MC per mouse, while the H^3-cytidine incorporation into RNA was less inhibited by a higher dose of 100 μg of MC per mouse and the S^{35}-methionine incorporation into protein was scarcely affected by 100 μg of MC.

Ehrlich ascites tumor cells cannot proliferate in culture media, however they are still capable of appreciable DNA synthesis in a devised medium (Harbers, 1959). Using these conditions studies were made on the effect of MC on nucleic acid synthesis of the cells in the culture medium, as a preliminary to *in vitro* studies with cell-free DNA synthesizing system.

The tumor cells which had been treated intraperitoneally with 100 μg of MC per mouse for 3 hours were withdrawn from the peritoneal cavity and erythrocytes were eliminated. Then the cells were resuspended at a concentration of about 10% in Robinson's medium containing 0.2% glucose, 1/3 volume of ascitic serum and 2 μc/ml of H^3-thymidine or H^3-cytidine, and 2.5 ml aliquots of the cell suspension in 50-ml conical flasks were incubated at 37°C for 30 minutes with gentle shaking. Analysis was performed as described above.

As shown in Fig. 3, in the culture medium the incorporation of H^3-thymidine into DNA in cells pretreated with MC was evidently reduced whereas that of H^3-cytidine into RNA was much less inhibited by MC-pretreatment.

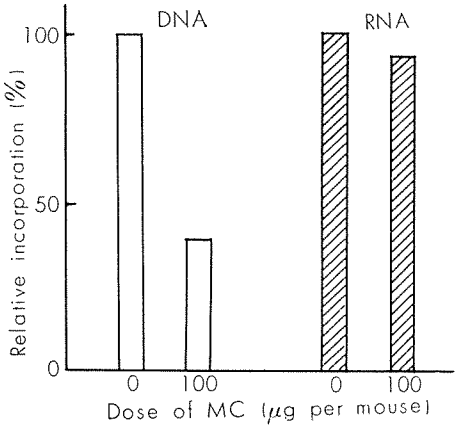

Fig. 3. Effects of MC on H^3-thymidine and H^3-cytidine incorporation into DNA and RNA respectively in Ehrlich ascites tumor cells in culture medium.
The tumor cells were treated intraperitoneally with the indicated doses of MC for 3 hours prior to incubation. The incubation was carried out at 37° C for 30 minutes in the presence of 2 μc/ml of H^3-thymidine or H^3-cytidine. The height of the bars indicates the specific radioactivity relative to that of untreated cells.

2. *Effect of MC on DNA synthesis and the mitotic cycle of tumor cells*

Since the inhibitory action of MC on DNA synthesis in the tumor cells appeared

to have some lag period between the contact of the cells with MC and manifestation of the inhibition, a question arose as to the action of MC that the inhibition of DNA synthesis *per se* might be a secondary effect as a consequence of interruption of the mitotic cycle. Then investigations were made on the kinetics of DNA synthesis in relation to the mitotic cycle using autoradiography and analysis of mitotic frequency.

The time relationship between DNA synthesis and mitotic division in Ehrlich ascites tumor cells have been shown by Howard (1956) and Baserga (1962); the mitotic cycle consists of a mitotic period of 1/2 or 1 hour, a period of DNA synthesis (called the S period) lasting 11 or 12 hours, and a post-synthetic rest period of 6 hours followed by the next mitosis.

Autoradiography: The ascites tumor cells were treated with MC for 3 or 6 hours and then labeled by intraperitoneal injection of H^3-thymidine. After the labeling period the ascitic fluid was withdrawn and autoradiographed.

A reduced uptake of H^3-thymidine into nuclei of MC-treated cells was apparent from autoradiograms. The result of counting of the silver grains is given in Table 1. The total number of the grains in the labeled cells decreased to about 40% of that of the controls after MC-treatment for 3 hours, and decreased further after 6 hours' treatment. This result was reasonably comparable with that obtained by determining the radioactivity of nucleic acid in the cells as is shown in Fig. 1.

