# Effect of Various Antitumor Agents on Electrokinetic Behaviour of Ehrlich' Ascites Tumor Cells and Mouse Erythrocytes

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## INTRODUCTION

A Simple and direct approach to cell surface properties is provided by the microscopic method of electrophoresis measuring migration of cells in an electric field.

It has been known that under the condition of physiological pH and ionic strength, the erythrocytes from various species exhibit a negative electrophoretic mobility and thus have a negative surface charge. Furchgott and Ponder [12] have found the erythrocyte to be isoelectric near pH 2.0 and have attributed its negative surface character to phosphoric acid groups of cephalin. More recently it has been shown that charge on the human erythrocyte derives from very acidic carboxyl groups of a sialic acid-containing component of the ultrastructure. [8] [10] Klenk and Uhlenbruck [14] have shown that neuraminidase acts upon erythrocyte stroma by releasing N-acetylneuraminic acid.

Previous investigations [2][18] have indicated that the electrophoretic mobility of certain tumor cell is greater than that of the corresponding normal homologous cell and that this increase might be related to the invasive properties of the tumor cell. Further comparative studies [6][13][19] have indicated that the elevation of electrophoretic charge density may be associated with growth in general rather than malignancy in particular. Bangham and Pethica [5] studied some of the electrophoretic properties of the Ehrlich' ascites tumor cell and concluded that phospholipids were an important component of the surface ultrastructure. Moreover, Ruhenstroth-Bauer, Kübler, Führmann and Rueff [20] published a study on the role of sialic acids which were an important part of tumor cell membranes and the membranes of blood cells.

Vasser [29] studied on the surface charge density of a variety of fresh human tumor tissues and indicated that mesenchymal tumor cells had a higher surface charge density than normal or neoplastic epithelial cells, while no significant difference was found in the charge densities between normal and malignant epithelial cells.

In the present experiment the effects of several antitumor agents on electrophoretic mobility of Ehrlich' ascites tumor cells and mouse erythrocytes were examined in vitro and vivo.

## MATERIALS AND METHODS

*Animals :*

Adult male mice from a $NA_2$-strain of 20—25g weight were used. The animals were fed chows and given water ad lib.

*Tumor cells :*

Experiments caried out with Ehrlich' ascites tumor cells in vitro and in vivo. The ascites tumor cell, which is relatively free-growing in vivo, can be readily obtained without excessive handling and therefore represents a convenient model tumor system for electrokinetic analysis. The ascites tumor cells were grown intraperitoneally in the old male mice following inoculation of 0.1 ml. of peritoneal fluid containing tumor cells diluted with double volume of physiological saline solution. The ascites tumor cells were obtained on ninth or tenth day following inoculation. Microscopic examination revealed that the ascites tumor cells obtained are heterogenous. About 80 per cent of the cells appeared to be Ehrlich' ascites tumor cells and the remainder was comprised with lymphocytes and other non-malignant mesodermal cells.

*Buffer Solutions :*

For studying the effect of several antitumor agents on the electrophoretic mobility of the ascites tumor cells and mouse erythrocytes, stocked phosphate saline buffer (a mixture of 90 volumes of 1 per cent NaCl, 2 volumes of M/15 $KH_2PO_4$ and 8 volumes of M/15 $Na_2HPO_4$) was used. For studying the effect of pH on the mobility, a series of buffers essentially constant ionic strength and tonicity was prepared. For pH values from 5.6 to 7.3, 10 ml. of suitable mixtures of M/15 $KH_2PO_4$ and M/15 $Na_2HPO_4$ were diluted to 100ml with 1 per cent NaCl. For pH values from 3.6 to 5.6, 20ml of suitablemixtures of 0.2 M sodium acetate and 0.2 M acetic acid were diluted to 100ml with 0.96 per cent NaCl. For use, these solutions were diluted with 3 volumes of 5.4 per cent glucose solution, giving isotonic solution of approximately 0.043 ionic strength.

For studying the effect of ionic strength on the mobility, various dilutions of stocked phosphate saline buffer with 5.4 per cent glucose were prepared.

