

# Regulation of Cyclic Adenosine 3':5'-Monophosphate-binding Protein in N-18 Mouse Neuroblastoma Cells

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## ABSTRACT

The regulation of cyclic adenosine 3':5'-monophosphate (cAMP)-binding protein in N-18 neuroblastoma cells in tissue culture was studied by the covalent incorporation of 8-azido-cyclic adenosine 3':5'-[<sup>32</sup>P]monophosphate, together with the techniques of sodium dodecyl sulfate:polyacrylamide gel electrophoresis and autoradiography. Greater than 95% of the total cAMP binding activity of N-18 neuroblastoma cells was identified as being regulatory subunits of the type I (R<sub>i</sub>) and type II (R<sub>ii</sub>) species, with R<sub>i</sub> being the predominant form of the two (R<sub>i</sub>:R<sub>ii</sub> = 3:1). The specific activity of R<sub>i</sub> but not of R<sub>ii</sub> increased 3-fold when cells were grown in medium containing 1% rather than 10% fetal calf serum. Under the same conditions, the specific activity of acetylcholinesterase increased 3- to 5-fold. The increase in R<sub>i</sub> was inversely related to the serum concentration in the medium and was specific for cells at the stationary phase of growth. An increase in intracellular cAMP, concomitant with the increase in R<sub>i</sub>, was also observed. Morphological examination of stationary-phase neuroblastoma cells maintained in medium containing 1% fetal calf serum suggested the presence of a high proportion of highly-differentiated cells. It is proposed that the regulatory control of R<sub>i</sub> cAMP-binding protein by serum may involve modulation of intracellular cAMP and that the expression of R<sub>i</sub> may be used as a biochemical index of differentiation in mouse neuroblastoma cells.

## INTRODUCTION

N-18 neuroblastoma cells grown in culture are predominantly round and highly refractile (1, 2). Under appropriate experimental conditions, these cells can undergo differentiation, a process grossly defined by increases in neurite outgrowth and acetylcholinesterase activity. A vast variety of agents or treatments have been shown to induce differentiation in neuroblastoma cells (3, 9, 12, 14, 16-18, 23, 26-28, 33, 34). In general, these agents or treatments also cause a slowing of cell growth. The precise relationship between cell growth and differentiation is not clear. It has been suggested that (a) morphological differentiation may be a direct function of the inhibition of cell growth rate (3), (b) inhibition of cell division is necessary, although by itself not sufficient, for cell differentiation (28), (c) the inhibition of cell growth is not the cause of neurite extension but rather is itself a result of the induction of differentiated functions incompatible with rapid growth (12), or finally (d) it is possible to dissociate inhibition of cell growth from biochemical differentiation of neuroblastoma cells (14).

The N-18 neuroblastoma cells undergo morphological and

biochemical changes characteristic of differentiated neurons when grown in medium supplemented with 1% rather than the normal 10% FCS<sup>3</sup> (3, 34). These changes are accompanied by a decreased growth rate and lower saturation density. Studies on a variety of mammalian cell systems have indicated an inverse relationship between cellular growth and intracellular cAMP concentration (25). It has been suggested that lower levels of cAMP in rapidly dividing cells may have a significant role in the increased growth rate (24). Previous studies on the regulation of cAMP binding activity in mouse neuroblastoma cells have demonstrated the induction of cAMP-binding protein either by cAMP or by agents which increase intracellular cAMP (29, 30, 35).

The purpose of this paper is to (a) characterize the possible relationships between serum concentration and the levels both of cAMP-binding protein(s) and of intracellular cAMP in N-18 neuroblastoma cells and (b) determine whether these relationships can be explained merely as functions of altered growth properties induced by changes in the serum concentration or whether they are in fact a characteristic property of the differentiated state in N-18 neuroblastoma cells.

## MATERIALS AND METHODS

**Materials.** cAMP, IBMX, neostigmine, and other fine biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Acid phosphatase (potato) was obtained from Calbiochem-Behring Corp., La Jolla, Calif. [<sup>3</sup>H]cAMP and [<sup>35</sup>S]methionine were obtained from New England Nuclear, Boston, Mass. 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP was obtained from ICN Pharmaceuticals, Inc., Irvine, Calif.; purity of the compound was checked by thin-layer chromatography using Cellulose F<sub>254</sub>-coated thin-layer chromatographic sheets (EM Laboratories, Inc., Elmsford, N. Y.) in a solvent system of *N*-butyl alcohol:acetic acid:H<sub>2</sub>O [5:2:3 (v/v/v)]. 1-[<sup>14</sup>C]Acetylcholine was obtained from Amersham/Searle Corp., Arlington Heights, Ill. Tissue culture supplies were obtained from Grand Island Biological Co., Grand Island, N. Y.

**Cell Cultures.** N-18 mouse neuroblastoma cells were grown as monolayer cultures in Dulbecco's modified Eagle medium (with 4500 mg glucose per liter, without sodium pyruvate) supplemented with 10% FCS. Cells were maintained at 37° in

<sup>3</sup> The abbreviations used are: FCS, fetal calf serum; cAMP, cyclic adenosine 3':5'-monophosphate; IBMX, 3-isobutyl-1-methylxanthine; 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP, 8-azido cyclic adenosine 3':5'-[<sup>32</sup>P] monophosphate; NB<sub>10%</sub> cells, neuroblastoma cells grown in medium supplemented with 10% fetal calf serum; NB<sub>1%</sub> cells, neuroblastoma cells grown in medium supplemented with 1% fetal calf serum; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; 8-Br-[<sup>32</sup>P]cAMP, 8-bromo cyclic adenosine 3':5'-[<sup>32</sup>P]monophosphate; MES, 2-(*N*-morpholino) ethane sulfonate; R<sub>i</sub>, regulatory subunit of the type I cyclic adenosine 3':5'-monophosphate-dependent protein kinase; R<sub>ii</sub>, regulatory subunit of the type II cyclic adenosine 3':5'-monophosphate-dependent protein kinase; Bt<sub>2</sub>cAMP, N<sup>6</sup>,O<sup>2</sup>-dibutyryl cyclic adenosine 3':5'-monophosphate; PBS, phosphate-buffered saline [0.15 M NaCl:10 mM sodium phosphate (pH 7.4)].

