ATTACHMENT AND CULTURE OF DISSOCIATED CELLS FROM RAT EMBRYO CEREBRAL HEMISPHERES ON POLYLYSINE-COATED SURFACE

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INTRODUCTION

Cultivation of neural tissue in vitro has been employed in the recent years for studying brain metabolism, isolated from influence by the whole organism. The complex intercellular relationship of the heterogeneous cell types present in the neural tissue have led to the use of several experimental approaches such as tissue explants (1–3) or dissociated single cells prepared by either enzymatic or mechanical means (4–6). The rationale behind the latter approach is based upon the ability of cells to reassociate and to form well organized aggregates which may acquire some of the characteristics of the original neural tissue (7–10). However, one of the major disadvantages of the dissociation technique is possible selection for cell types. This could be due either to the dissociation treatment itself or to the inability of certain cell types to attach to the surface when monolayer cultures are employed (11, 12). In a previous publication (13) we reported that reaggregation of cells from rat cerebral hemispheres occurs before the attachment process. A high percentage of cells which fail to aggregate and to adhere to the plastic surface will then be eliminated operationally at the first change of medium.

In this report we present a method for circumventing this selection problem by allowing the majority of cells to adhere. This is achieved by the use of a polylysine pretreated surface, and it is based upon previous observations on the electrostatic interaction of the polycation with the negative charges of the cell membrane (14).

MATERIALS AND METHODS

The procedure for preparation of dissociated cells has been previously described (13). In essence the cerebral hemispheres from 16 to 18-day old rat embryo were treated with a solution of 0.125% (wt/vol) trypsin for 5 min, and the resulting cell suspension was aspirated through a Pasteur pipette to complete the dissociation. The cells were centrifuged at 400 *g* for 5 min and the supernate was discarded. The pellet was then resuspended in the growth medium (13), and the suspension was centrifuged for 1 min at 200 g. The supernate containing the dissociated single cells was transferred to a sterile bottle, final cell concentration was adjusted to 0.1–0.3 $\times$ 10^6 cells per 1 ml medium, and 3 ml of the above suspension was placed into polylysine-coated Petri dishes. After 30 min incubation at 37°C in an atmosphere of 95% air, 5% CO_2, the medium containing the nonadhered cells was removed and replaced by fresh medium which was then changed every 3–4 days, depending on its acidity. Phase-contrast microscopy was employed for observation of cultures during their growth period.

Preparation of Polylysine-Coated Petri Dishes

Poly-L-lysine (mol wt 400,000, Sigma Chemical Co. St. Louis, Mo.) was employed unless otherwise mentioned; a stock solution was prepared in distilled water and filtered through a millipore filter (0.22 μm, Millipore Corp., Bedford, Mass.). 10 μg poly-L-lysine in 2 ml vol were added aseptically to 60-mm plastic Petri dishes (surface area 28 cm^2, Microbiological Associates, Inc., Bethesda, Md.) and removed after exposure for 1 h at ambient temperature. The plates were then washed twice with 3 ml Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.).

Measurement of Cell Attachment

Aliquots of cell suspension containing 0.3–0.6 $\times$ 10^6 cells in 3 ml growth medium were added and the polylysine-treated dishes incubated at 37°C in an atmosphere of 95% air, 5% CO_2 for the time periods designated in the experiments. The nonattached cells were carefully

removed and the Petri dish was washed twice with 1.5 ml solution of osmosol (Modified Eagle's solution, Harleco, Philadelphia, Pa.). For adhesion studies the attached cells were detached after 10-min incubation at 37°C in the presence of 0.125% trypsin. Cells were counted using a Coulter Counter, Model FM (Coulter Electronics, Inc., Hialeah, Fla). Recoveries of nonattached and attached cells was greater than 90%.

RESULTS

The time course of adhesion of brain cells to poly-L-lysine treated and untreated Petri dishes is depicted in Fig. 1. After 20-min incubation, between 85 and 90% of cells attached to the polylysine-treated surface, while the remaining cells did not attach even after prolonged incubation (24 h). Furthermore, these cells did not attach when transferred to another Petri dish, either coated or uncoated with polylysine. After 30 min of incubation the attached cells were firmly bound to the surface. In contrast to cells plated on uncoated Petri dishes, no detachment was observed even after incubating the cells with a solution of Eagle's modified medium (Ca- and Mg-free) containing 5 mM EDTA. Detachment could only be achieved following a 5–10-min incubation in the presence of 0.125% trypsin (wt/vol).