Preparation of antitumor agent solution : Several antitumor agents such as Toyomycin, Mitomycin, Endoxan, Nitromin and Tespamin were dissolved with physiological saline solution. Final concentrations of each drug in use were as follows :

| | in vitro | in vivo |
|---|---|---|
| Toyomycin (Chromomycin $A_3$) | 5γ/ml. | 0.5mg/kg |
| Mitomycin (Mitomycin—C) | 20γ/ml. | 2mg/kg |
| Endoxan (Cyclophosphamide) | 1mg/ml. | 100mg/kg |
| Nitromin (Nitrogen mustard N—oxide) | 0.5mg/ml. | 50mg/kg |
| Tespamin (N, N′, N″—triethylenethiophosphoramide) | 50γ/ml. | 5mg/kg |

In vitro, these antitumor agent solutions were added into 0.1—0.2 % V/V suspension of the ascitic tumor cells in physiological saline solution.

In vivo, a single intraperitoneal injection of each antitumor agent was performed and no further treatment was applied. The control groups were treated with solvent of these agents.

Preparation of the samples for electrophoresis : The ascitic fluid was centrifuged for 5 minutes at 500 rpm in order to eliminate all other cells except Ehrlich' ascites tumor cell. The resulting pellet was washed 4 times with physiological saline solution and then was made up to a concentration by volume of 0.1—0.2 per cent.

Finally, the pellet was washed once more with buffer solution. The pellet was uniformly suspended by forcing the suspension through a syringe needle. Approximately 90 per cent of the cell concentration appeared as gingle cells. The ascitic fluid were never mixed so as to avoid any possible immunological interactions. It is obvious that variation among samples prepared in this manner will be high. However, for all cell concentrations suitable for microelectrophoresis (approximately 0.1 to 0.4 per cent V/V), the mobility was found to be independent of the cell concentration. Measurements of the electrophoretic mobility of the ascites tumor cells were continued until 4 hours after the addition of antitumor agents in vitro and until 6 hours after the injection of the agent in vivo, while interval of each measurement had an hour.

Red blood cells from the same mouse were used as internal controls.

Electrophoresis apparatus and technics : The tumor cell mobility was measured by direct microscopic observation in a microelectrophoresis apparatus with a rectangular cell oriented in the horizontal position as described by Abramson.[1] The orientation, in combination with an eyepiece reticle having a rectangular guard, allows the measurement of the horizontal component of travel. Contamination with heavy metal ions was minimized by the use of reversible nonpolarizing electrodes in saturated copper sulfate solution which were separated from the electrophoretic cell by plaster of Paris plugs saturated with sodium sulfate and by 2 per cent agar plomb made with buffer solution between plaster and electrophoretic cell.[21)23)] Convection currents were almost insignificant so that particles with very small mobility could be measured accurately. The apparatus was cleaned with chromium sulfate solution and then with distilled water, further with buffer solution. Bubbles were easily flushed out from the

system. The electrophoretic cell was observed under an ordinary light microscope with a ×20 objective and ×15 ocular. The optical system gave a significant magnification and was calibrated aganist a 1.0 mm standard micrometer scale divided to 0.01 mm. The all mobility measurements were made at the first stationary level which was calculated by Smoluchowski's equation.[26]

A monochromatic green light source was conveniently mounted behind the water-bath in order to avoid heat. The all mobility measurements were performed at room temperature. At currents from 0−3 mA. satisfactory data were obtained in the present experiments as long as the tumor cell concentration was held within the range recommended above. The horizontal component of the travel of each individual cell was determined with the aid of a stop watch with 1/10 sec. division. The travel times of 10 individual cells were measured, then the electric field was reversed and a similar set of measurements was made on 10 different cells. The specific conductance was previously calculated from the conductivity of the cell suspension. The electrophoretic mobility unit is defined as $\mu$/sec. /volt/cm.

*Calculation:*

The electrophoretic mobility of the cells was calculated by the following equation.[22)27)]

$$U = -\frac{\mu \cdot c\hat{m} - \Omega}{\text{sec.} \cdot \text{Amp.}}$$

where $\mu$=distance in microns traversed, $c\hat{m}$=cross−sectional area in sq. cm., $\Omega$=specific conductance of the solution, sec.=time in seconds to traverse $\mu$ microns and U=mobility unit.

## RESULTS

Ehrlich' ascites tumor cells exhibit a moderately high negative charge as judged by electrophoretic mobility. The mean mobility of 20 different preparations of the ascites tumor cells was $-1.06 \pm 0.04\mu$/sec. /volt/cm.