Table 1. Effect of MC on H^3-thymidine uptake into nuclei

Time after MC-injection	Total No. of grains	Percent of labeled cells*	Mean No. of grains per cell
0 hr.	5338	68	157
3 hrs.	2144	64	67
6 hrs.	1504	64	47

The tumor cells were treated intraperitoneally with 50 μg of MC per mouse for the indicated periods, and then were labeled by intraperitoneal injection of 20 μc of H^3-thymidine per mouse for an hour. The numbers of grains were calculated from counts on 50 cells. Correction was not made for back ground.

* Cells containing more than 6 grains were regarded as labeled cells from the distribution.

However, no appreciable difference was observed in the population of the labeled cells between the MC-treated and the control cells. Consequently the average number of grains in the individual cells was decreased by MC-treatment.

These results may be interpreted by supposing that the rotation of the mitotic cycle is not directly interrupted by MC within 6 hours, and the MC-treated cells also seem to be able to enter the S period and begin to synthesize DNA ,but the rate of the DNA synthesis *per se* in individual cells is reduced to a low level during the S period.

Mitotic frequency: The mitotic frequency of Ehrlich ascites tumor cells was determined as a function of time after MC-treatment.

As shown in Fig. 4, the average number of mitotic indices of the untreated cells was 2.4%, and there was no change in the mitotic frequency after 3 hours' exposure to MC, while a decrease appeared after 6 hours and further decreased to an average index of 0.47 % after 9 hours' exposure.

Since the suppression of DNA synthesis was already apparent after 3 hours' exposure to MC, when the mitotic division was not yet disturbed, the suppression of DNA synthesis in MC-treated cells may not be a secondary phenomenon induced by mitotic arrest.

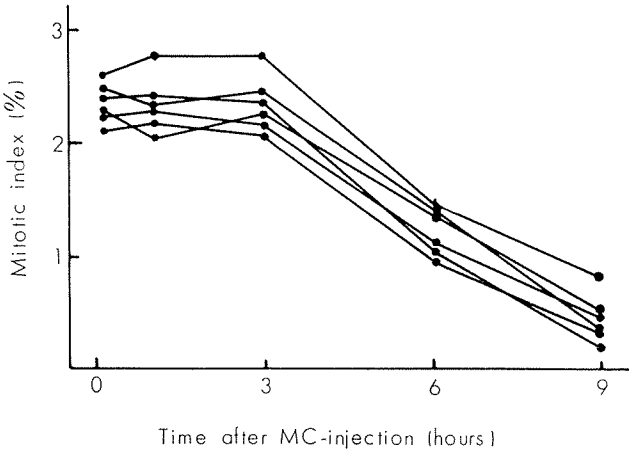

Fig. 4. Effect of MC on the mitotic frequency of Ehrlich ascites tumor cells.
The ascitic fluid was withdrawn from the peritoneal cavity of each mouse at the indicated time intervals after the injection of 50 μg of MC per mouse. The proportions of cells in mitotic phases were calculated from counts on 2000 cells.

3. *Action of MC on enzymic DNA synthesis by cell-free extracts of tumor cells*

An enzyme system capable of incorporating H^3-thymidine into DNA in cell-free extracts of Ehrlich ascites tumor cells has been demonstrated by Smellie *et al.* (1959). Using this cell-free system, investigations were made on the mode of inhibitory action of MC on DNA synthesis in tumor cells.

These extracts contain a multi-enzyme system involved in incorporating thymidine into DNA. This includes the kinases necessary for conversion of thymidine to thymidine triphosphate and the polymerase for condensation of the deoxynucleoside triphosphates to the polynucleotide. Thus the incorporating activity of the extracts in the present experiments shows the overall reaction from thymidine to DNA.

Influence of MC on the enzymic reaction in vitro: In examining the *in vitro* effect of MC on the enzymic reaction, MC was added to reaction mixtures at a final concentration of 100 μg/ml.

As presented in Table 2, even in the presence of such a high concentration of MC, no *in vitro* effect on the enzymic reaction was observed.

DNA, which is required as a primer in the thymidine incorporating reaction, was pretreated *in vitro* with 100 μg/ml of MC or actinomycin S at 37°C for 60 minutes and was then added to the reaction mixtures. The incorporation was markedly inhibited in the presence of actinomycin-pretreated DNA, whereas no inhibition was observed in the presence of MC-pretreated DNA.