The majority of the attached cells were spherical in shape, but morphologically not identical to each other after 2 h incubation (Fig. 2 *a*). After 24 h they varied in size and some already possessed small processes which multiplied and enlarged (Fig. 2 *b*). The formation of aggregates interconnected by bundles of neurites was highly dependent upon the initial number of cells plated; best results were obtained when a suspension containing 1×10^6 cells per Petri dish was employed (Fig. 2 *c*). A montage of a culture cultivated for 2 wk on polylysine is shown in Fig. 3. The bundles of fibers connecting the aggregates were in some cases 2 mm long. The small, dark-phase cells possessing bipolar or pseudounipolar processes were confined mainly to the aggregates and most likely represent immature neurons or oligodendroglia cells (5, 13). The majority of the larger neuronal-like cells (Fig. 2 *d*) could not be detected by using phase-contrast microscopy since most of them were located within the aggregates. The processes arising from those

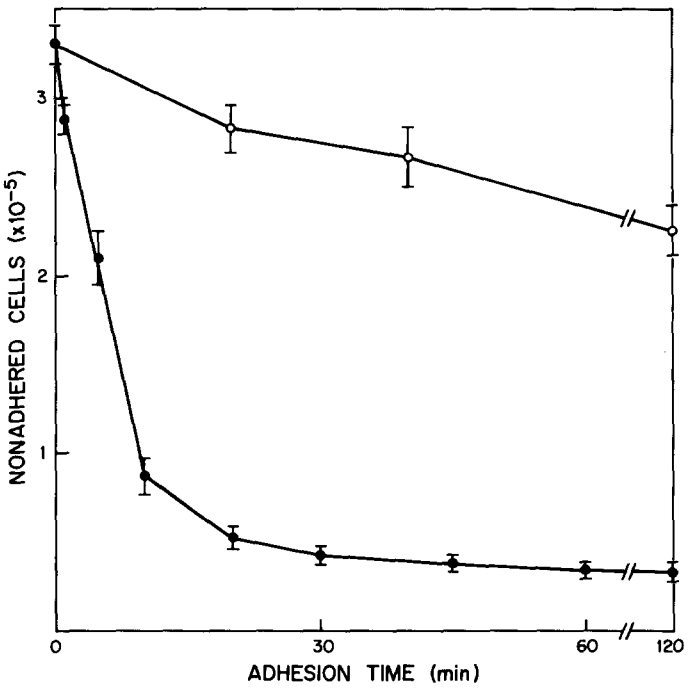

FIGURE 1 Time course of adhesion of dissociated brain cells. Aliquots of cell suspension containing 0.33 $\times 10^6$ cells in 3 ml growth medium were plated on poly-lysine-coated Petri dishes and incubated at 37°C for the time periods indicated. The number of nonadhered cells was determined as described in Materials and Methods. The results represent mean values of three experiments. O——O, untreated Petri dish; ●——●, polylysine-treated Petri dish.