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a water-jacketed CO<sub>2</sub> incubator (95% air:5% CO<sub>2</sub>). During the initial phase of this study, cells were kept in medium containing penicillin (50 units/ml) plus streptomycin (50 µg/ml). Control experiments demonstrated that the presence of these antibiotics in the culture medium had no effect on either the cell morphology or the cAMP binding activity. Periodic tests of the cultures for *Mycoplasma* and bacteria were negative. Upon reaching stationary phase of growth, cells were subcultured by flushing the cells off their growing substratum with a stream of medium, diluting the cell suspension 1:10 to 1:30, plating in Petri dishes, and incubating as described above.

**Differentiation.** In this study, differentiation, defined as the morphological appearance of neurites >50 µm in length and the biochemical expression of increased acetylcholinesterase activity, was induced by growing cells in medium supplemented with 1% rather than the usual 10% FCS. Morphological studies demonstrated that >95% of the cells grown in medium containing 10% FCS exist in the round undifferentiated form, while >95% of the cells extended lengthy neurites when grown in medium containing 1% FCS. However, it should be noted that these 2 morphologically distinct cell forms can and do coexist in any given culture; the ratio of these 2 forms varies as a function of cell growth and serum concentration in the medium. In view of this consideration, we decided to label populations of neuroblastoma cells according to the serum concentration in the growth media. Thus, NB<sub>10%</sub> and NB<sub>1%</sub> represent neuroblastoma cells grown in medium supplemented with 10% and 1% FCS, respectively. The term differentiated cells, when used, refers to NB<sub>1%</sub> cells at stationary phase which have elevated levels of acetylcholinesterase activity and extensive neurite outgrowth.

Fig. 1 is a photomicrograph of NB<sub>10%</sub> (A) and NB<sub>1%</sub> (B) cells at early stationary phase of growth. Two features should be noted. (a) The density of NB<sub>10%</sub> cells was approximately 2.5 times that of NB<sub>1%</sub> cells. The saturation densities of NB<sub>10%</sub> and NB<sub>1%</sub> cells were approximately  $5 \times 10^6$  and  $2 \times 10^6/25$  sq cm, respectively. (b) The high proportion of cells with neurites and the length of neurites (50 to 200 µm in length) were distinct features of the NB<sub>1%</sub> cells. It is of interest that in SDS:polyacrylamide gel electrophoresis, no distinct difference in the Coomassie blue staining pattern of proteins from NB<sub>10%</sub> and NB<sub>1%</sub> cells was detected.

**Cell Growth.** Cell growth was measured by the number of viable cells attached to the growing surface. It should be noted that this represents a balance of cell multiplication and shedding of cells into the medium. Occasionally, cell growth was also studied by the incorporation of [<sup>35</sup>S]methionine into TCA-insoluble material. Cells grown in 60-mm Petri dishes were labeled for 30 min with 5 µCi [<sup>35</sup>S]methionine per ml. The cell layer was then washed 5 times with cold PBS, and 0.7 ml of 10% TCA was added. The amount of radioactivity present in the TCA-precipitable material was determined by liquid scintillation spectrometry. For cells in the logarithmic phase of growth, the incorporation of [<sup>35</sup>S]methionine gave a fairly good index of the rate of increase in cell number.

**Cell Fractionation.** Unless otherwise stated, cells at an early stationary phase of growth were used; all procedures were conducted at 4°. Cells were rinsed twice with 5 ml of PBS and scraped off the Petri dishes with a rubber policeman. Cells were sedimented by centrifuging at 1000 × g for 2 min and washed once with PBS. The cell pellet obtained from ten 100-

mm Petri dishes was resuspended in 1.5 to 2.0 ml of a buffer containing 0.32 M sucrose, 1 mM EDTA, 50 µg phenylmethylsulfonyl fluoride per ml, and 10 mM Tris-HCl (pH. 7.4). Cells were broken by sonicating the cell suspension for 1 min at 4° with a Heat Systems-Ultrasonics sonicator equipped with a stepped microtip (50-watt power output; 50% duty cycle, pulsed). The 100,000 × g supernatant obtained from cell homogenate was defined as the cytosol fraction. Occasionally, cells were broken by forcing the cell suspension through a 27-gauge 7/8-inch needle. No difference was noted in the cytosol preparations obtained by these 2 methods. For routine analysis, all cytosol preparations were either passed through a Sephadex G-25 column or dialyzed against 10 mM Tris-HCl buffer (pH. 7.4) containing 50 µg phenylmethylsulfonyl fluoride per ml, 1 mM EDTA, and 1 mM dithiothreitol at 4° for 16 hr to remove low-molecular-weight endogenous substances. Protein concentration was determined by the method of Lowry *et al.* (22) using bovine serum albumin as standard.

**Covalent Binding of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP.** Covalent binding of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP was performed as described previously (36). The standard reaction mixture (final volume, 0.1 ml) contained 50 mM MES (pH. 6.2), 10 mM MgCl<sub>2</sub>, 50 µM ATP, 0.1 nM to 1 µM 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP (specific activity, 4 to 10 Ci/mmol), and various amounts of cytosol protein up to 200 µg. For studying the specificity of the incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP, 10 µM cAMP and 1 mM IBMX were added to the assay mixture. The presence of 1 mM IBMX by itself had no effect on the incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP. Samples were incubated for 60 min at 4° in the dark and were then photolyzed for 10 min with a mineralite UVS-11 hand lamp at a distance of 8 cm. To each sample 25 µl of a SDS stop solution containing 12% SDS, 0.5 M Tris-HCl buffer (pH. 8.0), 10% mercaptoethanol, 5 mM EDTA, 25% glycerol, and a small amount of pyronin Y (a tracking dye for electrophoresis) was added. Samples were heated at 100° for 5 min. The entire sample was then subjected to SDS:polyacrylamide (5 to 15%) gel electrophoresis (20). Gels were stained for protein with Coomassie blue, dried, and subjected to autoradiography. Apparent molecular weights of the radioactive bands were estimated by the method of Fairbanks *et al.* (11). Standard proteins (and their molecular weights) used were cytochrome c (12,500), chymotrypsinogen (25,000), aldolase (40,000), ovalbumin (45,000), catalase (57,500), bovine serum albumin (65,000), and phosphorylase a (95,000). Autoradiographs were scanned with a Schoeffel SD-3000 spectromicrodensitometer, and the peak heights of the optical tracings were used as a quantitative measure of the incorporation of <sup>32</sup>P. In most experiments, the results obtained by the densitometer method were compared with results obtained by liquid scintillation counting of the dried gel slices. Similar results were obtained by the 2 methods.

