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# NEURITE FORMATION AND MEMBRANE CHANGES OF MOUSE NEUROBLASTOMA CELLS INDUCED BY VALINOMYCIN

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## Summary

A clonal cell line of mouse neuroblastoma cells was found to undergo morphological differentiation in the presence of a $K^+$ ionophore, valinomycin, in the assay medium. This effect was blocked by increasing the concentration of KCl of the medium, suggesting that the changes in resting membrane potential and ion fluxes may be involved in the mechanism of the formation of neurites. No enhancement of the neurite formation was observed in salines containing high concentrations of KCl in the absence of valinomycin. Depolarizing agents including veratridine, gramicidin and ouabain did not stimulate the outgrowth of neurites. Neither electrophoretic mobility of the cells nor molecular anisotropy of fluorescence probes in the membranes was modified by the treatment of valinomycin. Instead, it modified the slow binding phase in kinetics of the interaction of 1-anilinonaphthalene-8-sulfonate (ANS) with the cells, which is related to the penetration process of the probe into membranes. Valinomycin also enhanced the fluorescence intensity of ANS by increasing the binding sites in neuroblastoma cells.

## Introduction

Mouse neuroblastoma cells possess a number of the properties characteristic of the matured neuron; these cells, for example, can be induced to extend neurites by the treatment of serum-free media, dibutyryl cyclic AMP, cytosine arabinoside, prostaglandins, dimethyl sulfoxide, glial factors and so on, and even of X-ray and hypertonic media (see refs. 1 and 2 for review). Nevertheless, the mechanism of the action of these treatments has not yet been fully understood in relation to the morphological differentiation of neuroblastoma cells.

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Abbreviations: 12-PS, 12-[4-(pyrenyl)butyryloxy]stearate; ANS, 1-anilinonaphthalene-8-sulfonate.

Recently, the mean resting membrane potential of the cells in neural plate of the axolotl has been described to increase from −25 mV to −45 mV during the transition from early to midneural plate stages of development [3]. Miyake [4] also made similar observations using mouse neuroblastoma cells that the resting membrane potential increased from −22 mV to −55 mV associated with the development from round dividing cells to differentiated cells. These observations suggest a possible relationship between the development of nerve cells and the permeability changes of ions asymmetrically distributed across the nerve membranes. Cone [5] offered a hypothesis of the role of the resting membrane potential in cell division by observing that the sustained depolarization of matured neuron resulted in the initiation of thymidine incorporation and also in the increase of mitotic activity.

It is, thus, anticipated that the change in the membrane properties leading to the hyperpolarization could facilitate the morphological differentiation of neuroblastoma cells under appropriate conditions. Spector et al. [6] observed rapid enhancement of electrical excitability of differentiating neuroblastoma cells induced by valinomycin. We now present evidence that a $K^+$ ionophore, valinomycin, is able to induce the formation of neurites of neuroblastoma cells. This effect was blocked by increasing potassium concentration of the medium, which suggests that mechanism relating to the changes in membrane potential and ion fluxes may play a role in the morphological differentiation of the cells. In addition, changes in surface membranes induced by valinomycin were also observed by fluorescence spectroscopy.

## Materials and Methods

Chemicals were obtained from the following sources: valinomycin and gramicidin from Sigma; veratridine from Aldrich; ouabain from Merck; fetal calf serum and Dulbecco's modified Eagle's minimum essential medium (H-16) from Gibco; amino acids and vitamins for Eagle's essential medium from Nissui Seiyaku Co., and crystalline bovine serum albumin from Armour. 12-[4-(pyrenyl(butyryloxy]stearate (12-PS) was synthesized as described [7]. Purified perylene and ANS were gifts of Drs. Y. Kawasaki and N. Wakayama, and Dr. Y. Uratani [8], respectively. Other chemicals used in this study were of reagent grade purity.

*Cell culture.* A clonal cell line (N18) derived from mouse neuroblastoma C1300 was obtained from Dr. T. Amano [9]. Cells (passage number 35-50) were grown in exponential phase in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 10% $CO_2$/90% air [7,10]. Other conditions were as described previously [7,10]. Cells were exposed to the medium without serum for one week [11], and then serum was added to grow the surviving cells. This cycle was repeated twice so as to get the cells that responded to the serum withdrawal resulting in the maximal extension of neurites.