Table 1.
Mouse erythrocyte
(phosphate buffer, pH 7.3, ionic strength 0.043, at room temperature)

| | 1 hour | 2 hours | 3 hours | 4 hours |
|---|---|---|---|---|
| Control | −1.31±0.04 | −1.31±0.04 | −1.31±0.04 | −1.31±0.03 |
| Toyomycin (5$\gamma$/ml) | −1.32±0.05 | −1.32±0.05 | −1.31±0.06 | −1.31±0.05 |
| MMC (20$\gamma$/ml) | −1.32±0.04 | −1.32±0.04 | −1.31±0.04 | −1.31±0.04 |

Table 2.

Effect of various antitumor agents on the electrokinetic behaviour of Ehrlich' ascites tumor cell in vitro

(phosphate buffer, pH 7.3, ionic strength 0.043)

| (No, of case) | 30 min. | 1 hour | 2 hours | 3 hours | 4 hours |
|---|---|---|---|---|---|
| Control (10) | 1.07±0.04 | 1.06±0.02 | 1.06±0.03 | 1.06±0.04 | 1.06±0.04 |
| Endoxan (6) | 1.09±0.04 | 1.09±0.02 | 1.08±0.04 | 1.08±0.02 | 1.08±0.06 |
| Nitromin (6) | 1.03±0.03 | 1.05±0.03 | 1.09±0.07 | 1.09±0.05 | 1.10±0.04 |
| Tespamin (5) | 1.02±0.04 | 1.04±0.04 | 1.04±0.03 | 1.04±0.02 | 1.08±0.06 |
| Mitomycin(10) | 0.96±0.04 | 0.97±0.02 | 0.00±0.02 | 0.99±0.02 | 0.99±0.02 |
| Toyomycin (10) | 0.94±0.02 | 0.96±0.03 | 0.99±0.03 | 1.01±0.02 | 1.08±0.03 |

The mobility was unaltered by five-fold washing of cells with physiological saline solution and phosphate buffer, further it remained constant up to 7−8 housr after collection as reported by Straumf jord.[27]

In mouse erythrocyte used as internal controls and measured with phosphate buffer in the same way as described above, the mobility was $-1.31 \pm 0.04\mu$/sec./volt/cm. for 4 hours and unaffected by the addition of various antitumor agents, but in the addition of Nitromin the cells accepted easily hemolysis.

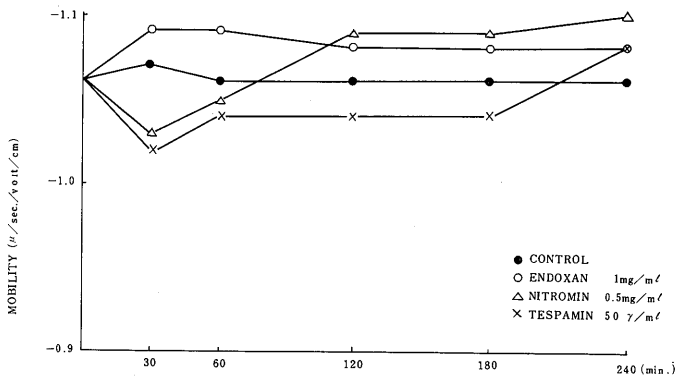


Fig. 1. (a)

Fig. 1. (a) (b)

Effect of various antitumor agents on electrophoretic behaviour of Ehrlich' ascites tumor cell in vitro at room temperature. Buffer used here was phosphate buffer with pH 7.3 and ionic strength 0.043. The ordinate represents the differences in the electrophoretic mobility between various antitumor agents-treated and untreated tumor cells.

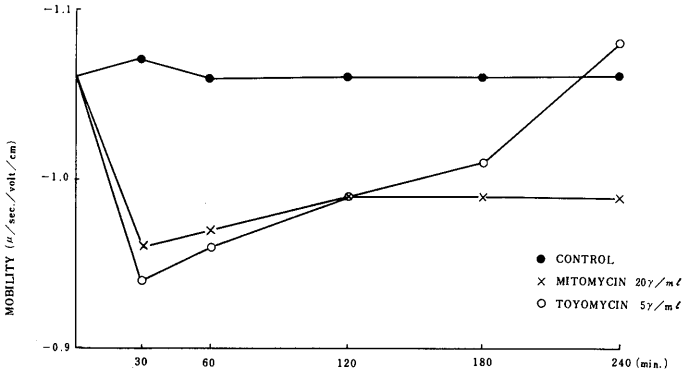


Fig. 1. (b)