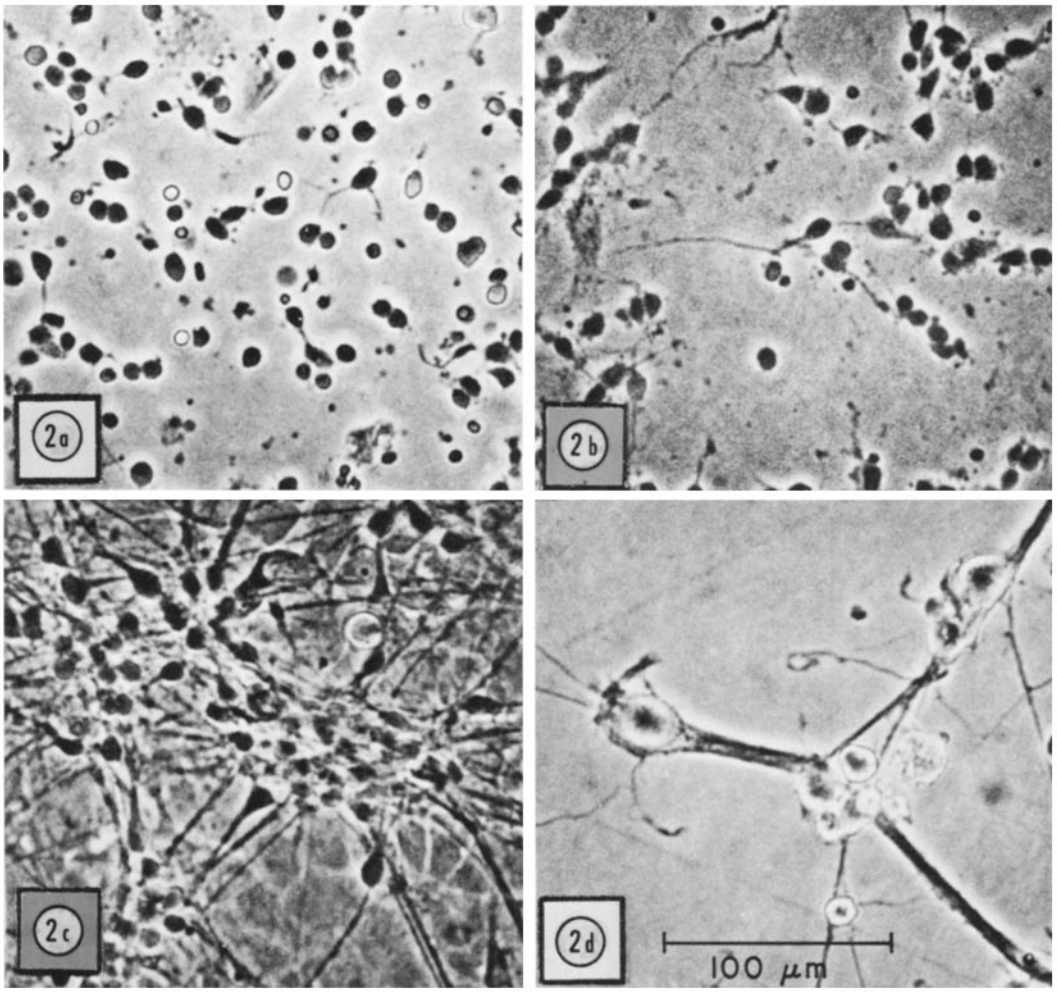

FIGURE 2 Phase-contrast microscopy of culture of dissociated cells from rat embryo cerebral hemispheres on polylysine-coated Petri dishes. After 2 h in culture the presence of small processes may be seen (2 *a*). At 24 h, cells have extended their processes and small cell aggregates are visible (2 *b*). After 4 days in culture, an extensive network of fibers interconnects aggregates as well as adjacent, single cells (2 *c*). After 16 days in culture, large cell bodies of neuron-like cells are interconnected by wide processes (2 *d*).

cells are definitely thicker than the ones observed in other cell types (Fig. 2 *d*). The presence of a polylysine surface seems to retard the growth of the background polygonal-type cells, in contrast to our previous observations employing the plastic surface (13). Incorporation of labeled thymidine into cells grown on polylysine proceeded to the same extent as found for those growing on the uncoated surface (unpublished observations).

The cells can be kept several months in culture. However, due to overgrowth the cells will eventually detach as large thin films from the surface.

Effect of Temperature and Various Inhibitors

The effect of temperature on the attachment of the brain cells to the polylysine-coated Petri dish is depicted in Fig. 4. When the adhesion was carried out for 20 min at 4°C, about 20% of the cells attached; while at ambient temperature or at 37°C more than 70% of the cells attached. This suggests that the attachment to polylysine is a temperature-dependent process with two different rates of binding and a transition point at about 23°C as

