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Effects of Dibutyryl Cyclic AMP in Cell Behavior: Studies on Glucosamine-Requiring Mutant Derived from Chinese Hamster Lung Cells

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ABSTRACT

The effect of dibutyryl adenosine cyclic 3′: 5′ monophosphate (db-cAMP) on growth, adhesion and motile properties of glucosamine-requiring mutant, G72-8 derived from Chinese hamster lung cells was studied. The properties of the mutant cells, which was characterized by a round morphology and a decreased adhesion, was further emphasized when treated with mannosamine. The mutant cells become spindle shape and look similar to parent cells when treated with db-cAMP, whereas the adhesiveness remains decreased. Further, when the mutant cells cultured with mannosamine was treated with db-cAMP, their cell behaviors showed no significant change in spite of recognizable morphological reversion. The mutant cells showed a slight decrease in the content of cyclic AMP when compared to the parent cells, but no significant differences between the mutant cells grown in presence and absence of N-acetylglucosamine were observed. On the other hand, N-acetylglucosamine restored to normal their cell behaviors of the mutant. Our results indicate that at least a defect in adhesion response for the change in cell surface components (glycoproteins and/or glycolipids).

Introduction

Morphological alterations are important criteria in distinguishing normal cells from their transformed counterparts. Malignant transformation of animal cells has been associated with several changes, such as biochemical changes in cell surface components (1–4), a decrease in adhesiveness (5), and morphological changes (6–7). All of these changes may be directly or indirectly related to modification of the cell surface membrane (8). From analysis of lactoperoxidase-catalyzed iodination we previously reported that the high-molecular weight cell surface protein detected in parental cells (CHL–36) was reduced on the mutant and restored reversibly to normal by addition of N-acetylglucosamine (GlcNAc) (9). A random migration of the mutant cells also was reverted to the directional moving of the parent cells by addition of GlcNAc.

It has also been demonstrated that Chinese hamster ovary cells grown in the presence of db-cAMP lose its typical transformation stigmata. This reaction, which was called 'reverse transformation', included changes in morphological and adhesive

characteristics, microfibrillar organization, growth, and biochemical properties (10–14). In this communication we have attempted to evaluate the role of db-cAMP in cell behaviours by exploiting the availability of glucosamine-requiring mutant clones of Chinese hamster lung cells.

Materials and Methods

Cell cultures

Chinese hamster lung cells (CHL–36) and the derivative mutant cells (G72–8) were cultured in Eagle's minimal essential medium with 10% fetal bovine serum (Microbiological Assoc., Bethesda Maryland), penicillin (100 units/m*l*) and Kanamycin (60 μg/m*l*) at 37°C in a CO_2 incubator. The mutant cells were routinely cultured in the medium containing with 400 μg/m*l* N-acetylglucosamine (GlcNAc). Unless other stated, the mutant cells usually were starved in the medium without GlcNAc for 2 days prior to the experiments. All cells were carried out in 15×60 mm plastic tissue culture dishes (Nunc, Denmark). Cell counts were performed in duplicate with a hemocytometer.

Cell locaomotion

A migration of the cells in Nunc plastic flask (40 m*l*/vol) was monitored with a phase contrast inverted microscope coupled to a Nikon 16 mm camera. The cells were maintained in a controlled thermobox at 37°C. Photographs were taken at 5 min intervals, starting 2–3 days after plating as sparse cells. Cell migration was analysed by print the film and making the location of the nucleous of each of the single cells every 15 min. Contact and mitotic cells were excluded from the analysis.

Cyclic AMP assay

The test procedure is based on the finding of Gilman. (15). The cells were planted in 17×100 mm Nunc plastic dishes and assayed 3 days after plating. Each plate was quickly removed from the incubator, medium was completly removed, and 1 m*l* of 6% trichloroacetic acid was added; this step finished less 15 sec. For each experiment three identical plate were pooled and kept 10 min at 0°C and the cells were removed with a rubber policeman and disrupted with a sonication. The extract was centrifuged for 20 min at 3,000 g to remove the TCA precipitate. 1 N HCl was added to the supernatant to a final concentration of 10% and placed in water bath at 80°C to completly exclude ethylether in the extract. Samples of the supernatants were then lyophilized to dryness and resolubilized in 1 m*l* of 50 mM acetate buffer (Ph 4.0) and stored -20°C until used. The samples were used for each cyclic AMP assay. Cyclic AMP contents were measured with a Kit of cyclic AMP test (Boehringer Mannheim GmbH, Biochemica). Protein was measured by the method of Lowry et al., (16).