As shown in Fig. 1 in vitro (a, b), the control groups added with the only solvent of these antitumor agents did not significantly alter the electrophoretic mobility and it remained constant during a period of 4 hours after the beginning of experiment. Addition of Endoxan, Nitromin and Tespamin to the Ehrlich' ascites tumor cells did not significantly change the electrophoretic mobility, however, Toyomycin and Mitomycin decreased the mobility. The maximal decrease in the mobility was observed at 30 minutes after addition of these antitumor agents and the rate of decrease was about 12 and 10 per cent respectively relative to the controls at that time. Though the decrease in the mobility of the Toyomycin-treated cells was recovered for 4 hours after treatment that of the Mitomycin-treated cells was still as evident as about 7 per cent even at that time.

Table 3.

Effect of various antitumor agents on the electrokinetic behaviour of Ehrlich' ascites tumor cell in vivo

(phosphate buffer, pH 7.3, ionic strength 0.043)

| (No. of case) | 1 hour | 2 hours | 3 hours | 4 hours | 5 hours | 6 hours |
|---|---|---|---|---|---|---|
| Contrl (10) | 1.06±0.04 | 1.05±0.04 | 1.05±0.02 | 1.05±0.04 | 1.06±0.03 | 1.05±0.04 |
| Endoxan (6) | 1.05±0.03 | 1.06±0.03 | 1.06±0.03 | 1.06±0.02 | 1.03±0.03 | 1.05±0.02 |
| Nitromin (6) | 1.01±0.05 | 1.01±0.05 | 1.00±0.04 | 1.04±0.03 | 1.05±0.05 | 1.07±0.05 |
| Tespamin (5) | 1.04±0.05 | 1.04±0.04 | 1.06±0.03 | 1.06±0.03 | 1.07±0.06 | 1.07±0.12 |
| Mitomycin (10) | 0.94±0.04 | 0.99±0.02 | 1.00±0.02 | 1.04±0.03 | 1.05±5.02 | 1.06±0.05 |
| Toyomycin (10) | 0.93±0.03 | 0.96±0.04 | 1.01±0.05 | 1.03±0.02 | 1.04±0.04 | 1.06±0.06 |

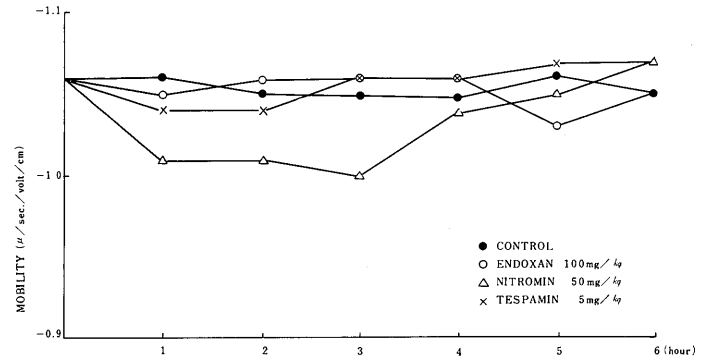


Fig. 2. (a)

Fig. 2. (a) (b)

Effect of various antitumor agents on the electrophoretic behaviour of Ehrlich' ascites tumor cell in vivo.

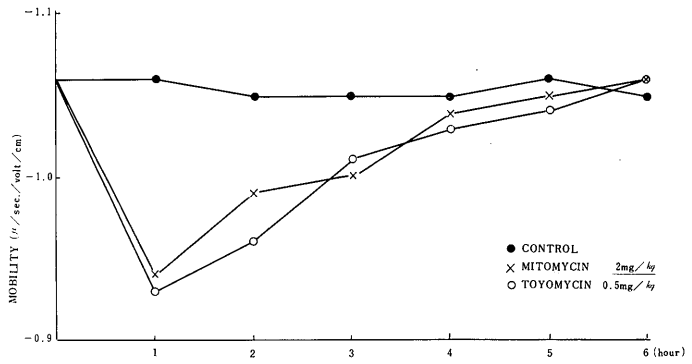


Fig. 2. (b)

As shown in Fig. 2 in vivo (a, b), the control groups remained constant during a period of 6 hours after the beginning of experiment. The electrokinetic behaviour of Endoxan-, Nitromin- and Tespamin- treated tumor cells appeared to be identical with the control groups during a period of 6 hours after treatment as entirely same as in vitro. However, Toyomycin and Mitomycin decreased the mobility at the minimal level of which was reached at 1 hour after treatment with these antitumor agents and gradually recovered until its normal level along the course of experiment. The decrement was 12 and 11 per cent respectively relative to the control group at their minimal level.