Table 2. Effect of MC on enzymic H^3-thymidine incorporation

Expt.	Conditions	Relative incorporation
1.	complete	100
	minus cell extracts*	0
	minus dAMP, dGMP, dCMP	33.1
	minus DNA**	3.2
	added MC 100 μg/ml	102
2.	complete	100
	added pyrophosphate***	6.1
	added actinomycin-treated DNA	8.3
	added MC-treated DNA	125
	added MC 100 μg/ml	105

The reaction mixture and the assay method are described in Materials and Methods. The incubation time was 60 minutes.
* Bovine serum albumine was substituted for cell extracts.
** Yeast RNA was substituted for salmon sperm DNA.
*** Pyrophosphate was added to a final concentration of 20 μ moles/ml.

Enzymic activity of extracts prepared from tumor cells treated with MC in vivo: Smellie *et al.* (1959) reported that a high enzymic activity for DNA synthesis was observed in extracts of proliferating tissues while only very slight activity was seen in extracts of non-proliferating tissues. Measurements were made on the H^3-thymidine incorporating activity of extracts of tumor cells following MC-treatment, which leads to a considerable suppression of H^3-thymidine incorporation *in vivo*.

The tumor cells were exposed intraperitoneally to 50 μg of MC per mouse for 6 hours. By this treatment the H^3-thymidine incorporating capacity of the cells *in vivo* was suppressed to 13% of the control value (Fig. 1). Extracts were prepared from these cells and their enzymic activity in the incorporation of H^3-thymidine was measured. As shown in Table 3, the H^3-thymidine incorporating activity of extracts prepared from MC-treated cells was only slightly reduced and this itself would not account for the suppression of the H^3-thymidine incorporating capacity of cells *in vivo* after MC-treatment.

Priming activity of DNA isolated from the tumor cells treated with MC in vivo: It is known that the priming activity of DNA changes following a moderate degradation by DNase *in vitro* (Sarkar, 1961), and that DNA subjected to thermal denaturation is more effective as a primer than native DNA (Bollum, 1959). If the cellular

Table 3. Enzymic activity of extracts prepared from MC-treated cells

Expt.	Treatment *in vivo*	H^3-thymidine incorporated per mg protein
1	none	17200 cpm
	MC	13100
2	none	15600 cpm
	MC	10400
3	none	13400 cpm
	MC	9800
4	none	0.10 mμ mole
	MC	0.09

The assay method is described in Materials and Methods. The incubation time was 60 minutes in expt. 1, 2, 3, and 40 minutes in expt. 4. Radioactivity was measured in a gas-flow counter in expt. 1, 2, 3, and in a liquid scintillation spectrometer in expt. 4.

DNA is degraded to any extent by MC-treatment, the DNA isolated from MC-treated cells would differ from the DNA of intact cells in its priming activity.

Ascites tumor cells pretreated intraperitoneally with 120 μg of MC per mouse for 9 hours were collected and the DNA was isolated by the method described above. The control DNA preparation was obtained from intact cells in the same way.

As shown in Table 4, the priming activity of the DNA isolated from the MC-treated cells did not differ from that of the DNA isolated from intact cells and was almost at the same level as that of commercial salmon sperm DNA.

Following thermal denaturation the priming activity of tumor cell DNA was markedly increased; it becomes about 10 times as much as that of native DNA. By thermal denaturation also the DNA of MC-treated cells as a primer was activated similarly to the DNA of intact cells.

Table 4. Priming activity of DNA isolated from MC-treated cells

Source of primer-DNA	Specific radioactivity (mμ moles per μ mole DNA-P)
salmon sperm	1.94
EATC	1.73
MC-treated EATC	1.62
EATC (heated)	14.4
MC-treated EATC (heated)	15.2

The tumor cells were treated intraperitoneally with 120 μg of MC per mouse for 9 hours. Methods of the isolation and the heat denaturation of DNA and the composition of the reaction mixture are described in Materials and Methods. The incubation time was 40 minutes. Radioactivity was measured in a liquid scintillation spectrometer.

EATC: Ehrlich ascites tumor cells

DISCUSSION

The present studies demonstrate that MC inhibits the incorporation of H^3-thymidine into DNA in proliferating Ehrlich ascites tumor cells. Preliminary experiments (Terawaki *et al.*, 1960) showed that, consistent with the present observation, the increase in the net DNA amount of the tumor cells within the peritoneal cavity of mice subjected to MC-treatment is considerably less than that in the control animals.