Materials

Eagle's minimal essential medium was purchased from Nissui Pharmaceutical Co., Japan, and N-acetylglucosamine and D-ManN from Sigma, Chemical Co.. N6, O^2-Dibutyryladenosine 3: 5-cyclic phosphate, sodium salt (db-cAMP) was obtained from P–L Biochemical, Inc. Milwaukee, Wis. and lyophilized trypsin from Beohringer Mannheim GmbH.

Results

1. *Effect of db-cAMP on cell growth*

To determine whether db-cAMP has any effect on the growth rate, we attempted to evaluate the effect of this agent on the growth of the mutant and parent cells. Both the cell types were compared for their proliferative response to amino sugars as described previously (17). The mutant cells divided very slowly in absence of GlcNAc, whereas the growth rate was markedly enhanced by addition of GlcNAc (Table 1). On the contrary, ManN caused severe inhibition on growth of the mutant cells. These compounds under the similar condition had little effect on the growth rate of the parent cells. Db-cAMP at the concentration of 10^{-3}M significantly inhibited the growth rate of the mutant as well as the parent cells.

Table 1. Effect of db-cAMP on growth of the mutant and parent cells.

Compounds	Addition of db-cAMP	Cell number/dish ($\times 10^{-4}$)	
		Mutant	Parent
None	+	53	328
	−	52	188
GlcNAc	+	183	332
	−	128	212
ManN	+	2	304
	−	4	160

The parent and mutant cells were seeded in the medium containing 10% fetal bovin serum at 1.5×10^5 and 3.0×10^5 cells per dish, respectively. Then db-cAMP at a concentration of 1.0 mM were added to half of the cultures. GlcNAc (400 μg/m*l*) or ManN (25 μg/m*l*) also was added simultaneously to the each culture. Experiments were carried out in 60 mm plastic dishes and cell counts were determined at 5 days after incubation.

2. *Morphological alteration by db-cAMP*

Change in morphology was perhaps the most striking feature of this mutant

(Fig. 1a). Most of the mutant cells exhibited rounded form, while at the presence of GlcNAc the morphological changes had reverted to a fibroblast-like shape (Fig. 1e). Treatment with ManN exhibited more rounded form in the mutant cells (Fig. 1c). After the addition of 1.0 mM db-cAMP, the cells began to show an altered morphology: the cell bodies were elongated, and numerous long, narrow-processes are distinctly appearent (Fig. 1b). Similar effect of db-cAMP was observed in the mutant cells treated with ManN (Fig. 1d). In agreement with previous observation (16), the colony appearence of the mutant cells (Fig. 2) were found to be quite different from the morphology of the parent cells. The mutant cells became more disorganized pattern and large multicellular clumps or spheres were observed at the surface of the mutant

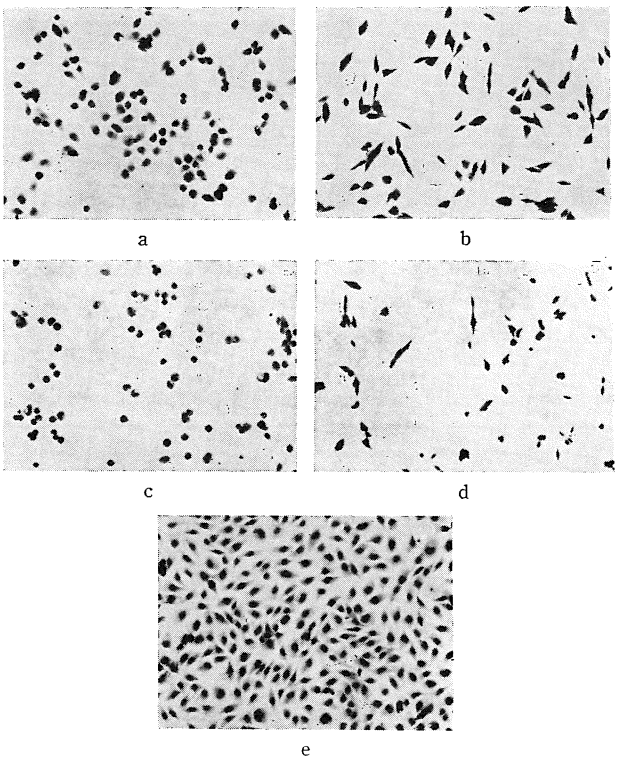
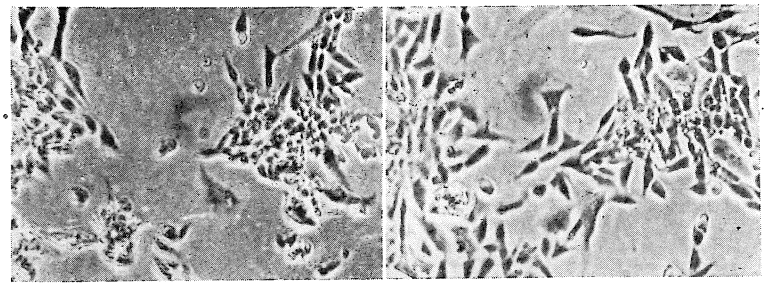