Table 4.

pH-mobility relationship of various antitumor agents-treated and untreated Ehrlich' ascites tumor cells

Buffert : For pH valves from 5.8 to 7.3, phosphae buffers
For pH valves from 3.6 to 5.6, acetate buffers
at ionic strength 0.043

| | Control | MMC (20γ/mℓ) | Toyomycin |
|---|---|---|---|
| pH 7.3 | −1.07 ± 0.04 | −0.96 ± 0.04 | −0.94 ± 0.03 |
| 5.8 | −1.02 ± 0.05 | −0.97 ± 0.03 | −0.91 ± 0.05 |
| 5.2 | −0.77 ± 0.04 | −0.73 ± 0.05 | −0.73 ± 0.04 |
| 4.4 | −0.32 ± 0.07 | −0.35 ± 0.09 | −0.35 ± 0.11 |
| 3.6 | −0.73 ± 0.04 | +0.75 ± 0.06 | +0.75 ± 0.05 |

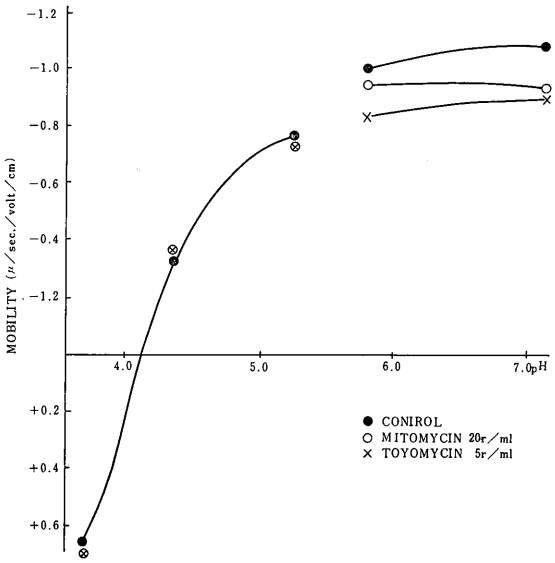


Fig. 3.

The influence of pH of the medium upon the electrophoretic mobility of Ehrlich' ascites tumor cells at 0.043 ionic strength. The left-hand portion of the curves was obtained with the use of acetate buffer; to the right-hand portion of the discontinuity, the values were obtained with phosphate buffers. The curve of untreated cells was contrasted with that of treated cells with Toyomycin or Mitomycin.

Fig. 3 depicts the pH-mobility relationships for washed Ehrlich' ascites tumor cells obtained in M/15 phosphate and M/5 acetate buffer at a constant ionic strength of 0.145. It could be shown that down to a pH of about 5.8, the

mobility did not almost alter, whereas it declined rapidly with a further decrease in pH, reaching the isoelectric point at pH 4.1 as found by Eisenberg[9] and Cook.[7] A further decrease in pH brought about a reversal of the electrokinetic charge. The appearance of a positive branch in the pH-mobility relationship below pH 4.1 also indicated the presence of cationic groups in the electrophoretic plane of sphere. Therefore, it was suggested that the rate and direction of migration of ascites tumor cells were dependent upon pH and the amphoricity of their surfaces. Straumfjord[27] observed in microscopic examination that the cells were unaffected by acid media, but above pH8.0 some lysis occurred. As observed in the present experiments, the cells did not significantly alter the pH of media from pH 3.6 to 5.8, but lowered by 0.3 pH units the pH of the buffered media in pH 7.3. When the ascites tumor cells were treated with Toyomycin or Mitomycin during 30 minutes in vitro, the reduction in mobility was not observed and the mobility consisted approximately with that of control group over pH 5.2 to 3.6. Otherwise treated tumor cells indicated gradual discharge running parallel with control group over pH 7.3 to 5.2. The type of cure with isoelectric point at pH 4.1 shown by the electrophoretic mobility as a function of the pH of the medium is similar to that of protein.