Results demonstrated the presence of three 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP-binding proteins in extracts of N-18 neuroblastoma cells. The apparent molecular weights of these proteins on SDS:polyacrylamide gel electrophoresis were 47,000, 52,000, and 54,000. In a previous study on the incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP into proteins from several rat tissues, the M.W. 47,000 and M.W. 54,000 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP-binding proteins were identified as regulatory subunits of the type I and type II cAMP-dependent protein kinases, respectively (36). Studies on the cAMP-binding proteins of bovine cardiac muscle indicated the occurrence of both a M.W. 54,000 and a M.W.

52,000 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP-binding protein as regulatory subunits of the type II kinase of bovine heart. Furthermore, the electrophoretic mobility of the M.W. 54,000 component was affected by the state of phosphorylation of the protein (31).

In N-18 neuroblastoma cells, the quantitative incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP into the M.W. 47,000, M.W. 52,000, and M.W. 54,000 proteins and the electrophoretic mobility of these proteins were unaffected by the presence of ATP (50 μM to 1 mM) or by preincubation of the samples with acid phosphatase for 20 min at 30°. DEAE-cellulose column chromatography demonstrated the coelution of the M.W. 47,000 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP-binding protein with the type I cAMP-dependent protein kinase peak, while the M.W. 52,000 and M.W. 54,000 proteins were eluted with the type II cAMP-dependent protein kinase.<sup>4</sup> Due to similarity in the apparent affinity of the M.W. 52,000 and M.W. 54,000 proteins for 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP, their close proximity on SDS:polyacrylamide gel electrophoresis, and their coelution in DEAE-cellulose column chromatography, the amount of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP incorporated into the M.W. 52,000 plus M.W. 54,000 proteins was often summated (Charts 1, 2, 5A, 5B, and Table 1). The terms R<sub>I</sub> and R<sub>II</sub> were used to designate the M.W. 47,000 and the M.W. 52,000 and M.W. 54,000 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP-binding proteins, respectively. No reference was made to the possible physiological functions of these proteins, *e.g.*, associating with and inhibiting the catalytic activity of cAMP-dependent protein kinase.

The incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP into R<sub>I</sub> and R<sub>II</sub> is both specific and quantitative. That the incorporation is specific is supported by the ability of a 10-fold excess of cAMP, in the presence of 1 mM IBMX, to displace the 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP. That the incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP is quantitative was supported by the following experiments. In Experiment 1, at saturating concentrations of the ligand (1 μM), the amount of specifically bound [<sup>3</sup>H]cAMP or 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP, as determined by the reversible (noncovalent) binding procedure of Gilman (13), was the same as the total amount of covalently incorporated 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP. In Experiment 2, removal of endogenous sources of cAMP by overnight dialysis or by incubation at 30° for periods of up to 60 min had little effect on the amount of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP incorporated. It should be noted that, in quantitating cAMP-binding protein by the incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP, the presence of endogenous sources of cAMP does not present an insurmountable barrier, due to its hydrolysis by phosphodiesterase (present in abundant amounts in extracts of N-18 neuroblastoma cells). That 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP was indeed relatively resistant to hydrolysis by phosphodiesterase was verified by the following observations. (a) Incubation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP with crude bovine heart phosphodiesterase for 10 min at 30° had little or no effect on the chromatographic behavior of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP on thin-layer chromatography (occasionally, certain batches of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP obtained from ICN Pharmaceuticals contained trace amounts of [<sup>32</sup>P]cAMP and 8-Br-[<sup>32</sup>P]cAMP, which could be separated from 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP on thin-layer chromatography). (b) The incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP was not significantly affected by the presence of a phosphodiesterase inhibitor, IBMX, in the assay mixture. In Experiment 3, the rate of exchange of protein-bound ligand with free ligand in solution was investigated by

studying the exchange of protein-bound [<sup>3</sup>H]cAMP with free 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP. Greater than 90% of the protein bound [<sup>3</sup>H]cAMP exchanged with the 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP in solution in a time course of less than 60 min at 4°.

**Reversible Binding of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP or [<sup>3</sup>H]cAMP.** An alternative approach for the determination of cAMP binding activity present in extracts of neuroblastoma cells was the noncovalent binding of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP or [<sup>3</sup>H]cAMP (13). The standard reaction mixture (final volume, 0.1 ml) contained 50 mM MES (pH 6.2), 10 mM MgCl<sub>2</sub>, 10 pmol of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP (specific activity, 2 to 3 Ci/mmol) or [<sup>3</sup>H]cAMP (specific activity, 30 to 50 Ci/mmol), and various amounts of protein up to 200 μg. Samples were incubated for 60 min at 4°. To each sample, 3 ml of 50 mM MES (pH 6.2) was added. The entire sample was filtered through a Millipore filter (Type HAWP; 0.22 μm) followed by washing twice each with 5 ml of MES buffer (pH 6.2). The amount of [<sup>3</sup>H]cAMP bound to protein and hence retained by the Millipore filter was determined by liquid scintillation spectrometry. All results were corrected for nonspecific binding, determined from the amount of radioactivity retained in the presence of 10 μM cAMP and 1 mM IBMX.