Fig. 1. Effect of db-cAMP on cell morphology. The mutant cells were seeded at 2.5×10^5 cells per dish and incubated for 3 days. Then, ManN or GlcNAc was added into each culture. Db-cAMP (1.0 mM) was added to a part of the cultures 24 hr prior to the end of incubation. a), the mutant cells untreated; b), the mutant cells treated with db-cAMP for 24 hr; c), the mutant cells grown with ManN (25 μg/m*l*) for 3 days; d), the mutant cells grown with ManN (25 μg/m*l*) for 3 days and db-cAMP (1.0 mM) added 24 hr prior to experiments; e), the mutant cells grown with GlcNAc (400 μg/m*l*) for 3 days.



a b

Fig. 2. Cell morphology in colony of the mutant. The colonies which grew in medium containing GlcNAc (400 μg/m*l*) were morphologically monitored under various condition. Prior to the experiments, the growth medium were replaced with GlcNAc-free medium and thereaf ter incubated for 4 days. a), the cells at 4 days after removal of GlcNAc; b), the cells which GlcNAc (400 μg/m*l*) was added again to (a) the culture at 4 days after removal of GlcNAc and further incubated for 24 hr.

cells, while by addition of GlcNAc the cells elongated to the spindle shape as well as those of db-cAMP-treated cells and tend to line up in parallel fashion to produce a highly oriented colony typical of the fibroblast.

3. *Effect of db-cAMP on cell adhesion*

We also examined the effect of db-cAMP on cell adhesiveness. Cell-to-substratum adhesion can be quantified by measuring the detachability of the cells, using the common technique of trypsinization. When the cells are treated with trypsin in PBS, a difference of adhesion between the parental strain and the mutant cells is made obvious by varing the time of incubation (Fig. 3). Conditions which detach 10–15% of the parental cells detach 65–80% of the mutant cells. In addition, when the cells was cultured in the presence of ManN, this loss of adhesiveness was further emphasized. Although db-cAMP caused the mutant to flattern and reverted partially to the parent-type morphology, the strength of adhesion to the substratum was not modified in the mutant cells. Similar results also were observed with the mutant cells treated with ManN. When the cells was fed with GlcNAc, the adhesive rate of the cells was recovered to a value close to that of the parent cells, and the adherence was further increased by addition of db-cAMP. In the case of the parent cells, db-cAMP also increased cell adhesion.

4. *Cyclic AMP content*

Cyclic AMP levels in both cell types were examined with or without GlcNAc. Cellular levels of cyclic AMP were higher in the parent cells compared with the mutant cells (Table 2). Little change in cyclic AMP levels occured at the mutant cells grown with GlcNAc, which exhibit normal growth, indicating GlcNAc had little effect on the