*Assay method.* Cells were dissociated with EDTA ($5 \cdot 10^{-4}$ M) in phosphate-buffered saline (NaCl 8.0 g/l, KCl 0.20 g/l, $CaCl_2$ 0.10 g/l, $MgCl_2 \cdot 6H_2O$ 0.10 g/l, $Na_2HPO_4 \cdot 2H_2O$ 1.15 g/l, $KH_2PO_4$ 0.20 g/l) supplemented with 0.54% glucose, pH 7.2 and allowed to attach to glass surface (60 mm) for 20 h in the medium

supplemented with 10% fetal calf serum before being placed in control or modified media. Assay medium used is either Dulbecco's modified Eagle's minimum essential medium supplemented with 1% dialyzed fetal calf serum, pH 7.2, or Eagle's minimum essential medium containing 1% dialyzed fetal calf serum and also various concentrations of KCl (5, 15, 25, 35 and 50 mM), pH 7.9, where osmolality was adjusted to approx. 310 mosM by adding glucose and ionic strength was kept constant by decreasing the corresponding amount of NaCl. Valinomycin (lot no. 66C-0281), veratridine and gramicidin were all dissolved in ethanol and added to the assay medium in concentrations in which ethanol did not exceed 0.1%. Ouabain was dissolved in phosphate-buffered saline, pH 7.0. The initial cell concentration was approximately $3 \cdot 10^3/cm^2$ for convenience of cell counting. After 2 h incubation in control or modified media at 37°C, cells were evaluated for the presence of neurites from photographs or directly under microscope. Cells having one or more neurites with length longer than cell body (average, 15 $\mu$m) were counted, and compared with the total number of cells.

*Labeling of cells and binding experiments.* Cells were labeled either with 12-PS or with perylene at 37°C for 15 h as described [7]. Dye content in cells was estimated spectrophotometrically [7]. Protein content was assayed according to the method of Lowry et al. [12]. The amount of dye labeled was less than $1 \cdot 10^{-8}$ mol/mg protein. Cells were incubated at 37°C for 30 min with or without valinomycin ($10^{-5}$ M) before fluorescence measurements. For binding studies using ANS, control or valinomycin-treated cells were incubated with ANS (final concentration, 7.9 $\mu$M unless otherwise stated) in phosphate-buffered saline, pH 7.2 for 60 min, and then centrifuged. ANS remaining in supernatant was estimated by its fluorescence in the presence of saturated amount of bovine serum albumin [13]. Cell volume ($1.2 \cdot 10^{-8}$ ml/cell) was corrected in this calculation. Binding constant and number of sites were obtained according to the method of Scatchard [14].

*Fluorescence measurements.* Fluorescence measurements were carried out with a Shimadzu RF502 fluorescence spectrophotometer equipped with a constant-temperature (37°C) cell holder connected to a temperature-controlled water bath. Labeled or non-labeled cells were suspended in phosphate-buffered saline containing 0.54% glucose, pH 7.2, or in modified salines in concentrations where absorbance of the suspensions did not exceed 0.1 so as to minimize the effect of light scattering. Cell suspensions were gently mixed before measurements. Care was taken not to damage the cells by minimizing the period of application of excitation beam. Unless otherwise indicated, fluorescence of 12-PS, perylene and ANS was excited at 346, 411 and 380 nm and measured at 398, 473 and 480 nm, respectively. Slit width was 4 or 5 nm for both excitation and emission wavelengths. Anisotropy measurements were made using Polacoat ultraviolet polarizing filters. Time-averaged fluorescence anisotropy, $\bar{r}$, is defined as;

$$\bar{r} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed through a polarizer oriented parallel and perpendicular, respectively, to the plane of polarization of

the excitation beam. Excitation energy transfer from aromatic amino acids to ANS was evaluated by measuring the relative value of fluorescence intensities at 470 nm, being excited at 290 nm (protein) and at 380 nm (ANS) [7,15].

*Measurements of electrophoretic mobility.* Cells were scraped off from the glass surface by pipetting with or without preincubation in $5 \cdot 10^{-4}$ M EDTA in phosphate-buffered saline, pH 7.2, and suspended in saline in the absence or presence of valinomycin ($10^{-6}$ M). Mobility was measured by a Zeiss cytopherometer equipped with a temperature-controlled bath (37°C). Mobility, $u$, ($cm^2 \cdot s^{-1} \cdot V^{-1}$) is expressed as;

$$u = \frac{v}{E}$$

where $v$ denotes the rate (cm/s) of the cells at the electric strength $E$ ($V \cdot cm^{-1}$). Only cells in the stationary layer of the electrophoresis chamber were evaluated for their mobility at electrical field strength of below 10 $V \cdot cm^{-1}$.