**Measurement of cAMP.** Measurements of intracellular cAMP were done according to the method described by Brown *et al.* (7). The assay is based on the competition of binding of [<sup>3</sup>H]cAMP and cAMP to a preparation of cAMP-binding protein (the type II cAMP-dependent protein kinase eluted from DEAE-cellulose column) obtained from bovine heart. Briefly, 2 ml of ice-cold 6% TCA was added to neuroblastoma cells grown on 100-mm Petri dishes. A rubber policeman was used to scrape the cell debris off the Petri dish. The protein precipitate was removed by centrifugation. The supernatant thus obtained was extracted 3 times each with 5 ml of water-saturated ether, evaporated to dryness, and taken up in 100 μl of 50 mM MES (pH 6.2). The standard assay mixture (total volume, 0.1 ml) contained 50 mM MES (pH 6.2), 10 pmol [<sup>3</sup>H]cAMP (specific activity, 30 to 50 Ci/mmol), an aliquot of the cAMP unknown, and 30 to 40 μl of the binding protein (containing approximately 5 to 7 pmol of cAMP binding capacity). Samples were incubated at 4° for 60 min. The method used for separation of bound cAMP from free cAMP was identical to that described for determining [<sup>3</sup>H]cAMP binding activity in cell extracts. The amount of cAMP present in the unknown was calculated from a standard curve generated by adding known concentrations of cAMP to the assay mixture.

**Acetylcholinesterase Assay.** Acetylcholinesterase activity was assayed by a radiometric method described previously (10, 21). All assays were carried out at 37° with 5 μM 1-[<sup>14</sup>C]-acetylcholine, unless otherwise stated. The standard reaction mixture contained 0.1 mM Tris-HCl, 1 mM histidine hydrochloride (pH 7.6), 5 μM [<sup>14</sup>C]acetylcholine, and various amounts of protein up to 200 μg/ml. At predetermined time intervals (usually 10 min apart), a 1-ml aliquot was removed from the assay bath, and the enzyme reaction stopped with 0.1 ml of 1 μg neostigmine per ml solution. The entire mixture was loaded onto a column of cation exchange resin (Bio-Rad AG 50 W-X8; 200 to 400 mesh) equilibrated in 0.1 M sodium phosphate buffer (pH 7.2). The amount of 1-[<sup>14</sup>C]acetate present in the column eluate was determined by liquid scintillation spectrometry. Results are expressed in nmol of 1-[<sup>14</sup>C]acetate generated per min per mg protein for a substrate concentration of 10<sup>-4</sup> M.

<sup>4</sup> A. Y.-C. Liu, T. Chan, and K. Y. Chen, manuscript in preparation.

## RESULTS

Cytosolic cAMP-binding proteins of NB<sub>10%</sub> and NB<sub>1%</sub> cells were studied by the photoactivated incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP and were characterized by: (a) the pattern of incorporation (Fig. 2); (b) the apparent affinity of this incorporation (Chart 1); and (c) a quantitative comparison of the amount of covalently incorporated 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP to the amount of reversibly bound 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP or [<sup>3</sup>H]cAMP (Table 1).

Three protein bands, with apparent molecular weights of 47,000, 52,000, and 54,000, present in cytosols of NB<sub>10%</sub> and NB<sub>1%</sub> cells incorporated 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP. The incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP into cytosolic proteins of NB<sub>1%</sub> cells differed from that of NB<sub>10%</sub> cells in that there was a 2- to 4-fold increase in the amount of radioactivity incorporated into the M.W. 47,000 protein. Under the same experimental conditions, no significant difference in the amount of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP incorporated into the M.W. 52,000 or M.W. 54,000 proteins of NB<sub>10%</sub> and NB<sub>1%</sub> cells was observed (Fig. 2).

The concentration-dependent photoactivated incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP into R<sub>I</sub> and R<sub>II</sub> cAMP-binding proteins of NB<sub>10%</sub> and NB<sub>1%</sub> cells is shown in the form of semilog dose-response plots in Chart 1. No significant difference in the affinity of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP for either the R<sub>I</sub> (M.W. 47,000) or the R<sub>II</sub> (M.W. 52,000 and M.W. 54,000) cAMP-binding protein of NB<sub>10%</sub> and NB<sub>1%</sub> cells was observed. The apparent dissociation constants (K<sub>d</sub>) for the incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP into R<sub>I</sub> and R<sub>II</sub> cAMP-binding proteins were 1 × 10<sup>-8</sup> M and 1 to 2

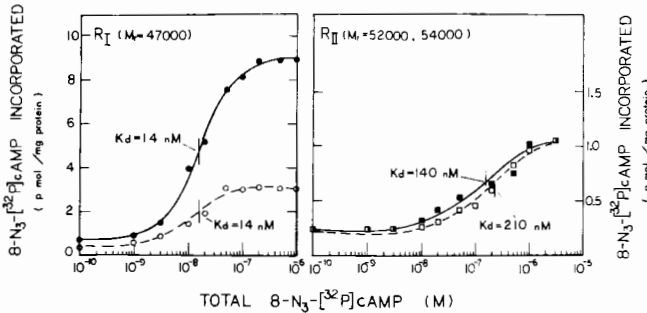


Chart 1. Semilog dose-response plots showing the concentration-dependent photoactivated incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP into R<sub>I</sub> (M.W. 47,000) and R<sub>II</sub> (M.W. 52,000 and M.W. 54,000) of NB<sub>10%</sub> (○, □) and NB<sub>1%</sub> (●, ■) cells. Cells at early stationary phase were harvested, and cytosols were prepared. The incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP was performed under standard conditions. The amounts of radioactivity incorporated into the M.W. 47,000 and the M.W. 52,000 plus M.W. 54,000 proteins were determined by liquid scintillation counting of the dried gel slices. Results are presented in pmol of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP incorporated per mg of cytosol protein. Note the difference in scale of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP incorporation for the R<sub>I</sub> and R<sub>II</sub> cAMP-binding proteins.

Table 1

Comparison of covalent binding of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP to reversible noncovalent binding of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP or [<sup>3</sup>H]cAMP

The photoactivated incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP into cytosolic R<sub>I</sub> and R<sub>II</sub> cAMP-binding proteins of NB<sub>10%</sub> and NB<sub>1%</sub> cells was performed under standard conditions. The reversible binding of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP and [<sup>3</sup>H]cAMP was done according to methods described. All assays were performed with 1 μM of the radioactive ligand and 200 μg of cytosol protein. Results are expressed per mg of cytosol protein.

