Proc. Nat. Acad. Sci. USA
Vol. 73, No. 2, pp. 462–466, February 1976
Biochemistry

Maturation of neuroblastoma cells in the presence of dimethylsulfoxide

(electrical differentiation/neurospecific enzymes/neurite outgrowth/cyclic 3′:5′-AMP)

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Communicated by David Nachmansohn, December 5, 1975

ABSTRACT **Addition of dimethylsulfoxide at concentrations of 1% and 2% (vol/vol) to cells of mouse neuroblastoma clone NIE-115 in the confluent phase of growth resulted in the production of morphologically differentiated cultures with extensive process formation. Cells maintained in 2% dimethylsulfoxide remained in a stable nondividing condition for periods of up to 4 weeks. A high degree of electrical excitability was found in these cells, but there was no clear correlation of this property with the level of induction of either acetylcholinesterase (acetylcholine hydrolase; EC 3.1.1.7) or tyrosine hydroxylase [L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2]. In addition, intracellular levels of cyclic 3′:5′-AMP were not elevated in fully morphologically and electrically differentiated cells. While cell division was markedly inhibited by 2% or higher concentrations of dimethylsulfoxide, at 1% growth continued at a somewhat slowed rate and such cultures exhibited enhanced process formation and electrical activity for a relatively short period. High concentrations (3% or 4%) of dimethylsulfoxide totally suppressed process formation and did not result in increased excitability, but cells maintained high resting potentials. The results suggest that the development of the excitable membrane in neuroblastoma cells may be expressed independently of neurospecific enzyme induction, and does not require a sustained elevation of cyclic 3′:5′-AMP levels.**

Nerve cells are characterized by their unique morphological appearance, the possession of an excitable membrane, and a specialized biochemical machinery. In order to study the expression of specific neuronal properties during the maturation process it is necessary to obtain sufficient quantities of a relatively homogeneous differentiating population. Cloned cell lines isolated from the C-1300 mouse neuroblastoma may serve as a useful system for exploring certain aspects of nerve cell differentiation. The transition of a culture of neuroblastoma cells from the actively dividing state to the confluent one is characterized by the synthesis of various enzymes involved in neurotransmitter metabolism (1), an enhancement of electrical excitability (2, 22), and some degree of process formation. To achieve a further expression of these properties, cells have been treated with various agents such as aminopterin (3, 4) or dibutyryl cAMP (5–7).

In the present study, we show that in the presence of dimethylsulfoxide (Me_2SO) neuroblastoma cells will extend neurites and develop a highly excitable membrane. It appears that this method offers certain advantages over those most commonly used, especially in that cells appear to reach a higher level of electrical differentiation and can be maintained in this state for extended periods.

MATERIALS AND METHODS

Mouse neuroblastoma clones NIE-115, N-18, NS-20, and NIA-103 as well as the hybrid cell lines NG108-5 and NG-108-15 formed by Sendai virus-induced fusion of mouse neuroblastoma clone N18TG-2 and rat glioma clone C6BU-1, were all obtained from Dr. M. Nirenberg, N.I.H. The human neuroblastoma cell line IMR-32 was obtained from the American Tissue Culture Collection (8).

Culture Conditions. Cells were grown at 37° in Dulbecco's modified Eagle's medium (DMEM) containing 0.12% $NaHCO_3$ and supplemented with 10% fetal calf serum (Gibco). The cultures were maintained under a humidified atmosphere containing 5% CO_2. After the cultures were maintained for 12–18 days in the confluent phase, cells were trypsinized and seeded at a density of 5×10^5 per 60 mm dish in 3 ml of growth medium containing various concentrations of Me_2SO.

In experiments where logarithmically growing cells were used, cultures were trypsinized before attaining confluency and replated in 60 mm dishes at a density of 1×10^5 cells per dish. Two days later, the medium was replaced with Me_2SO containing medium. Cell counts were performed using a hemacytometer, and growth curves were constructed without regard to floating cells.