## Results

The addition of valinomycin ($10^{-6}$ M) to the assay medium resulted in enhancement of neurite formation of mouse neuroblastoma cells compared with the control culture (Fig. 1). The enhancement was further studied with varying concentrations of valinomycin. Fig. 2 shows there was a maximum enhancement of the formation of neurites at a valinomycin concentration of

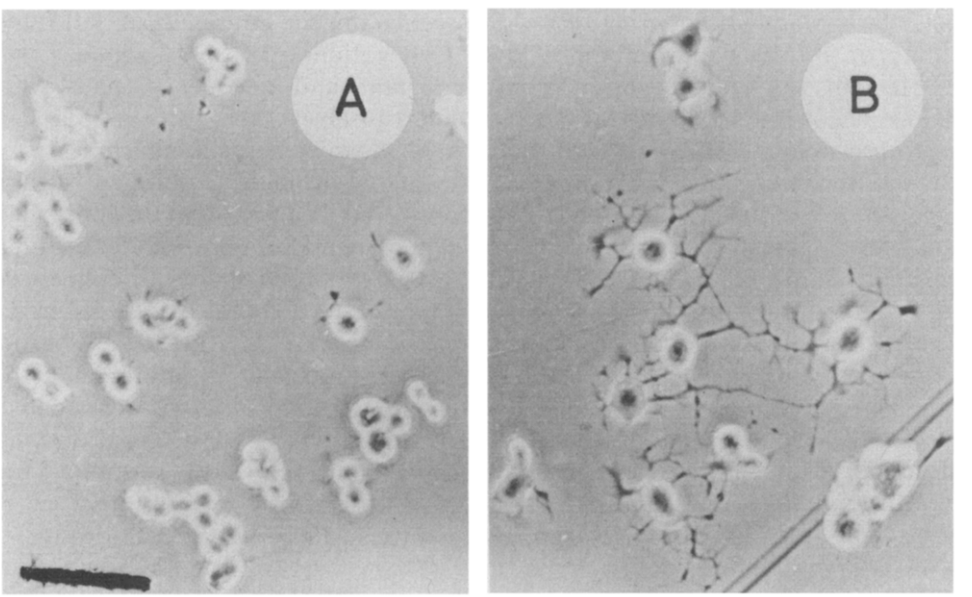


Fig. 1. Photographs of control and valinomycin-treated cells. Cells were incubated for 2 h at 37°C in the assay medium, Dulbecco's modified Eagle's minimum essential medium supplemented with 1% dialyzed fetal calf serum, in the absence (A) or presence (B) of valinomycin ($10^{-6}$ M). Differences in activity of valinomycin were observed between bathes [6], so that the same lot number described in the text was used throughout the work. The bar is 100 $\mu$m.