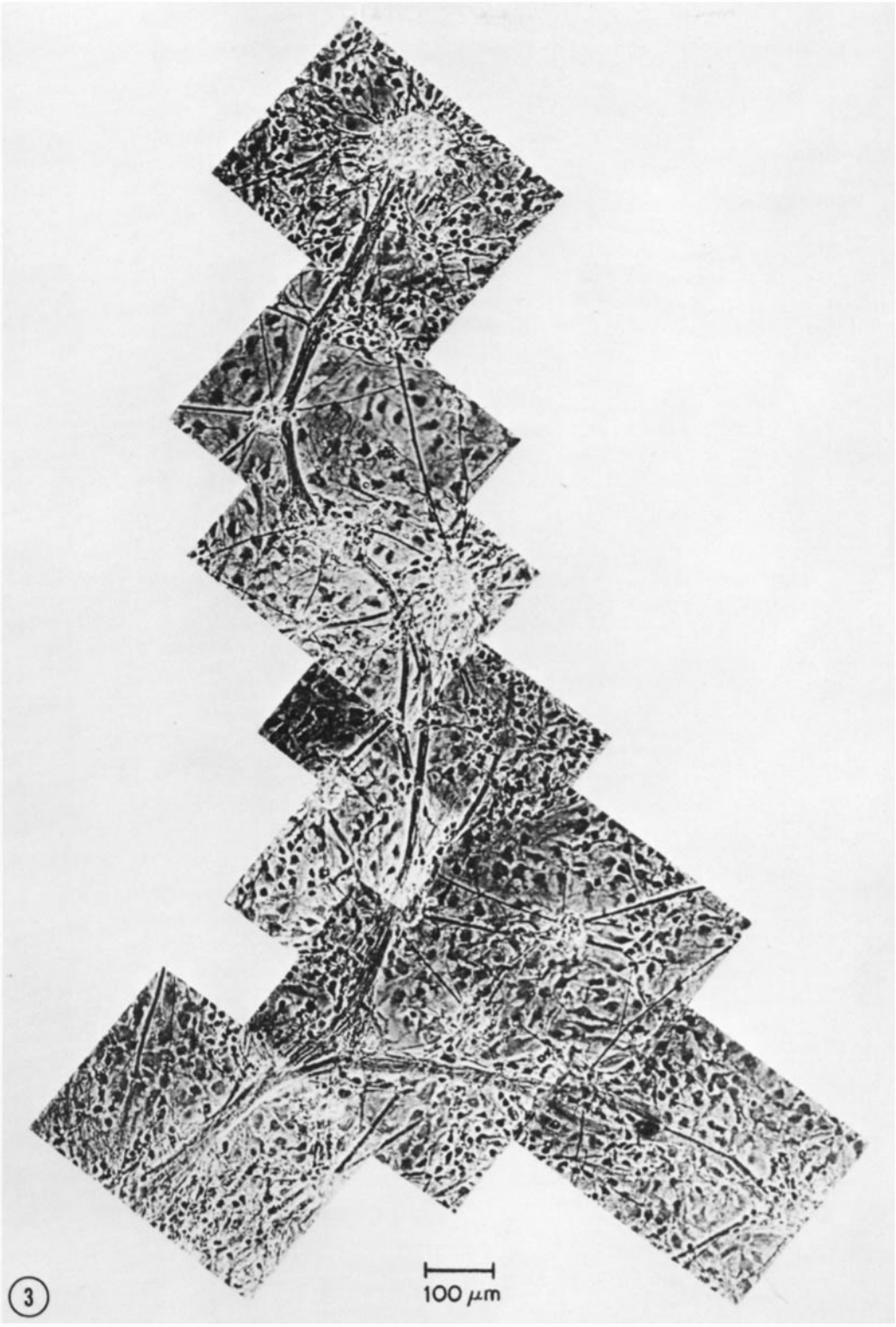

FIGURE 3 Phase-contrast microscopy of dissociated cells from 14 to 16-day rat embryo cerebral hemispheres after 14 days in culture; montage shows the extent of fibers interconnecting the cell aggregates. Note the relatively low occurrence of background, ependymal-like cells.

revealed by the Arrhenius plot (inset, Fig. 4). It may also indicate that the heterogeneous brain cell population is not homogeneous with respect to its attachment to polylysine.