Electrophysiological Measurements. Electrophysiological measurements were performed using standard intracellular microelectrode recording techniques, as described (4). To obtain comparable results, only large cells of diameter of more than 40 μm were studied.

Biochemical Measurements. Acetylcholinesterase (AChE) (acetylcholine hydrolase; EC 3.1.1.7) activity was determined using sonicated cell suspensions as described by Blume *et al.* (9). One unit of activity corresponds to 1 nmol of acetylcholine hydrolyzed/mg of protein per min. Tyrosine hydroxylase (TH) [L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] activity was assayed by the method of Nagatsu *et al.* (10) as described by Richelson (11), with the following modifications: the final concentration of tyrosine was 0.1 mM, the final concentration of the cofactor (2 amino-5,7-dimethyltetrahydropteridine, Aldrich Chem. Co.) was 1 mM, and the dihydropteridine reductase-NADPH regenerating system was replaced by 40 mM 2-mercaptoethanol. Reactions were run in duplicate at 37° for 10 min. One unit of activity corresponds to 1 pmol of ^3H_2O released/mg of protein per min. Some discrepancy in the specific activity of this enzyme in the NIE-115 clone as measured by two different groups has been previously noted (11, 12); our results agree more closely with the lower values reported by the second group.

Similar results were obtained for both enzymes whether cells were removed from the plate with trypsin or by scraping. In both cases cell pellets were stored in liquid air prior to assay.

Cyclic 3′:5′-AMP (cAMP) content was determined using the protein binding assay of Gilman (13), in a final reaction volume of 0.1 ml. The rabbit skeletal muscle protein kinase

Abbreviations: Me_2SO, dimethylsulfoxide; cAMP, cyclic 3′:5′-AMP; RP, resting potential; max. dV/dt, maximum rate of increase of action potential; TH, tyrosine hydroxylase; AChE, acetylcholinesterase.

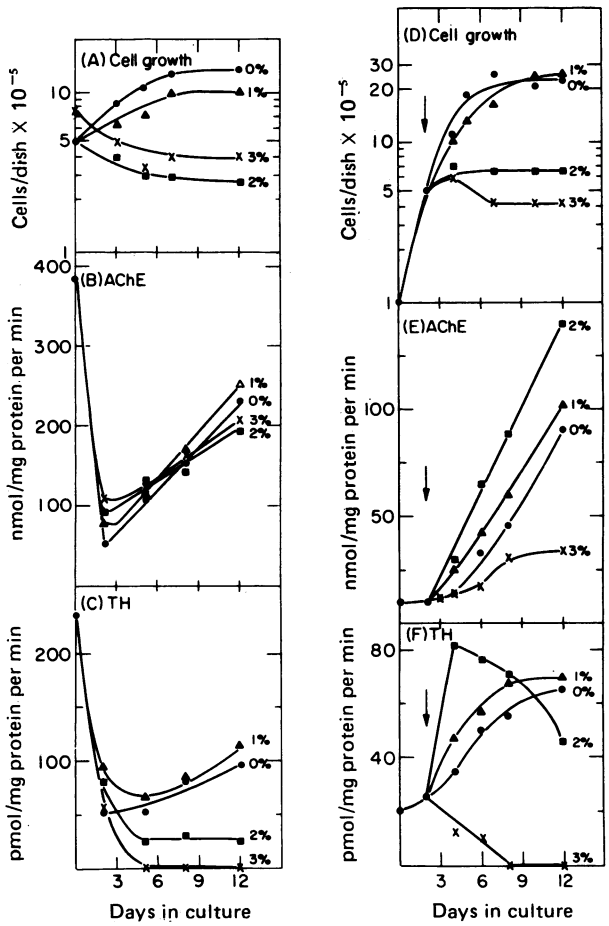

FIG. 1. Responses of replated confluent (A–C) and exponentially growing (D–F) cells of clone NIE-115 to Me_2SO (1%, 2%, and 3%). (A) and (D) growth curves, (B) and (E) AChE specific activity (note different scales on ordinate), (C) and (F) TH specific activity. In (D–F) the arrow represents the time of Me_2SO addition to the cultures; in (A–C) Me_2SO was added at zero time.