	Covalent binding (pmol/mg protein)		Noncovalent binding (pmol/mg protein)	
	R <sub>I</sub>	R <sub>II</sub>	8-N <sub>3</sub> -[ <sup>32</sup> P]cAMP	[ <sup>3</sup> H]cAMP
NB <sub>10%</sub>	3.05 ± 0.8 <sup>a</sup>	1.1 ± 0.4	4.42 ± 1.07	3.86 ± 0.78
NB <sub>1%</sub>	9.21 ± 1.41	1.23 ± 0.27	11.75 ± 1.98	9.86 ± 1.47

<sup>a</sup> Mean ± S.E. of 3 independent determinations.

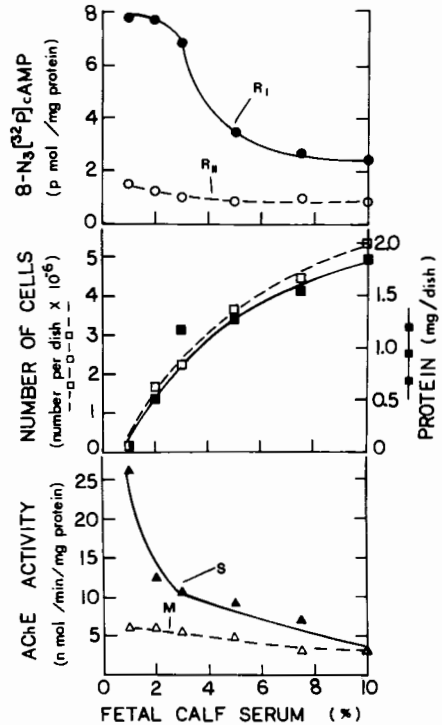


Chart 2. Effects of serum concentration on the amount of cytosolic R<sub>I</sub> cAMP-binding protein, cell growth, and acetylcholinesterase activity in N-18 neuroblastoma cells. Cells were seeded at a concentration of 1 × 10<sup>5</sup> cells/25 sq cm and were grown in medium supplemented with various concentrations of FCS as indicated. Cells were harvested at an early stationary phase of growth (2.1 days after plating). Cell counts were performed using a hemocytometer and were expressed in number of cells × 10<sup>6</sup> per 25 sq cm of growing surface. The incorporation of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP into cytosol proteins was done under standard conditions, using 1 μM 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP. The activity of acetylcholinesterase present in the 100,000 × g supernatant (S) and pellet (M) obtained from cell homogenate was determined according to methods described. Results shown are representative of 3 independent experiments.

× 10<sup>-7</sup> M, respectively. The K<sub>d</sub> values obtained for the R<sub>I</sub> and R<sub>II</sub> cAMP-binding proteins of N-18 neuroblastoma cells were in excellent agreement with values obtained for similar proteins from various rat and bovine tissues (36, 37).

At saturating concentrations of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP, the amounts of radioactivity incorporated (and the percentage of incorporation of total radioactivity) into R<sub>I</sub> and R<sub>II</sub> of NB<sub>10%</sub> cells were 3.18 pmol/mg protein (71%) and 1.05 pmol/mg protein (23%), respectively. The corresponding values of NB<sub>1%</sub> cells were, for R<sub>I</sub>, 9 pmol/mg protein (86%) and, for R<sub>II</sub>, 1.07 pmol/mg (10%).

In both the NB<sub>10%</sub> and NB<sub>1%</sub> cells, the total amounts of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP covalently incorporated into R<sub>I</sub> and R<sub>II</sub> cAMP-binding proteins were in agreement with the amount of specifically bound 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP or [<sup>3</sup>H]cAMP, as determined by the reversible binding procedure of Gilman (Table 1).

In Chart 2, the amount of R<sub>I</sub> cAMP-binding protein present in cytosol of N-18 neuroblastoma cells was studied as a function of serum concentration in the growth media. The rates of cell growth and acetylcholinesterase activity under these experimental conditions are also presented. In N-18 neuroblastoma cells, cell growth at logarithmic phase and final cell density at stationary phase are directly proportional to serum concentration. In the experiment illustrated in Chart 2, cells at early stationary phase were used. Morphological observations of these cultures indicated an inverse relationship between serum concentration and the proportion of cells with extended neu-

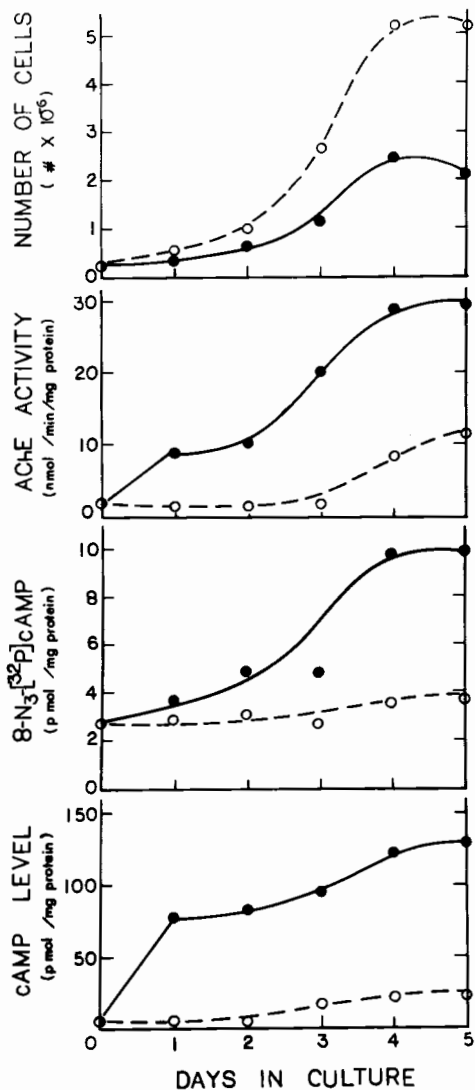


Chart 3. Cell growth, cytosolic acetylcholinesterase activity, incorporation of 8-N<sub>3</sub>[<sup>32</sup>P]cAMP into R<sub>i</sub> cAMP-binding protein, and intracellular cAMP concentration of NB<sub>10%</sub> (○) and NB<sub>1%</sub> (●) cells. Cells were plated on Day 0 at 2 × 10<sup>5</sup> cells/25 sq cm. On Days 1, 2, 3, 4, and 5 after plating, groups of ten to twenty 100 mm plates were harvested, and cytosols were prepared. All assays were performed according to methods described. Results shown are representative of 2 independent experiments.

rites. The incorporation of 8-N<sub>3</sub>[<sup>32</sup>P]cAMP into R<sub>i</sub> was (a) inversely related to serum concentration in the growth medium, (b) an inverse function of cell growth, and (c) correlated with an increased expression of acetylcholinesterase activity.