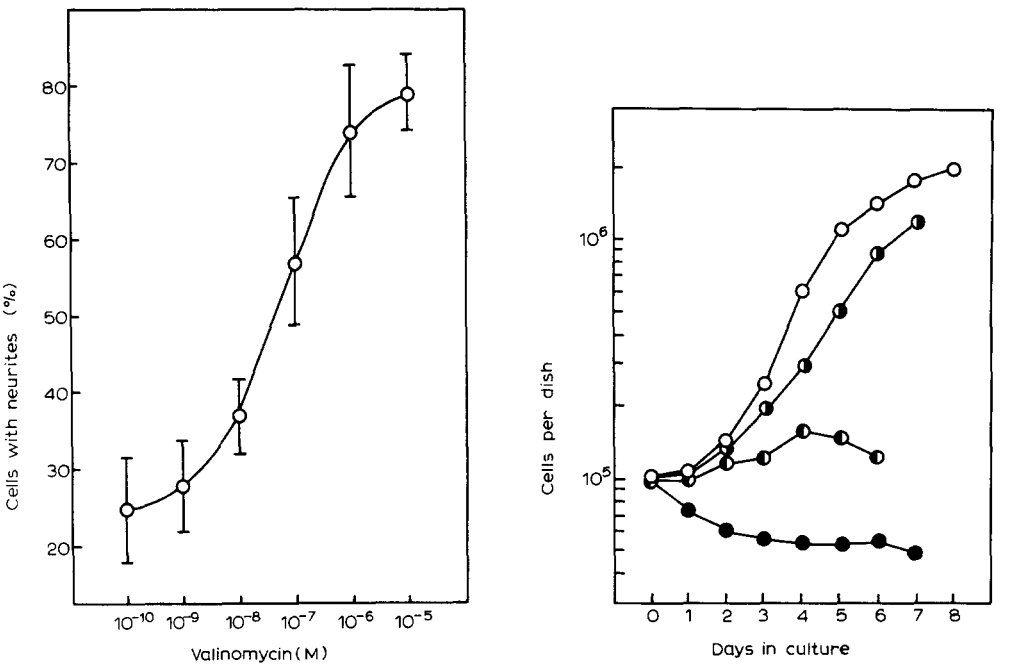


Fig. 2. Effect of valinomycin concentration of the neurite formation of neuroblastoma cells. Assay method is described in the text. 4—5 independent experiments, each consisting of the measurements of 200—800 cells for the presence or absence of neurites, were performed and the limits of standard deviation are indicated. In control cultures, thirteen percent of the cells (±5%) were found to have the neurites under the assay conditions.

Fig. 3. Effect of valinomycin on the growth of neuroblastoma cells. Cells were cultured in glass plates (60 mm) in the medium supplemented with 10% fetal calf serum in the absence (○) or in the presence of $10^{-10}$ M (◑), $10^{-9}$ M (◐) and $10^{-8}$ M (●) valinomycin. Duplicates of culture were performed and viable cells were counted with a hemocytometer.

$10^{-5}$ M in our conditions. At concentrations of $10^{-9}$ and $10^{-8}$ M, the formation was partially stimulated with less than or almost equal to 30% of the effect observed at $10^{-5}$ M valinomycin. Between $10^{-8}$ and $10^{-6}$ M there was a large increase in the enhancement. A slight effect was observed at even $10^{-10}$ M valinomycin compared with the control culture where 13 ± 5% of the cells had the neurites. Viability of the cells exposed to assay medium containing $10^{-5}$ M valinomycin for more than 2 h did not decrease appreciably within our experimental limitations (±15%). Valinomycin was also known to inhibit growth of bacteria [16], lymphocytes [17] as well as protein synthesis of reticulocytes [18]. Growth of neuroblastoma cells in the presence of valinomycin was examined and reproduced in Fig. 3. The proliferation was slightly inhibited at a concentration of $10^{-10}$ M, while valinomycin at $10^{-8}$ M totally blocked the growth; some cells were detached from the glass surface.

Evidence suggesting that valinomycin may exert its effect at the cell membranes was provided by increasing the potassium concentration of the external medium. Assay was made in Eagle's minimum essential medium containing dialyzed fetal calf serum and also varying concentrations of KCl, pH 7.9. Fig. 4 shows that the enhancement of neurite formation induced by