preparation used had a pH optimum for cAMP binding of 4.5, and 50 mM Na acetate buffer was used at this pH to prepare the reactants. The standard curve was linear from 2 to 20 pmol of cAMP. In some experiments extracts were partially purified through columns of Dowex-AG1X8 (formate form). After application of the neutralized extracts, the column was washed with 10 ml of water, and cAMP was eluted with 6 ml of 1 M formic acid. Similar results were obtained using either crude or partially purified extracts.

RESULTS

Growth and Morphology. Most of the following experiments were carried out using the adrenergic neuroblastoma clone NIE-115. In normal growth medium, confluent cultures of this clone comprised a heterogeneous cell population. The majority of the cells were round, some had poorly developed neurites, and only a small percentage of the population were larger in size (>40 μm), with a well differentiated morphology. When such a culture was replated into Me_2SO-free medium, the cells showed a slower rate of multiplication (4–6 days doubling time) as compared to exponentially growing cells (about 24 hr doubling time) and reached a lower plateau density. Replated confluent cultures also showed a transient increase (at 2–4 days) in the proportion of cells bearing neurites.

Addition of 1 or 2% (vol/vol) Me_2SO to replated confluent cultures induced morphological differentiation, with about 75% of the cells extending long neurites. As shown in Fig. 1A, 1% Me_2SO caused only a partial inhibition of cell multiplication, with some cells remaining in a dividing state throughout the treatment. Neurite extension under these conditions took place after a lag of 1–2 days and reached a maximum on day 3–6 when about 75% of the cells had long thick processes (Fig. 2B). Subsequently, a decline in the proportion of differentiated cells occurred due to proliferation of the dividing cells.

In contrast, in medium containing 2% Me_2SO, cell proliferation was completely inhibited (Fig. 1A) and a longer delay in the appearance of neurites was noted. The lag period was extended to 2–4 days and long processes were observed by day 4–8 (Fig. 2C). These morphologically differentiated cultures, unlike the cells treated with 1% Me_2SO, remained in a stable condition for periods of over 4 weeks. In addition, these cultures appeared to produce much less acid, and required less frequent changes of medium than 0% or 1% Me_2SO cells. The longer the period in which the cells were maintained in the confluent state prior to replating in Me_2SO, the higher was the percentage of differentiated cells and the shorter the lag in process formation. Under these conditions about 25% of the cells remained round as aggregates, while others were embedded in the neurite network. This prevented a more quantitative determination of their relative amount in the population.

Cell division was rapidly arrested in the presence of either 3% or 4% Me_2SO. Under these conditions no neurites were extended, and cultures consisted of a homogeneous population of round cells (Fig. 2D). The initial period after addition of 2, 3, or 4% Me_2SO to the medium was characterized by a larger fraction of cell detachment than that seen in the controls; this accounting for the drop in cell number seen at the outset of the growth curve at these concentrations (Fig. 1A).

Me_2SO was also able to induce morphological differentiation when added to logarithmically growing cells. The normal generation time of 24 hr was slightly increased in medium containing 1% Me_2SO, but cells ultimately reached a similar saturation density (i.e., approximately 2.6×10^6 cells per dish). Addition of 2–4% Me_2SO rapidly arrested growth, and the number of cells remained constant over a period of several days (Fig. 1D). Neurite outgrowth was enhanced in 1% and 2% Me_2SO cultures, but a longer delay than that seen in replated confluent cultures was observed, maximal process formation occurring in 2% Me_2SO after 8 days of exposure to the agent.