In the previous studies, Shiba *et al.* (1958) observed that MC shows an exceedingly preferential inhibition on the DNA synthesis in *Escherichia coli* B. In Ehrlich ascites tumor cells also, RNA synthesis was little suppressed and protein synthesis was almost unaffected by doses of MC which inhibited DNA synthesis markedly. However, in the tumor cells the inhibitory action of MC was not so highly selective for DNA synthesis as observed in the case of *Escherichia coli* B.

As presented in Fig. 1, the incorporation of H^3-thymidine into DNA was reduced 3 hours after exposure of tumor cells to MC, but was not reduced after 1 hour. This means that there may be some lag period between the time of contact of the cells with the drug and the expression of its effect. A similar lag period was also seen in the inhibition of the H^3-thymidine incorporation in MC-treated *Escherichia coli* B (Shiba *et al.*, 1961). Though the lag was much longer in the tumor cells than in the bacteria, these two phenomena, measured by a time scale of life cycle, seem to be of the same kind.

This delayed expression of the effect of MC leads to a question that the inhibition of DNA synthesis *per se* might not be caused directly by MC, but be induced secondarily by an interruption of the rotation of the division cycle, as was reported with the cases of Ehrlich ascites tumor cells (Howard, 1956) and L cells (Whitmore *et al.*, 1961) subjected to X-ray irradiation. However, from the results shown in Table 1 and Fig. 4, the population of cells in the S period was not changed at a time when the DNA synthesis was markedly suppressed, and the reduction of the mitotic frequency appeared later than the inhibition of the H^3-thymidine incorporation. It may well be inferred that the rotation of the mitotic cycle and the entrance of cells into the S period are not primarily interrupted by MC, and that the inhibition of DNA synthesis precedes the mitotic disturbance, which appears rather as a consequence of the former. The rate of DNA synthesis in individual cells during the S period is at a reduced level. Therefore, what is closely concerned with the process of DNA synthesis *per se* seems to be directly affected.

No explanation for the inhibitory action of MC on DNA synthesis observed *in vivo* has been obtained from *in vitro* experiments using a cell-free DNA synthesizing system of the tumor cells.

Even in the presence of a high concentration of MC, no *in vitro* effect on the enzymic reaction was observed. Sekiguchi and Takagi (1960) similarly reported that the incorporation of labeled precursors into DNA by a cell-free extract from

Escherichia coli was not suppressed in the presence of MC.

Furthermore, there was no sufficient loss of enzymic activity in the extracts prepared from the tumor cells subjected to MC-treatment which leads to a remarkable suppression of DNA synthesis *in vivo*. If the enzyme activity present in the extracts reflects the actual enzyme content involved in DNA biosynthesis in the tumor cells, it may be concluded that MC does not block enzyme formation or damage the enzyme activity in the cells. This result is comparable with those in which an almost unaltered DNA synthesizing capacity in cell-free extracts was shown in *Escherichia coli* subjected to chloramphenicol, amino acid deficiency, or X-rays (Billen, 1962) and UV-light (Doudney and Billen, 1961).

It has been shown that MC causes depolimerization of bacterial DNA (Reich *et al.*, 1961; Kersten and Rauen, 1961) and that an activation of DNase is involved in the depolimerization (Nakata *et al.*, 1962; Kersten, 1962). In mammalian cells also fragmentation of the nuclear apparatus has been reported (Shatkin *et al.*, 1962; Kersten and Themann, 1962), though in mammalian cells the breakdown seems to appear far later than the decrement in DNA synthesis and cell death. If the cellular DNA of Ehrlich ascites tumor should be broken down to an appropriate degree by the action of MC, the DNA isolated from MC-treated cells would differ from that of intact cells in its priming activity. As shown in Table 4, however, in this cell-free system the priming activity of the DNA isolated from MC-treated cells showed no difference from that of intact cells.

On the other hand, recently Iyer and Szybalski (1963) reported the formation of links between complementary DNA strands by the action of MC and interpreted this as a primary and sufficiently lethal lesion to induce cell death. However, such a delicate change as occurs in the structure of the DNA molecule could not be represented in the system used in the present study.

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