Table 5.

Ionic Strength-Mobility relationship

phosphate buffer at pH 7.3

$\mu$/sec./volt/cm

| ionic strength | Straumfjord et al. (1959) | in present experiment |
|---|---|---|
| 0.172 | −0.67 | |
| 0.086 | −0.90 | −0.92 |
| 0.056 | | −1.01 |
| 0.043 | −1.04 | −1.07 |
| 0.0286 | | −1.20 |
| 0.0215 | | −1.31 |
| 0.0172 | −1.27 | −1.32 |
| 0.0086 | −1.56 | |

Fig. 4 indicates the electrophoretic mobility of Ehrlich' ascites tumor cells being suspended in media of various ionic strength at pH 7.3. It was observed that the net surface charge was gradually decreased with the lowering of ionic strength in phosphate buffer.

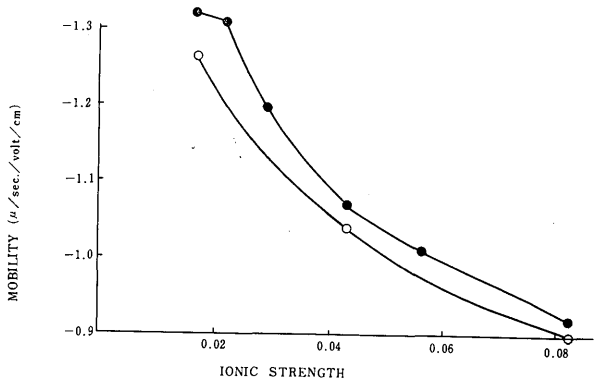


Fig. 4.

Fig. 4.

Electrophoretic Mobility of Ehrlich' ascites tumor cells as influenced by the ionic strength at pH 7.3
Lower curve indicates that which was obtained dy STRAUMFJORD in the same condition.

## DISCUSSION

Although the mobility of mouse erythrocyte was unaffected by the antitumor agents, in this study Ehrlich' ascites tumor cells treated with some of these agents, especially Toyomycin and Mitomycin, indicated the temporary decrease of electrophoretic mobility of 10 to 12 per cent respectively as compared with untreated cells for 1/2 to 1 hour after treatment in vitro and in vivo. The remainder did not significantly exert any alteration on ionogenic behaviour of the cell surface.

Previous investigation [4] indicated that when MTK 11 and Yoshida sarcoma cells were treated with tespamin (2 mg/kg) and Mitomycin (1 mg/kg) in vivo, these both agents led to decrease of about 16 to 29 per cent in electrophoretic mobility and the decrease was considerable in accordance with that on the mitotic frequency examined by Umeno. [28] However, Cook Heard and Seaman[7] suggested that although Ehrlich' ascites tumor cells were in various phases of mitosis, the electrophoretic mobility of individual cells indicated the small scatter, so that the electrokinetic properties of the tumor cell surface were independent of the state of the nucleus.

The antitumorigenic mechanisms of these agents used here have been reported previously. [15][17][25][30][31] Alkylating agents (Nitromin, Tespamin and Endoxan) and antibiotics (Toyomycin and Mitomycin) are known as antimetabolic substances against nucleic acid compounds. It has been reported in application of certain cytochemical techniques that the principle effects of alkylating agents are to

damage only DNA synthesis; Mitomycin impedes mainly DNA synthesis in the resting and further produces any changes in the nature of RNA; Toyomycin produces mainly only change in the nature of RNA.

Eisenberg, Ben-Or. and Doljanski[9] suggested that an increase in the average mobility of liver cells following the partial hepatectomy may be similar process with an immediate stimulation of RNA synthesis as indicated by the increased incorporation of glucose$-1-^{14}C$ into the RNA fraction.[24] It is very interesting that, in the present experiments, the remarkable decrease in the electrophoretic mobility of Ehrlich' ascites tumor cells is observed in the series with the antitumor agents which may impede RNA synthesis.