In general, logarithmic and confluent cells treated with either 1% or 2% Me_2SO were larger and more refractile than in the control cultures. There was a tendency towards the formation of thread-like structures in which the cells were apposed in a linear array. Moreover, the processes were much thicker and with larger growth cones than those observed in aminopterin-treated or serum-removed cultures.

When replated confluent cells maintained in 2% Me_2SO for 2–3 weeks were transferred to a Me_2SO-free medium, growth was resumed after a delay of 1–2 weeks. In addition, Me_2SO treated cultures still exhibited malignancy when injected into A/J mice, although a delay in the appearance of the tumor was noted (Y. Danon, unpublished observations). It remains to be determined whether the resumption of growth and ability to induce tumors arose from differentiated cells or from a small yet undifferentiated subpopulation.

Electrophysiological Properties. Resting potential (RP) and maximum rate of rise of action potential (max. dV/dt)

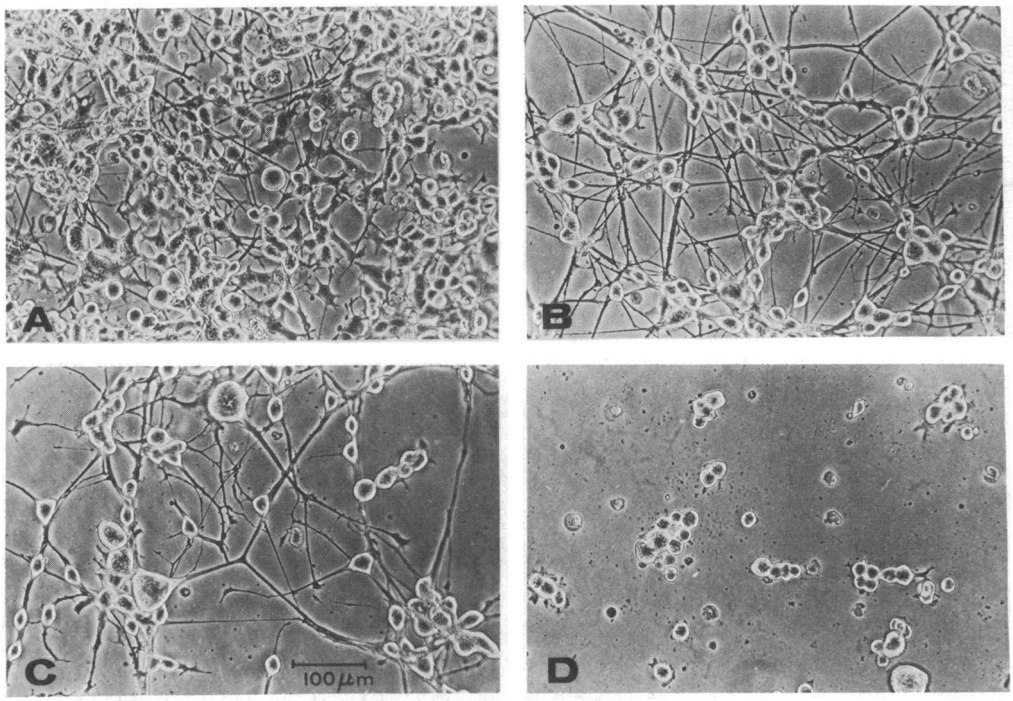

FIG. 2. Morphological appearance of confluent NIE-115 cells replated into various concentrations of Me_2SO. (A) Control; (B) 1% Me_2SO; (C) 2% Me_2SO; (D) 4% Me_2SO. Phase contrast photomicrographs taken 3 days after subculture. Bar represents 100 μm.