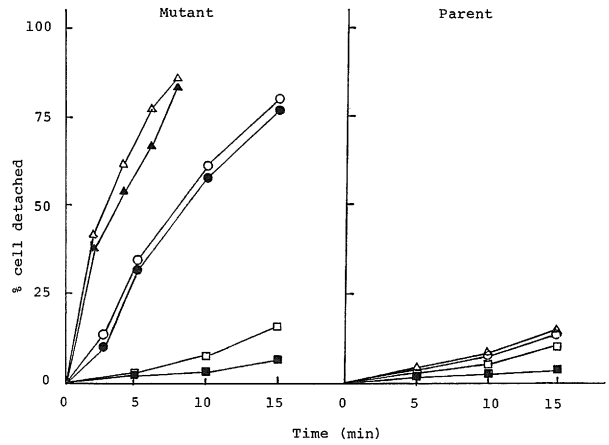


Fig. 3. Kinetics of detachment of the mutant and parent cells. The parent and mutant cells were seeded at 1.5×10^5 and 3.0×10^5 cells per 60 mm Nunc plastic dish, respectively and incubated at 37°C in CO_2 incubator. Detachment assays were performed 3 days after plating as described previously (17). ○, no addition; ●, cells grown with db-cAMP (1.0 mM), for 24 hr prior to the experiment; △, cells grown with ManN at 25 μg/m*l* for 48 hr before the end of incubation; ▲, cells grown with ManN at 25 μg/m*l* for 48 hr and db-cAMP at 1.0 mM for 24 hr before the end of incubation; □, cells grown with GlcNAc at 400 μg/m*l* for 48 hr prior to the experiment; ■, cells grown with GlcNAc 400 μg/m*l* and db-cAMP at 1.0 mM for 24 hr under same condition.

Table 2. Cyclic AMP levels in the mutant and parent cells

Cell line	Addition to the growth medium	cAMP concentration, pmol/10^6 cells
Parent	None	6.7±0.3
	GlcNAc	6.4±0.5
Mutant	None	4.9±0.2
	GlcNAc	5.3±0.3

Cyclic AMP contents were determined in exponentially growing cells. The values are the averages from two independent experiments in duplicate.

cyclic AMP levels. The effect was similar for both cell types.

5. *Motility*

A difference of cell motility may reflect actual changes in the composition or comformation of cell surface structures. We previously described that the mutant cells

differed from the parent-type fibroblasts by an absence of direction of motion. The migration pattern of the mutant appeared to be random, whereas the parent cells showed a high degree of persistence of direction. To test the effect of db-cAMP on cell locomotion, we cultivated the mutant cells in the presence of amino sugars. Figure 2 shows that GlcNAc restores the direction of locomotion in the mutant cells, and on the contrary, ManN repress the cell motility. Furthermore, db-cAMP slightly affect the motility of the mutant cells, but not in the treatment with ManN.

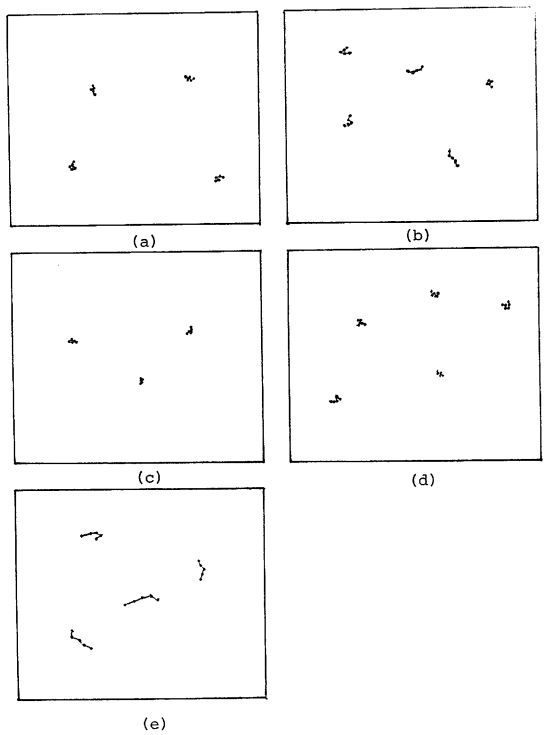


Fig. 4. Locomotion pattern of the mutant cells. Db-cAMP was added to each culture for 24 hr prior to experiments. Each tracing represents the locomotion of a cell recorded every 15 min. a), the mutant cells untreated; b), the mutant cells grown with db-cAMP (1.0 mM); c), the mutant cells grown with ManN (25 μg/m*l*); d), the mutant cells grown with ManN (25 μg/m*l*) and db-cAMP (1.0 mM); e), the mutant cells grown with GlcNAc (400 μg/m*l*). The photographs were taken 3 day after plating.