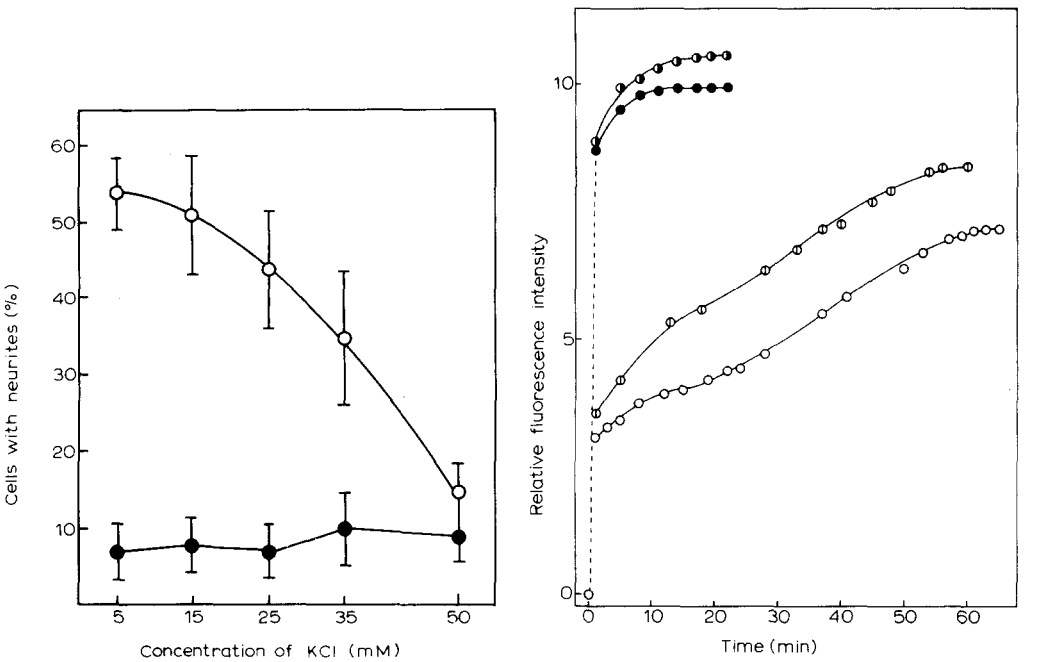


Fig. 4. Effect of potassium concentration on the neurite formation of neuroblastoma cells. Assay was done using Eagle's minimum essential medium, pH 7.9, where the potassium concentration was changed. The results obtained in the absence (●) or presence (○) of $10^{-6}$ M valinomycin are included. The limits of standard deviation of 3 independent experiments are also described.

Fig. 5. Kinetics of the fluorescence enhancement of ANS interacted with neuroblastoma cells. Fluorescence increase was observed, as described in the text, with cells ($4.9 \cdot 10^5$/ml) in normal saline in the absence (○) or presence (●) of $5 \cdot 10^{-6}$ M valinomycin and also in high-potassium-containing saline in the absence (Φ) or presence (◉) of $5 \cdot 10^{-6}$ M valinomycin at 37°C.

valinomycin was gradually diminished upon increasing the concentrations of $K^+$ from 5 mM to 50 mM; the effect was totally blocked to the level of the control cultures at 50 mM KCl where the resting potential was expected to depolarize. Increase of $K^+$ concentration itself did not enhance the neurite formation.

In order to check the specificity of the effect of valinomycin, we examined the effect of agents which are also known to act at cell membranes by changing the permeability or ion fluxes. These depolarizing agents, including gramicidin [19], veratridine [19,20] and ouabain [21], whose concentration range examined was 0.5—100 $\mu$M, showed no enhancing effect on neurite formation suggesting the role of hyperpolarization of membrane potentials in the enhancement of neurite formation.

In an attempt to confirm the action of valinomycin at the membranes, membrane changes induced by this ionophore were searched for using fluorescence technique. On addition of ANS to a suspension of neuroblastoma cells a rapid rise in fluorescence was observed followed by gradual time-dependent increase (Fig. 5). The fast phase was too fast to follow with our apparatus. The half time of the first phase was reported to be less than 5 ms [13]. The slow binding phase, consisting of more than one kinetically distinguishable site, was observed in salines containing both normal (4.2 mM)

TABLE I

CHARACTERISTICS FOR THE INTERACTION OF ANS WITH CONTROL AND VALINOMYCIN-TREATED NEUROBLASTOMA CELLS

Each value except that of $K_{diss.}$ represents the mean ± S.D. of three or four experiments. n.d., not determined.

| Cells | Concentration of $K^+$ in saline, pH 7.2 (mM) | Enhancement of fluorescence intensity * | Proportion of fast phase ** (%) | Anisotropy *** ($\bar{r}$) | $I_{pro.}/I_{dye}$ | Bound ANS ($\mu g/10^6$ cells) | $K_{diss.}$ (mM) |
|---|---|---|---|---|---|---|---|
| Control cells | 4.2 | 14.3 ± 0.7 | 43 ± 4 | 0.149 ± 0.008 | 1.67 ± 0.02 | 2.5 ± 0.3 | 0.083 |
| Valinomycin-treated cells | 4.2 | 19.9 ± 1.0 | 87 ± 9 | 0.148 ± 0.008 | 1.53 ± 0.02 | 3.8 ± 0.2 | 0.119 |
| Control cells | 50 | 16.8 ± 0.8 | 40 ± 4 | 0.152 ± 0.009 | 1.70 ± 0.02 | 3.7 ± 0.7 | n.d. |
| Valinomycin-treated cells | 50 | 21.4 ± 1.1 | 83 ± 8 | 0.145 ± 0.008 | 1.53 ± 0.03 | 4.7 ± 0.6 | n.d. |

* Fluoresence intensity of ANS (final concentration 7.9 $\mu$M) added to the cell suspensions ($4.9 \cdot 10^5$ cells/ml) in respective salines, was measured after equilibration and compared with that of the solution of ANS without cells.