were determined as indicators of passive and active membrane properties, respectively. Changes in electrical properties at various times after Me_2SO treatment of replated cells of clone NIE-115 are summarized in Table 1. A further account of the electrophysiological parameters of these cells will be presented in detail elsewhere (I. Spector, in preparation). In Me_2SO-free medium, the average RP was relatively high on the second day after plating (−37.8 mV) and did not change significantly by day 8. In cells exposed to 1, 2, or 4% Me_2SO, RP values were significantly lower than controls at day 2. Thereafter, treated cells exhibited a rise in RP which reached a maximum for all Me_2SO concentrations after 7–8 days. This rise was most marked in the case of 4% Me_2SO cells. These cells had an average value of −66 mV 8 days after replating despite the maintenance of a round morphological appearance throughout this period. Whereas in control and 1% cultures, RP values dropped in parallel with the rise in the proportion of morphologically undifferentiated cells, in 2% Me_2SO cells, which maintained a stable differentiated state from day 3 onwards, RP values remained essentially constant.

A marked enhancement of electrical excitability occurred after treatment with 1% or 2% Me_2SO as indicated by changes in the max. dV/dt values (Table 1). As compared with the highest average control values of 74.3 V/s obtained on day 2, the average max. dV/dt for 1% Me_2SO cells, rose to 103.6 V/s by day 8. In 2% cultures average max. dV/dt values continued to increase, reaching 132.5 V/s after 18 days. Thus, it appears that Me_2SO-treated NIE-115 cells can attain a high degree of electrical differentiation, a fact that is also reflected in the ability of most 1% and 2% treated cells to fire repetitively in response to prolonged depolarizing stimuli (Fig. 3). On the other hand, electrical excitability was poorly developed in 4% Me_2SO cells in spite of the high RP values seen in such cells.

Biochemical Measurements. Both AChE and TH activities have been shown to respond to changes in growth conditions in different neuroblastoma clones (9, 11). Fig. 1B shows that AChE specific activity decreased on trypsinization of confluent cells and subsequently increased at almost identical rates in control, 1%, 2%, and 3% Me_2SO cultures. The TH activity also fell on replating, and recovered slowly in control cells and in those exposed to 1% Me_2SO. However, TH activity was not regained in 2% treated cells (Fig. 1C).

Table 1. Electrical parameters of confluent NIE-115 cells at various times after subculture into Me_2SO-containing medium

Treatment	Measurement	Days in culture 2	8	18
Control	R.P.	37.8 ± 1.8	39.3 ± 1.9	30.4 ± 6.0
	dV/dt	74.3 ± 9.7	49.7 ± 9.3	15.3 ± 2.7
1% Me_2SO	R.P.	28.5 ± 2.5	45.6 ± 2.3	37.7 ± 1.6
	dV/dt	71.6 ± 10.0	103.6 ± 10.0	81.7 ± 3.1
2% Me_2SO	R.P.	30.0 ± 2.3	45.1 ± 2.9	41.0 ± 4.9
	dV/dt	65.0 ± 8.2	78.4 ± 11.0	132.5 ± 13.1
4% Me_2SO	R.P.	24.2 ± 6.7	66.0 ± 2.3	—
	dV/dt	32.7 ± 9.6	23.3 ± 3.3	—

RP = resting potential; dV/dt = maximum rate of rise of action potential evoked from a standard resting potential of −90 mV. Values are ±SEM.

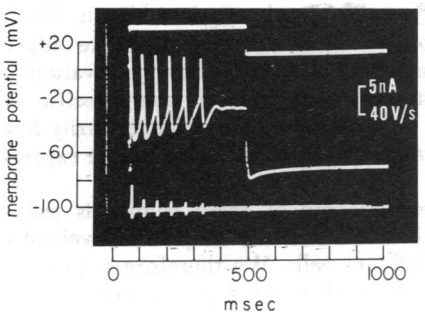

FIG. 3. Repetitive action potentials evoked in a cell of clone NIE-115 grown in the presence of 2% Me_2SO for 8 days. Upper trace represents applied depolarizing current; middle trace: transmembrane potential response; lower trace: time derivative of transmembrane potential. The resting potential was adjusted to −70 mV before stimulation.