Discussion

We have previously reported biological properties of mutant, G72–8, derived from Chinese hamster lung cells: the mutant is characterized by low adhesion to substratum, round shape, increase in spontaneous aggregation, and decrease in growth rate and

these altered properties restored to normal by addition of GlcNAC (17, 18). Lactoperoxidase-catalyzed iodination of the cell surface showed that the high-molecular weight protein detected in the parent cells was reduced on the mutant and restored reversibly to normal by addition of GlcNAc, and the altered motility of the mutant cells also was reverted to normal by adding this compound (9). Pouyssegur and Pastan (8) reported that a fibroblast mutant, AD_6, which has a low adhesiveness to the substratum due to a defect in cell surface carbohydrate synthesis has a random locomotion. When AD_6 was grown in the presence of GlcNAc, its directional locomotion pattern was fully restored to that of the wild-type cells. They also observed a general decrease in the biosynthesis of cell surface carbohydrates which affect the adhesive properties of the cells. Similar findings were reported by us in Chinese hamster lung cells and its mutant (9, 17).

In this studies, we indicated that the mutant cells treated with ManN showed morphologically profound round shape (19, 20). In spite of this dramatic change in shape there was no effect of db-cAMP on adhesion of the mutant cells to substratum. Similar results were observed in the untreated mutant cells. By contrast, when the mutant was fed with GlcNAc the high rate of the detachment was decreased to close that of the parent cells. The adhesion has a tendency to further increease when db-cAMP was added to the parent cells or to the GlcNAc treated mutant cells. The reduced adhesiveness in this mutant is not directly correlated with intracellular level of cyclic AMP, but rather appear to depend on the chemical nature of the cell surface, because the biochemical reversion of the biosynthesis of surface carbohydrates of the mutant cells restores normally these cell behaviors (9). Db-cAMP also has a considerable effect on directional locomotion of the mutant cells, but not the cells treated with ManN. It is known that transformed cells growing in presence of db-cAMP show morphological characteristics of untransformed cells. This phenomenon which was called "reverse transformation" is exhibited by several different transformed cells when treated with db-cAMP, or similar agents (21–24), and db-cAMP-treated cells increase in adhesion to substratum (25). Recently, Nielson and Puck (26) reported that Chinese hamster ovary cells, like other transformed cells, has lost the fibronectin deposit around its membrane. Treatment with cyclic AMP derivates restores the typical fibroblastic deposit of fibronectin. Cell elongation in the presence of db-cAMP also appear to be dependent on the polymerization of the intracellular microtubules and microfilaments (10, 27). Electron microscopic studies of 3T3 and L929 cells have confirmed that db-cAMP treatment alters the cellular distribution of the microfilaments and microtubules, leading to their aligment into paralleled patterns (6, 13). The defect in the biosynthesis of amino sugars in our mutants leads to incomplete glycosylation of glycoproteins, and one consequence of the defect appear to be a decrease in the exposure of glycoproteins at the outer surface of the cells. Adding GlcNAc to this mutant restores the synthesis of the carbohydrate portion of the glycoproteins to normal, and the cell surface glycoproteins become normally exposed. By contrast, ManN has an antago-

nistic function against GlcNAc (20). Very little is known about the basis for ManN inhibition. Our results support a hypothesis that the carbohydrate moieties of cell surface proteins has an important role in biological cell behavior, including in adhesion and motility.