** Kinetics of interaction of ANS with the cells consisted of fast and slow phases which correspond to surface binding and migration into the membranes, respectively. Fluorescence enhancement due to the fast phase is compared with the total fluorescence intensity.

*** Cells ($4.9 \cdot 10^5$ cells/ml) were treated with valinomycin ($10^{-6}$) as described in Materials and Methods. Ethanol (0.1%) did not change the molecular anisotropy measured.

and high (50 mM) concentrations of $K^+$. Contrary to this, the slow binding phase was diminished in cells treated with valinomycin ($5 \cdot 10^{-6}$ M). The first phase is thought to correspond to surface binding of ANS with membranes in which surface charge distribution plays a role, while the slow binding phase involves migration of the probe into the membranes and possibly its transport into the cells [22]. Therefore, our observation suggests that valinomycin modified the slow binding sites in neuroblastoma cells which may be related to penetration processes of ANS into cell membranes. The direct interaction of ANS with valinomycin was ruled out, since the fluorescence intensity finally reached the same plateau regardless the sequence of addition of ANS and valinomycin to the cell suspensions. Treatment with valinomycin did not change the viability of the cells at 37°C for at least 3 h in these conditions. Thus, the change in kinetics of fluorescence increase observed in this study was not due to possible deleterious effect of valinomycin on the cells in these assay conditions.

In view of the increase in the proportion of the fast binding phase in cells treated with valinomycin, we measured electrophoretic mobility of the cells in the absence or presence of this ionophore. The mobility of control cells and valinomycin-treated cells was 0.87 ± 0.11 (S.D.) and 0.88 ± 0.15 (S.D.) $cm^2 \cdot s^{-1} \cdot V^{-1}$, respectively, where about 20 cells were subjected to the measurements in both cases, indicating no change of surface charges under our conditions.

The results obtained with ANS are reproduced in Table I. Valinomycin treatment brought about fluorescence enhancement in salines containing both normal and high concentrations of $K^+$. Binding parameters in which a range of probe concentration was changed and the bound dye was evaluated by means of indirect method [13], revealed that this enhancement stemmed rather from the increase in the number of dye molecules bound, than from the increase in quantum yield of the bound dye. No shift of fluorescence emission maximum upon treating the cells with valinomycin also supports this view. Fluorescence anisotropy ($\bar{r}$) of ANS in the cells in normal saline was 0.149, suggesting considerable rotational flexibility of ANS in membranes, comparable to the values reported [13,23], smaller than that of electroplax [24], and it did not change upon the treatment. Some information about the environment of the probe could be obtained from the energy transfer measurements between membrane components and the probe. Relative values of $I_{pro.}/I_{dye}$ were evaluated and compared with that of ANS in 50% (v/v) ethanol (Table I). Energy transfer was decreased upon the treatment of valinomycin in both normal and high concentrations of $K^+$.

Molecular anisotropy of lipophilic fluorescent probes [7] was measured to see whether the membrane fluidity was changed by adding valinomycin to the medium. Molecular anisotropy ($\bar{r}$) of the cells labeled with 12-PS was 0.008 ± 0.002 (S.D.) and did not change upon the treatment with valinomycin ($10^{-5}$ M). No change of rotational flexibility was observed using perylene as a fluorescent probe even in saline containing high concentration of $K^+$.

## Discussion

Valinomycin is a cyclic oligopeptide which forms lipid-soluble complexes with monovalent cations [25,26]. This ionophore is highly specific for

potassium and forms an insulating cage around potassium leading to the transport across the lipid phase of the membranes. Valinomycin has been demonstrated to exert its effect at two sites at least in intact cells: by effect on intracellular membranes including mitochondria and even the nucleus, or by its action on the electrical properties of the cell membranes.

It has been confirmed that the treatment of valinomycin in normal saline results in hyperpolarization of the resting membrane potential of neuroblastoma cells (ref. 6 and Miyake, M., unpublished). Application of the constant field equation [27,28] predicts that such changes in the resting potential could be reversed by increasing the concentration of $K^+$ in the external medium. The validity of this prediction was brought about experimentally in skeletal muscles [29] and erythrocytes [30]. Our observation that a high concentration of $K^+$ (50 mM) completely abolished the effect of valinomycin on neurite formation suggests the action of valinomycin occurred at the cell membranes perhaps by changing electrical properties. It is also noted that high concentrations of $K^+$ themselves did not enhance the neurite formation in spite of the fact that the membranes behave like a potassium electrode in these regions (ref. 31 and Miyake, M., unpublished). No enhancement of neurite formation was observed by treating the cells with depolarizing agents including veratridine, ouabain and gramicidin, supporting the view that hyperpolarization was strongly associated with the neurite formation in these conditions.