The enzyme level remained significantly lower than that found for the control cultures even at day 12, when considerable morphological and electrical differentiation was evident. In cultures treated with 3% or 4% Me_2SO, TH activity dropped to undetectable levels after 5 days. Whether these changes are a result of decreased synthesis of active enzyme or an increased rate of degradation is not known.

Somewhat different effects of Me_2SO on the two enzymes were noted in exponentially growing cells (Fig. 1E). Untreated cultures exhibited a constant basal level of AChE activity (10 units) until growth rate started to decrease, when a virtually linear increase in enzyme activity ensued. These results are comparable to those previously obtained with clone N18 (9). Addition of 1% or 2% Me_2SO in mid-logarithmic phase led to an increase in activity over the control, with the higher concentration showing a more marked effect. These inductive effects closely paralleled the retardation or inhibition of growth. Nevertheless, Me_2SO at concentrations of 3% which inhibited growth to the same extent as 2%, did not lead to an increase in activity and AChE level was consistently lower than control values.

The pattern for TH resembled that for AChE in that activity in 1% and 2% Me_2SO showed early increases over untreated cells, while Me_2SO at 3% led to a rapid drop in activity comparable to that noted earlier for replated confluent cultures (Fig. 1F). However, the elevated level found after adding 2% Me_2SO gradually declined such that at day 10, when these cells had become morphologically and electrically differentiated, it was lower than that of control or 1% Me_2SO.

Treatment of neuroblastoma cells with dibutyryl cAMP or phosphodiesterase inhibitors has been reported to result in the appearance of differentiated functions in certain neuroblastoma clones including NIE-115 (5–7). Consequently, we checked endogenous cAMP levels in replated confluent cultures of NIE-115 cells that were induced to differentiate morphologically and electrically by Me_2SO. The results shown in Table 2 indicate that after Me_2SO treatment, cAMP concentrations were significantly lower than in the control cultures.

Effect of Me_2SO on Other Cell Lines. Me_2SO also induced morphological differentiation in a cholinergic clone NS-20 and in an inactive clone N-18. Both of these clones exhibit enhanced electrical activity in the presence of Me_2SO. Induction of differentiation in response to 2% Me_2SO occurred to a lesser extent in hybrid cell lines, NG108-5 and NG108-15, formed by Sendai virus induced fusion of mouse neuroblastoma and rat glioma clones (14).

Table 2. Cyclic AMP levels in replated confluent NIE-115 cells after various periods of exposure to Me_2SO

Me_2SO concentration %	Days in culture: 2	5	12
	pmol of cAMP/mg of protein		
0	11	15	16
1	7	14	9
2	10	12	7
3	9	9	10

On the other hand, the NIA-103 clone, which is unable to extend neurites when incubated in a medium without serum, did not respond to Me_2SO treatment.

We have also examined the effect of Me_2SO on a human cell line IMR-32 (8). Me_2SO at concentrations of 0.5–3% caused considerable cell detachment from the plate, but enhanced neurite outgrowth was not observed.

DISCUSSION

Treatment of neuroblastoma cells with Me_2SO provides a convenient way to obtain morphologically stable differentiated cultures exhibiting a high degree of electrical activity. Some characteristics of the Me_2SO-induced differentiation of neuroblastoma cells described in this report resemble those of erythroleukemic cells infected with Friend virus (15). When the latter cells are maintained in 2% Me_2SO, the number of morphologically and biochemically differentiated cells is markedly increased. The erythroleukemic cells remain viable, and after a short lag period continue to proliferate. Me_2SO-treated erythroleukemic cell cultures also retain their malignant potential, although tumor appearance is delayed.