Previous studies on the regulation of cAMP binding activity in mouse neuroblastoma cells demonstrated the induction of cAMP-binding protein by cAMP or by agents which increase intracellular cAMP (29, 30, 35). Studies on a variety of mammalian cells indicated an inverse relationship between cellular growth and intracellular cAMP concentration (25). For example, growth stimulated by protease treatment of untransformed 3T3 fibroblasts is correlated with a decline in intracellular cAMP levels and can be prevented by Bt<sub>2</sub>cAMP (8). The transition of 3T3-4 fibroblast from the logarithmic to the stationary phase of growth is accompanied by an increase in intracellular cAMP (24). Most significant of all, Kram *et al.* (19) demonstrated that the effects of serum deprivation on several

general cellular biochemical processes related to growth of normal fibroblasts can be mimicked by treatment of these cells with cAMP or agents which increase intracellular cAMP in the presence of serum. Based on these and other similar observations, it has been suggested that lower levels of cAMP in rapidly dividing cells may have a significant effect on the increased growth rate.

In our present study, cell growth was manipulated by controlling the serum concentration in the medium. To investigate the possibility of whether the effect of serum deprivation on the induction of R<sub>i</sub> might be related to changes in cAMP concentration, we studied changes of R<sub>i</sub> and cAMP concentration in NB<sub>10%</sub> and NB<sub>1%</sub> cells as a function of their time course of growth (Chart 3). In this experiment, measurement of acetylcholinesterase was used as a biochemical index for the transition of neuroblastoma cells from the logarithmic to the stationary phase of growth (3). For both NB<sub>10%</sub> and NB<sub>1%</sub> cells, cell division was rapid and logarithmic in the early phase of growth. The generation times of NB<sub>10%</sub> and NB<sub>1%</sub> cells were approximately 22 and 30 hr, respectively. As the cells reached higher population densities, there was a significant decrease in the rate of cell division. At the stationary phase of growth, there was no net increase in cell count due to the balancing of cell growth by the shedding of cells from the substratum (approximately 1 to 2% of the entire population over a 24-hr period). In both the NB<sub>10%</sub> and NB<sub>1%</sub> cells, the transition from the logarithmic to the stationary phase of growth was accompanied by an increase in acetylcholinesterase activity; the magnitudes of such increases for NB<sub>10%</sub> and NB<sub>1%</sub> cells were approximately 8- and 3-fold, respectively. At all time points studied, the absolute activity of acetylcholinesterase in NB<sub>1%</sub> cells was considerably higher than that of the NB<sub>10%</sub> cells.

Measurements of the R<sub>i</sub> cAMP-binding protein demonstrated a 3.6-fold (260%) increase in the R<sub>i</sub> cAMP-binding protein of stationary-phase NB<sub>1%</sub> cells as compared to the rapidly dividing counterpart in the logarithmic phase of growth (Chart 3). A qualitatively similar but quantitatively different observation was made in NB<sub>10%</sub> cells; the magnitude of increase in R<sub>i</sub> of NB<sub>10%</sub> cells, during their transition from logarithmic to stationary phase, was 1.4-fold (42% increase). Under identical experimental conditions, no significant fluctuation in the amount of R<sub>ii</sub> cAMP-binding protein was observed for either the NB<sub>10%</sub> or the NB<sub>1%</sub> cells.

The mechanism by which serum regulates R<sub>i</sub> cAMP-binding protein appears to be related to intracellular cAMP concentrations. As illustrated in Chart 3, increases in cAMP levels were observed both during the transition from logarithmic to stationary phase and when cells were grown in medium containing 1% rather than 10% FCS.

The experiments presented above demonstrate that the R<sub>i</sub> cAMP binding activity of N-18 neuroblastoma cells can be regulated by serum concentration in the growth medium. One possible interpretation of this result is that serum (or serum factors) have a direct inhibitory effect on the expression of R<sub>i</sub>. Alternatively, it is possible that the increased expression of R<sub>i</sub> at low serum concentrations is keyed to differentiation and/or decreased cellular growth. In order to manipulate cell growth and differentiation without introducing variables such as serum concentration or time of growth in serum-containing medium, N-18 neuroblastoma cells were plated at 6 different densities. At the end of a 3-day growth period, the densely seeded cells

reached the stationary phase of growth, whereas the sparsely seeded cells continued in logarithmic growth. The amount of  $R_i$  cAMP-binding activity was then studied and compared. Results are shown in Charts 4 and 5 and in Fig. 3.

Chart 4 illustrates the growth rate of  $NB_{10\%}$  (A) and  $NB_{1\%}$  (B) cells at different seeding densities. In addition to the aforementioned effects of serum on cell growth and saturation density, the rate of increase in cell count is also dependent on the seeding density. Thus, densely seeded neuroblastoma cells, compared to the sparsely seeded population, showed reduced growth rates. Morphological observations of  $NB_{1\%}$  cells showed a high proportion of cells with long neurites at the stationary phase of growth.