Spector et al. [6] reported no changes as to the intracellular concentration of $K^+$ upon the treatment of $10^{-8}$ M valinomycin for one hour, denying the possibility that the ionophore may act by exchanging intracellular $K^+$ for extracellular $Na^+$ or $H^+$. In addition, electrogenic pump $(Na^+ + K^+)$-ATPase did not contribute significantly to the resting membrane potential in neuroblastoma cells (Miyake, M., unpublished). These observations also support our view on the action of valinomycin. However, without a more precise knowledge of the relative concentrations and permeabilities of $Na^+$, and $K^+$ as well as $Cl^-$ in neuroblastoma cells, our results offer rather indirect evidence concerning the role of membrane potential and ionic fluxes in the process of neurite formation.

Cone [5,32] offered a hypothesis that for some somatic cells intracellular cation levels in association with the generation of electrical membrane potential may be involved in the control of mitogenesis and cell proliferation. Initiation of cell division was accompanied by depolarization of cell membrane by increasing extracellular potassium concentration in fibroblasts [23]. From these considerations, one attractive hypothesis is that the trigger for the morphological differentiation of neuroblastoma cells involves changes in membrane potential and ion conductance across the membranes.

As to the mode of action, Spector et al. [6] assumed it was possible that a structural rearrangement of the membranes took place because of the closing of passively conducting ionic pathways as well as the activation of membrane elements involved in the fast inward movement of $Na^+$. It is also suggested that the functional properties of the ionophore may be strongly concerned with the membrane organization such as surface charges, dipoles and fluidity [34]. In view of complex processes resulting in the formation of neurites, membrane changes triggered by valinomycin were searched for using fluorescent probes.

Our observation indicated valinomycin modified the environment of some of the 'slow' ANS-binding sites so that the affinity for the charged probes increased. ANS is known to be a probe for an apolar-polar interface, neutral or cationic in nature, which could be due to phospholipid aggregates or to hydrophobic regions in membranes [13,35—39]. The lack of a shift in ANS emission spectrum and the constant anisotropy values in conjunction with the unchanged fluidity of 12-PS and perylene in membranes make it unlikely that the lipid molecules are more densely packed by the action of valinomycin. Bessette and Seufert [40] indicated that the ionophore provoked a perturbation in the palisade arrangement of lipid molecules in the bilayer. It is also suggested that the change of slow binding sites involved alternations of structural water in the apolar-polar interphase [13]. Since ANS interacts with various sites in the membranes, it is hard for us to identify unequivocally the kind of structural changes induced by valinomycin.

It has been demonstrated that the formation of neurites of mouse neuroblastoma cells is associated with changes in the composition of membrane proteins [41,42], glycopeptides [43,44], cell-surface antigens [45,46] and membrane microviscosity [7]. It has also been suggested that microtubules and microfilaments are necessary for both the elongation and maintenance of the neurites [11,47,48,49]. Olmsted et al. [50] isolated microtubule protein from cultured mouse neuroblastoma cells. Morgan and Seeds [51] showed that there was no change in the concentration of turbulin subunits in both differentiated and undifferentiated neuroblastoma cells, suggesting the regulation of tubulin assembly associated with the neurite formation. These is an indication of the presence of membrane-bound tubulin in rat brain [52]. It is also shown that contractile proteins possibly concerned with cell motility were localized just beneath the plasma membranes of neurites and cell body of neuroblastoma cells [53]. These observations are in line with the view that membranes are the sites at which the assembly of tubulin and neurite formation may be regulated. There is a suggestion that the slow binding sites of ANS with the membranes may be related to the changes of structural water bound to microtubules [22]. However, it remains unclear whether or not the structural changes induced by valinomycin are concerned with the mechanism involved in the neurite formation of mouse neuroblastoma cells. It is also noted that fluorescence changes observed here rather represent common features of those induced by valinomycin, since these changes were more or less detected in both normal and high concentrations of KCl. Further investigations are necessary so as to delineate the changes of surface membranes responsible for triggering the formation of neurites of mouse neuroblastoma cells.

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