The effect of various inhibitors of respiration, SH binding reagents, and EDTA on cell attachment is shown in Table I. When cells were plated on polylysine-treated Petri dishes which contained such inhibitors of respiration as KCN, 2,4-dinitrophenol (DNP), and NaF, only slight inhibition of attachment occurred; while n-ethyl maleimide (NEM) and para-chloro-mercuribenzoate (PCMB) markedly affected cell adhesion. Preincubation of cell suspension in the presence of KCN (5 mM) or NEM (0.01 mM) before plating caused an almost complete inhibition of attachment (Table I). Cells treated with NEM, even though attached to the Petri dish (36.5%, Table I), did not grow and after 24 h died and detached from the surface.

Addition of $CaCl_2$ (10 mM) or the presence of ion chelators, such as EDTA (10 mM), did not affect cell adhesion, suggesting that the process of attachment to polylysine is not mediated through a divalent cation. The possibility that high concentrations of serum and egg ultrafiltrate could bind the EDTA was excluded by employing adhesion medium devoid of those compounds.

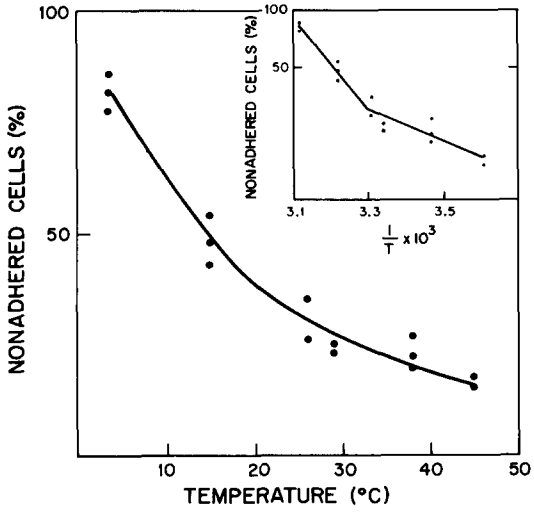

FIGURE 4 Temperature dependence of adhesion of dissociated brain cells. Aliquots of cell suspension containing 0.4 × 10^6 cells in 3 ml growth medium were plated on polylysine-coated Petri dishes and adhesion was measured after 30-min incubation at the designated temperatures. Each point represents the value obtained from one Petri dish.

TABLE I

Effect of Various Inhibitors on Brain Cell Adhesion

Effector	Final concentration	Percentage of cells attached	Percentage inhibition
	mM		
Control	—	75.5 ± 3.2	—
NaF	2	76.8 ± 0.5	0
2.4 DNP	0.5	68.4 ± 6.8	11.7
KCN	5	50.9 ± 10.2	34.3
KCN*	5	4.5 ± 2.1	94.0
DTT	1	78.1 ± 2.6	0
PCMB	0.5	26.8 ± 4.5	65.4
NEM	1	36.5 ± 4.6	52.9
NEM*	0.01	6.6 ± 0.8	91.2
EDTA	10	70.9 ± 3.4	8.5
$CaCl_2$	10	57.8 ± 2.7	25.4

Aliquots of cell suspension containing 0.5 × 10^6 cells in 2 ml growth medium were added to 60-mm polylysine-treated Petri dishes containing the various inhibitors. The cells suspended in a final volume of 3 ml were allowed to attach for 15 min at 37°C, after which the percentage of nonadhered cells was determined as described in Materials and Methods. The results are mean values of triplicates.

* Cells exposed to inhibitor 15 min at 37°C before adhesion. Cell suspension containing the inhibitors was centrifuged, the supernate was discarded, and the cells were resuspended to appropriate concentration and plated as described above.

Polyamino acids such as polyaspartate, polyglycine, and polycysteine are not suitable surfaces for brain cell attachment (Table II), and the residual percentage of cells that attached did not grow. D or L isomers of polylysine of various degrees of polymerization are all excellent surfaces for attachment as shown in Table II.

A marked reduction of cell attachment is observed when polylysine is treated with dansyl chloride, suggesting that the amino groups of the polycation could be involved in the adhesion process of the brain cells (Table II).