Ambrose[3] suggested a model of the altered membrane of the cancer cell according to Danielli' model. In the membrane of the normal cell, it consists of a double lipid layer with the polar groups facing outward into the aqueous phase. This layer is coated on each side with protein. In the tumor cell membrane, the outer protein layer is deficient in certain regions. In these region exposed phosphate groups of phospholipid are present, giving a higher net negative surface charge. It has been suggested by Furchgott and Ponder[12] that the electrokinetic behaviour of the human erythrocyte arises from a component containing ionizable phosphate groups which is probably associated with a complex phospholipid system. Bangham and Pethica[5] have also studied some of the electrokinetic properties of Ehrlich' ascites tumor cells and concluded that phospholipids were important components of the surface ultrastructure. They further concluded that phospholipid, notably phosphatidic acid and monophosphoinositide, was mainly responsible for the negative surface charge of erythrocyte.[10] Eylar, Madoff, Brody and Oncley.[11] concluded that the carboxyl group of sialic acid was more responsible for the negative surface charge rather than lipid phosphate and that the likely substances among these lipids were not lecithin, phosphatidylethanolamin or sphingomyelin, but rather phosphatidylserine, monophosphoinositide and phosphatidic acid which had much of negative charge.

It was reported by Kurata[16] that cobalt-porphyrin (COPP) as a new antitumor agent, which was not used in the present experiment, had a significant affinity for lipoprotein, especially lecithin in the membrane of Ehrlich' ascites tumor cell. However, any evidence that other antitumor agents might have the same biological activity as COPP for lipoprotein in the tumor cell membrane has not been described. Judging from the effect of these antitumor agents on the cytological appearance and the electrophoretic mobility of Ehrlich' ascites tumor cell, it is suggested that these antitumor agents might have some significant affinity for lipoprotein in the tumor cell membrane and lead to a decrease in the electrophoretic mobility. The electrophoretic mobility of the mouse erythrocyte was unaffected by these antitumor agents in the present experiment,

because the erythrocyte membrane had the same ionogenic behaviour as suggested by Abramson.

Recently it has been shown that the charge on a variety of erythrocytes derives from very acidic carboxyl groups of a sialic acid containing component of ultrastructure.[8] Moreover it has been demonstrated that some portion of the negative charge on the Ehrlich' ascites tumor cell may be due to a sialic acid system. It has been reported as to the action of neuraminidase on erythrocytes from various animal species and Ehrlich' ascites tumor cells that the reductions in surface charge are accompanied by the release of sialic acid system.[10] Cook et al[7] suggested that the electrokinetic behaviour of Ehrlich' ascites tumor cells appeared to arise from at least three ionogenic systems; two dissociable acid functions and one dissociable basic function; (a) a carboxyl group of sialic acid, pK about 2.8, (b) an unidentified acidic group, pK about 2.5 to 4.0—this group may be associated with a lipid system of the tumor cell, (c) a basic group, pK about 10.— this ionogenic component may be a free carboxyl group of an amino acid.

As shown in Fig. 3, Ehrlich' ascites tumor cells treated with Toyomycin and Mitomycin indicated the decrement of about 10 to 12 per cent respectively relative to that of control group over the pH range 7.3 to 5.2. Moreover it was observed that various antitumor agents did not yield any alteration on electrokinetic properties of mouse erythrocyte membrane. Therefore, assuming that these antitumor agents might have a significant affinity for lipoprotein in the membrane of Ehrlich' ascites tumor cell, it may be suggested that the decrement might be due to release phospholipid phosphate rather than sialic acid from the tumor cell membrane.

## SUMMARY

1. The effects of various antitumor agents on the electrophoretic mobility of Ehrlich' ascites tumor cells and mouse erythrocytes were examined in vitro and in vivo.
2. These antitumor agents had no estimable effect on the electrophoretic behaviour of mouse erythrocyte membrane, while significant decrease was obtained in the electrophoretic mobility of Ehrlich' ascites tumor cells treated with Toyomycin and Mitomycin.
3. The isoelectric point of Ehrlich' ascites tumor cell was supposed to be about pH 4.1.

These values indicate that the surfaces of the ascites tumor cells are amphoric and are strongly electronegative at neutral pH.

The significant decrease on the electrophoretic mobility of the ascites tumor

cells treated with Mitomycin and Toyomycin was obtained mainly on the alkali side of pH.

4. The electrophoretic mobility of the ascites tumor cells was influenced by the ionic strength at pH 7.3. The value decreased according to increase of ionic strength.

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