As in the Friend virus infected cells, not all the neuroblastoma cells are equally affected by Me_2SO. In cultures grown in 1% Me_2SO, and to a lesser extent in those maintained in 2%, a significant proportion of the cells remain in a morphologically undifferentiated state, often in the form of aggregates of round cells. These cells are probably those than can commence division rapidly upon replacement with Me_2SO-free medium, and may be responsible for the development of tumors *in vivo*. However, cell growth in this clone is arrested by 2% Me_2SO and in this respect behaves similarly to another erythroleukemic line (TSFAT-3-Cl-1), whose growth is markedly inhibited by 2% Me_2SO (16), and to a fibroblast line L-929 (17).

Certain effects of Me_2SO on mouse neuroblastoma cells have been previously reported (18, 19). These workers have shown that under conditions of serum deprivation short-term Me_2SO treatment causes an inhibition of neurite outgrowth that was later reversed. A definite lag period before neurite development occurred was also observed in the present work when 2% Me_2SO was added to replated confluent NIE-115 cells. This was more noticeable with cells in the logarithmic state of growth, which remained round for several days after application of 2% Me_2SO. In general, one should distinguish between the initial phase of neurite outgrowth and the establishment of the fully morphologically differentiated state. In the former, small thin neurites of about 50 μm are extended within a few hours, while the latter is a more protracted phenomenon involving thicker neurites that may reach a length of 500 μm or more. Thus, long-term treatment with 2% Me_2SO resulted in a culture with

well developed neurites, exhibiting a high degree of electrical activity, which was stable for periods of several weeks.

The response to Me_2SO varied among the individual mouse neuroblastoma clones. Me_2SO was found to induce differentiation in adrenergic (NIE-115), cholinergic (NS-20), and inactive clones (N-18), and to a lesser extent in hybrid cell lines of mouse neuroblastoma and rat glioma (NG108-5, NG108-15). On the other hand, another clone (NB60) has been shown to divide after a lag period in the presence of 2% Me_2SO (18). In addition, clone NIA-103, which is unable to extend neurites in the absence of serum, did not respond to Me_2SO. The cause for this defect is not clear, but it is interesting to note that the surface glycopeptides of these cells are significantly different from those obtained from axon-forming cells (20). The only human neuroblastoma clone tested so far (IMR-32) did not respond to Me_2SO. More clones have to be studied in order to determine whether this deficiency is characteristic of all human neuroblastoma cells.

It has been established that the excitable membrane of neuroblastoma cells in culture is expressed to varying degrees during different phases of growth (21, 22). The results obtained with 1% or 2% Me_2SO treated cultures in this study show that these cells reach a high degree of membrane differentiation as manifested by the consistent appearance of repetitive firing evoked by prolonged depolarizing stimuli. This behavior resembles that seen in cultures of dissociated dorsal root ganglia neurons (23) and indicates that neuroblastoma cells retain the ability to mature to a state characteristic of normal nerve cells.

In general, an increase in resting potential is paralleled by the attainment of a more electrically excitable membrane and differentiated morphology (3, 21). The properties of cells treated with 4% Me_2SO do not correspond to a simple model in which electrical and morphological differentiation are coupled. These cells do not extend neurites, yet exhibit the highest resting potentials so far reported in neuroblastoma cultures. Their poor excitability suggests that the membrane components determining passive and active permeability properties may not necessarily be under coordinated control.

Our findings concerning the lack of correlation between the levels of TH activity and the degree of electrical excitability induced by Me_2SO treatment reopens the more general question of the interrelationship of the morphological, biochemical, and electrical modes of differentiation in these cells. Previous investigations have suggested that under certain conditions morphological differentiation need not be associated with electrical or biochemical differentiation. An example of the former case is that of serum removal, which although inducing the formation of extensive neurites in a number of different neuroblastoma clones (24, 25), results in only a minor enhancement in active electrical properties (3). A corresponding dissociation of neurospecific biochemical function from changes in morphology occurs when clone NIE-115 cells are treated with sodium butyrate (7, 26), which causes an induction of TH while cells retain a round morphology. In the present work, the antithesis of this situation occurs where replated confluent cells with extensive neurite formation (1% and 2% Me_2SO) do not exhibit elevated levels of TH. These cells, however, do possess a fully developed excitable membrane. In addition, there was no clear correlation between the degree of neurite outgrowth and the level of induction of AChE. These observations lead us to the conclusion that induction of neurospecific enzymes and electrical differentiation are not necessarily linked and that all three modes of differentiation can be expressed independently.