DISCUSSION

While studying the effect of a protein growth factor on cell adhesion, Lieberman and Ove (16) observed that polylysine enhanced the attachment of cells to glass surfaces and suggested that the role

TABLE II

Effect of Various Polyamino Acids on Adhesion of Brain Cells

Polyamino acids	Mol wt	Adhesion
		%
Poly-S-CBZ-cysteine	13,100	8.7 ± 2.5
Poly-glycine	6,000	20.2 ± 2.6
Poly-aspartate	21,600	18.3 ± 1.6
Poly-D-lysine	40,000	83.3 ± 3.0
Poly-D-lysine	100,000	84.6 ± 4.6
Poly-L-lysine	4,000	87.6 ± 6.6
Poly-L-lysine	100,000	91.5 ± 3.9
Poly-L-lysine	400,000	89.4 ± 8.0
Dansylated poly-L-lysine*	400,000	48.4 ± 5.0

Aliquots of cell suspension containing 0.55×10^6 cells in 3 ml growth medium were added to 60-mm Petri dishes pretreated with various polyamino acids. The coating was prepared under similar conditions as described in Materials and Methods, except that 25 μg/ml (on weight basis) of each of the polyamino acids solutions were employed. The adhesion was carried out for 30 min at 37°C. The results are mean values of triplicates.

* Dansylation of polylysine was performed using a modification of the procedure described by Gray (15). In brief, 1 mg poly-L-lysine (mol wt 400,000) dissolved in 1 ml of 0.2 M $NaHCO_3$ was treated at 37°C for 2 h with 10 mg dansyl chloride (Sigma Chemical Co.) in 0.1 ml acetone, and the resulting solution was dialyzed extensively against water. The dialyzed solution (1.3 ml) was diluted 1:100 with water and employed for treatment in the Petri dishes. Approximately 35% of the initial fluorescence added to the dish was recovered in the solution after 1 h, and this value did not change significantly after 3 h.

of the polybase was to mediate between the negative charges of the glass and cell surfaces. In recent studies, Macieira-Coelho and Avrameas (17), using cultured fibroblasts, and Stulting and Berke (18), employing ascites tumor cells, provided evidence for binding of cells and formation of confluent monolayers on polylysine-coated surfaces. In this study we present evidence that the polylysine is an excellent surface that enables the majority of the dissociated embryonic rat brain cells to adhere (Fig. 1). The attachment is followed by cell proliferation, histiotypic organization, and prompt formation of neurites which are characteristic signs of neural tissue differentiation (Fig. 2, 3). The relative low abundance of the ependymal-like cells, as observed by phase-contrast microscopy, (Fig. 3) may indicate that the rate of growth of some cell types may be altered. However, the possibility that some cells may assume a different morphological appearance due to the presence of polylysine is not to be excluded.

Morphological observation of cultures by phase-contrast microscopy revealed that the growth of the oligodendroglia-like and neuron-like cell populations was unaltered. The formation of neurites at early stages after plating (Fig. 2) could most likely be attributed to the presence of polylysine which appears to be a highly efficient surface for growth and differentiation of embryonic brain cells.

The exact molecular mechanism of the adhesion of the brain cells to the polylysine is as yet not fully understood. Our preliminary observations suggest that the attachment to the polycation surface is an energy-dependent process completely inhibited by KCN and NEM (Table I). However, the latter reagent did not completely inhibit attachment when added concomitantly with the adhering cells even at high concentrations (1 mM). This finding could be explained by the relatively fast rate of adhesion of the brain cells to the polylysine surface, i.e., 75% in 15 min, as compared with the rate of inhibition caused by NEM. It may also explain the differences between our inhibition values and those obtained by Grinnell and Srere (19) employing rat hepatoma cells. The possibility that NEM could affect cell membrane thiol groups involved in adhesion (20) requires further investigation.

Attachment of brain cells to polylysines of different degrees of polymerization as compared with other polyamino acids containing carboxylic acid and SH groups (Table II) suggests that the free amino groups are required for cell adhesion. This is further supported by the inhibition of attachment when the polybase is treated with dansyl chloride, which is known to react with free amino groups.

In summary, our experiments indicate that the polylysine is an adequate surface for attachment of the majority of the brain cells and for enabling growth and morphological differentiation of neural tissue in vitro. Cell attachment is inhibited by cyanide and SH-binding reagents, suggesting that adhesion to polylysine is energy dependent. There was evidence that the amino groups of the polycation are involved in the process of attachment, making this model suitable for studying certain aspects of cellular adhesion.

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