References

1. Hynes, R. O. 1973. Alteration of cell surface proteins by viral transformation and by proteolysis. Proc. Natl. Acad. Sci. U.S.A. 70: 3170–3174.
2. Wickus, G. G. and Robbins, P. W. 1973. Plasma membrane proteins of normal and Rous sarcoma virus-transformed chick-embryo fibroblasts. Nature New Biol. 245: 65–67.
3. Vaheri, A. and Ruoslahti, E. 1974. Disappearence of a major cell-type specific surface glycoprotein antigen (SF) after transformation of fibroblasts by Rous sarcoma virus. Int. J. Cancer 13: 579–586.
4. Duksin, D. and Bornstein, P. 1977. Changes in surface properties of normal transformed cells caused by tunicamycin, an inhibitor of protein glycosylation. Proc. Natl. Acad. Sci. U.S.A. 74: 3433–3437.
5. Shields, R. and Pollock, K. 1974. The adhesion of BHK and PyBHK cells to the substratum. Cell. 3: 31–38.
6. Porter, K. R., Puck, T. T., Hsie, A. W. and Kelley, D. 1974. An electron microscope study of the effects of dibutyryl cyclic AMP on Chinese hamster ovary cells. Cell, 2: 145–162.
7. Pollack, R., Osborn, M. and Weber, K. 1975. Patterns of organization of actin and myosin in normal and transformed cultured cells. Proc. Natl. Acad. Sci. U.S.A. 72: 994–998.
8. Pouyssegur, J., Willingham, M. and Pastan, I. 1977. Role of cell surface carbohydrates and proteins in cell behavior: Studies on the biochemical reversion of an N-acetylglucosamine-deficient fibroblast mutant. Proc. Natl. Acad. Sci. U.S.A. 74: 243–247.
9. Onoda, T. and Nishigami, K. 1981. Effects of N-acetylglucosamine on cell surface glycoprotein and cell behavior of glucosamine reguiring mutant derived from Chinese hamster lung cells. Hiroshima Journal of Medical Sciences, 30: 29–34.
10. Hsie, A. W. and Puck, T. T. 1971. Morphological transformation of Chinese hamster cells by dibutyryl adenosine 3′: 5′-monophosphate and testosterone. Proc. Natl. Acad. Sci. U.S.A. 68: 358–361.
11. Hsie, A. W., Jones, C. and Puck, T. T. 1971. Further changes in differentiation state accompanying the conversion of Chinese hamster cells to fibroblastic form by dibutyryl adenosine cyclic 3′: 5′ monophosphate and hormones. Proc. Natl. Acad. Sci. U.S.A. 68: 1648–1652.
12. Grimes, W. J. and Schroeder, J. L. 1973. Dibutyryl cyclic adenosine 3′: 5′ monophosphate, sugar transport, and regulatory control of cell division in normal and transformed cells. J. Cell Biol. 56: 487–491.
13. Willingham, M. C. and Pastan, I. 1975. Cyclic AMP and cell morphology in cultured fibroblasts. J. Cell Biol. 67: 146–159.
14. Puck, T. T. 1977. Cyclic AMP, the microtubule-microfilament system, and cancer. Proc. Natl. Acad. Sci. U.S.A. 74: 4491–4495.
15. Gilman, A. G. 1970. A protein binding assay for adenosine 3′: 5′-cyclic monophosphate. Proc. Natl. Acad. Sci. U.S.A. 67: 305–312.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 24: 265–275.
17. Onoda, T. 1980. Altered cell behavior of glucosamine requiring mutant derived from Chinese

hamster lung cells. Hiroshima Journal of Medical Sciences. 29: 61–68.
18. Onoda, T., Saito, H., and Note, S. 1980. Growth and G_1 arrest of glucosamine requiring mutant cells derived from Chinese hamster lung cells. Hiroshima Journal of Medical Sciences. 29: 76–81.
19. Kajiwara, N. 1978. Inhibitory effect of D-mannosamine on the growth of D-glucosamine-requiring mutant strain of Chinese hamster lung cells. Hiroshima Journal of Medical Sciences. 27: 75–83.
20. Onoda, T., Kajiwara, N., and Matsuo, Y. 1978. Inhibitory effects of D-mannosamine and reversal of the D-mannosamine inhibition by D-glucosamine on growth of a glucosamine-requiring mutant of Chinese hamster lung cells. Hiroshima Journal of Medical Sciences. 27: 233–240.
21. Sheppard, J. R. 1972. Difference in the cyclic adenosine 3′: 5′-monophosphate levels in normal and transformed cells. Nature, New Biol. 236: 14–16.
22. Johnson, G. S., Friedman, R. M., and Pastan, I. 1971. Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine 3′: 5′-cyclic monophosphate and its derivatives. Proc. Natl. Acad. Sci. U.S.A. 68: 425–429.
23. Johnson, G. S., Morgan, W. D., and Pastan, I. 1972. Regulation of cell motility by cyclic AMP. Nature, New Biol. 235: 54–56.
24. Johnson, G. S. and Pastan, I. 1972. Role of 3′: 5′-adenosine monophosphate in regulation of morphology and growth of transformed and normal fibroblasts. J. Natl. Cancer Inst. 48: 1377–1387.
25. Johson, G. S. and Pastan, I. 1972. Cyclic AMP increase the adhesion of fibroblast to substratum. Nat, New Biol, 236: 247–249.
26. Nielson, S. E. and Puck, T. T. 1980. Deposition of fibronectin in the course of reverse transformation of Chinese hamster ovary cells by cyclic AMP. Proc. Natl. Acad. Sci. U.S.A. 77: 985–989.
27. Rubin, R. W. and Weise, G. D. 1975. Direct biochemical measurements of microtubule assembly and disassembly in Chinese hamster ovary cells. The effect of intracellular contact, cold, D_2O and N^6, $O^{2'}$-dibutyryl cyclic adenosine monophosphate. J. Cell Biol. 64: 42–53.