A detailed examination of the incorporation of  $8-N_3-[^{32}P]cAMP$  into cytosolic proteins of  $NB_{10\%}$  and  $NB_{1\%}$  cells seeded at various cell densities revealed the following. (a) For  $NB_{1\%}$  cells, there were basically 2 patterns of incorporation reflected by cells at the logarithmic and the stationary phase of growth. Stationary-phase  $NB_{1\%}$  cells, regardless of seeding cell concentration, had significantly higher amounts of  $R_i$  cAMP binding activity than cells at the logarithmic phase of growth (Fig. 3; Chart 5B). Under identical experimental conditions, little or no change in the  $R_{ii}$  cAMP-binding protein was observed. (b) Unlike the tight coupling of the expression of  $R_i$  cAMP-binding protein to the stationary phase of growth in  $NB_{1\%}$  cells, the specific activity of  $R_i$  cAMP-binding protein of  $NB_{10\%}$  cells did not show dramatic changes as a function either of seeding cell concentration or of logarithmic versus stationary phase of growth (Fig. 3; Chart 5A). These results suggested that the expression of  $R_i$  was specific for stationary-phase  $NB_{1\%}$  cells. The fact that significant increases of  $R_i$  were not observed in early logarithmic-phase  $NB_{1\%}$  or stationary-phase  $NB_{10\%}$  cells

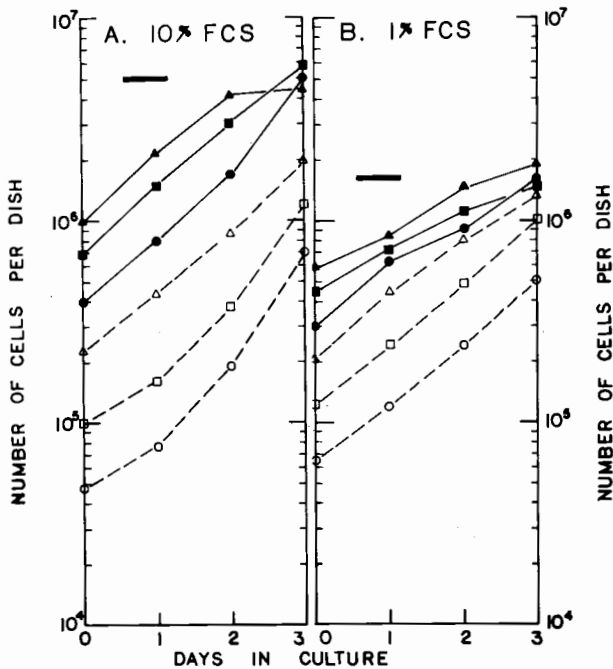


Chart 4. Growth rates of N-18 neuroblastoma cells at different cell and serum concentrations.  $NB_{10\%}$  (A) and  $NB_{1\%}$  (B) cells were seeded at 6 different concentrations. Cells were counted daily with a hemocytometer. Results are expressed in number of cells per dish (approximate growing surface, 25 sq cm). Bars, saturation densities of  $NB_{10\%}$  and  $NB_{1\%}$  cells.

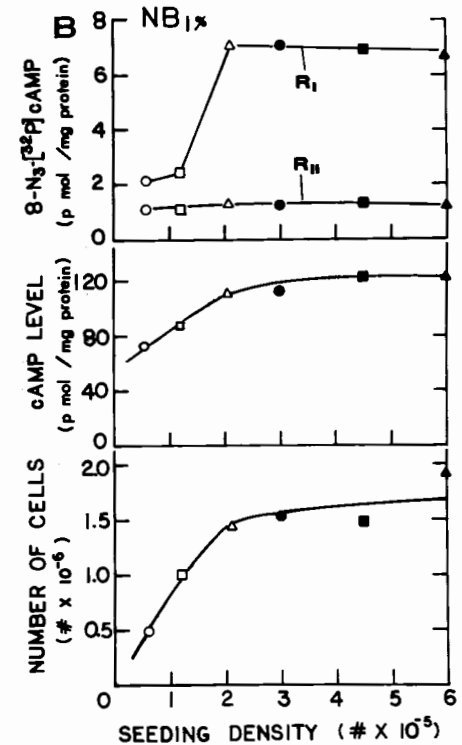
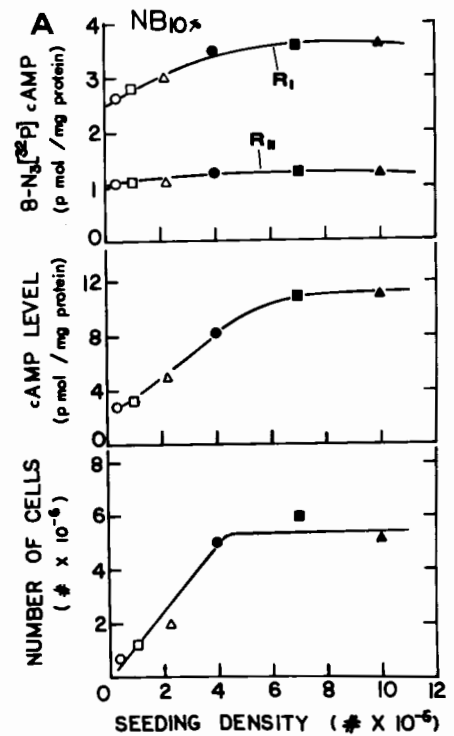


Chart 5. Studies on the incorporation of  $8-N_3-[^{32}P]cAMP$ , intracellular cAMP concentration, and cell growth as a function of seeding cell density. The various symbols used identify the corresponding cell populations depicted in Chart 4. Cells were harvested 3 days after plating. All assays were carried out according to the methods described. Note the difference in scales used for A and B.

suggested that neither the decrease in serum concentration alone nor the slowing of cell growth alone was sufficient to trigger the expression of  $R_i$  cAMP-binding protein.

The mechanism by which serum regulates  $R_i$  cAMP binding

activity in neuroblastoma cells is not known. Based on a number of studies (19, 32), as well as the results presented in this study, the possibility of cAMP serving as a second messenger seems plausible. Measurements of intracellular cAMP concentrations, expressed in pmol per mg protein, indicate elevated levels of cAMP in NB<sub>1%</sub> cells compared to the NB<sub>10%</sub> cells. Furthermore, in both the NB<sub>10%</sub> and the NB<sub>1%</sub> cells, there appears to be a relationship between cells at stationary phase of growth and increased intracellular cAMP (Chart 5).