Another outcome of these studies is the finding that Me_2SO did not raise the steady state levels of cAMP in replated confluent cells. We therefore suggest that sustained elevation of cAMP is not a precondition for morphological or electrical differentiation.

We thank Dr. M. Nirenberg, NIH, for the cell lines. This work was supported in part by the United States–Israel Binational Foundation. The excellent technical help of Mrs. D. Saya is gratefully acknowledged.

1. Amano, T., Richelson, E. & Nirenberg, M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 258–263.
2. Nelson, P. G., Ruffner, B. W. & Nirenberg, M. W. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 1004–1010.
3. Peacock, J., Minna, J., Nelson, P. & Nirenberg, M. (1972) *Exp. Cell Res.* **73**, 367–377.
4. Spector, I., Kimhi, Y. & Nelson, P. G. (1973) *Nature New Biol.* **246**, 124–126.
5. Chalazonitis, A. & Greene, L. (1974) *Brain Res.* **72**, 340–345.
6. Furmanski, P., Silverman, D. L. & Lubin, M. (1971) *Nature* **233**, 413–415.
7. Richelson, E. (1973) *Nature New Biol.* **242**, 175–177.
8. Tumilowicsz, J., Nichols, W. W., Cholon, J. J. & Greene, A. E. (1970) *Cancer Res.* **30**, 2110–2118.
9. Blume, A., Gilbert, F., Wilson, S., Farber, J., Rosenberg, R. & Nirenberg, M. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 786–792.
10. Nagatsu, T., Levitt, M. & Udenfriend, S. (1964) *Anal. Biochem.* **9**, 122–126.
11. Richelson, E. (1973) *J. Neurochem.* **21**, 1139–1145.
12. Lloyd, T. & Breakefield, X. O. (1974) *Nature* **252**, 719–720.
13. Gilman, A. G. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 305–311.
14. Klee, W. A. & Nirenberg, M. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3474–3478.
15. Friend, C., Presler, H. D. & Scher, W. (1974) *Curr. Top. in Dev. Biol.* **8**, 81–101.
16. Fujinami, N., Suemoto, Y. & Hagiwara, A. (1973) *Dev. Growth Differ.* **15**, 141–151.
17. Berliner, D. L. & Ruhmann, A. G. (1967) *Ann. N.Y. Acad. Sci.* **141**, 159–164.
18. Furmanski, P. & Lubin, M. (1972) *J. Nat. Cancer Inst.* **48**, 1355–1361.
19. Furmanski, P. (1973) *Differentiation* **1**, 319–322.
20. Glick, M. C., Kimhi, Y. & Littauer, U. Z. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1682–1687.
21. Nelson, P. G., Peacock, J. H., Amano, T. & Minna, J. (1971) *J. Cell. Phys.* **77**, 337–352.
22. Spector, I., Palfrey, C. & Littauer, U. Z. (1975) *Nature* **252**, 121–124.
23. Peacock, J., Nelson, P. G. & Goldstone, M. W. (1973) *Dev. Biol.* **30**, 137–152.
24. Seeds, N. W., Gilman, A. G., Amano, T. & Nirenberg, M. W. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 160–167.
25. Schubert, D., Humphreys, S., De Vitry, F. & Jacob, F. (1971) *Dev. Biol.* **25**, 514–546.
26. Waymire, J., Weiner, N. & Prasad, K. N. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2241–2245.