## DISCUSSION

One of the major contributions from cell culture studies was the demonstration that proteins involved in cAMP metabolism could be adaptive. Thus, in S-49 lymphoma cells, intracellular phosphodiesterase activity fluctuates as a function of cAMP concentration inside the cell; this effect of cAMP is mediated through the activation of a cAMP-dependent protein kinase (4-6, 15). The causal relationship of the cAMP-induced increase in phosphodiesterase activity to the cAMP-mediated cytotoxic effects in S-49 lymphoma cells is not clear. In mouse neuroblastoma cells, the addition of Bt<sub>2</sub>cAMP to the growth medium causes morphological differentiation (28); it also produces increases in cAMP phosphodiesterase and cAMP-binding protein (29). The precise relationship between these cAMP-induced changes has not yet been resolved. Studies aimed at delineating the role of cAMP-binding protein in the differentiation of neuroblastoma cells have been hindered by the dual action of Bt<sub>2</sub>cAMP in both inducing differentiation and inhibiting cell growth. The use of Bt<sub>2</sub>cAMP in the mM concentration range, its possible effects on the intracellular nucleotide pool, and the growth inhibitory effects of butyrate certainly serve to complicate the issue even further.

In this report, we studied the regulation of cAMP binding activity by serum concentration in the growth medium. The results showed that the R<sub>i</sub> cAMP binding activity is negatively regulated by serum and that the regulatory mechanism may involve modulation of intracellular cAMP. That the induction of R<sub>i</sub> by serum deprivation is specific for cells at the stationary phase of growth and was not observed in cells at the logarithmic phase of growth suggests the unlikelihood of a direct repression by serum or serum growth factor(s) on the expression of R<sub>i</sub>.

Attempts were also made to dissociate the effects of slowing of cell growth from the effects of differentiation, a phenomenon incompatible with rapid growth, on the expression of R<sub>i</sub> cAMP-binding protein. Using NB<sub>10%</sub> cells, a comparison was made between the rapidly dividing cells in logarithmic phase of growth and the predominantly quiescent cells in stationary phase of growth; although some increase (40%) in R<sub>i</sub> was noted in the stationary-phase cells, its magnitude was not nearly comparable to that observed in the NB<sub>1%</sub> cells. Furthermore, the addition of 2% dimethyl sulfoxide to the culture medium inhibited cell growth by >50% without producing an increase in R<sub>i</sub> cAMP binding activity.<sup>4</sup>

The increased expression of R<sub>i</sub> cAMP-binding protein in stationary-phase NB<sub>1%</sub> cells, coinciding with the appearance of a significant population of highly differentiated and nondividing cells, suggests an intimate relationship between these 2 parameters. Nevertheless, in view of the varying degrees to which a differentiated morphology (length of neurite, number of neurites

per cell) is expressed in cells in tissue culture, the direct relationship between neurite extension and the expression of R<sub>i</sub> cAMP-binding protein remains to be documented at a single-cell level.

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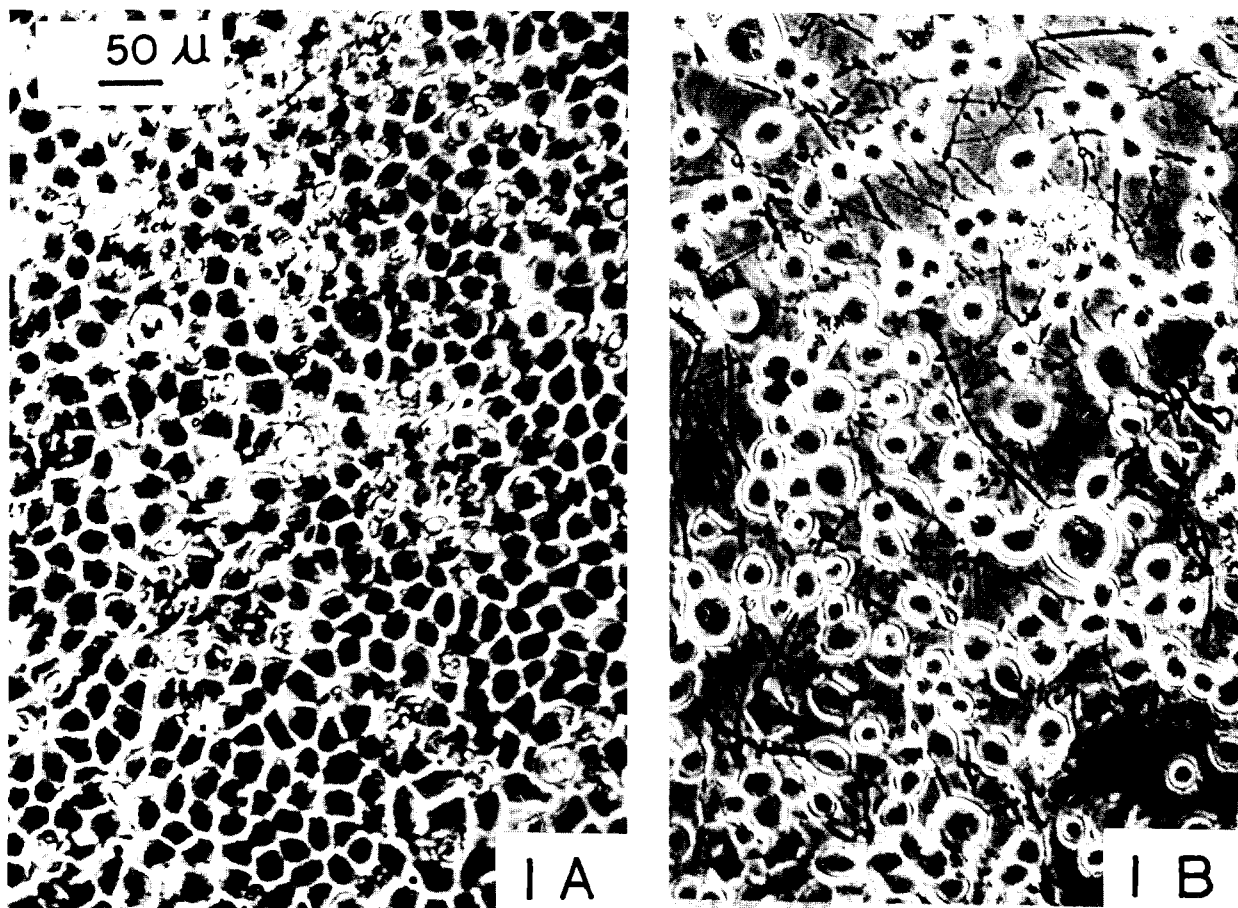


Fig. 1. Photomicrograph of stationary-phase NB<sub>10%</sub> (A) and NB<sub>1%</sub> (B) cells.