diabetes and 29 percent in all others including nondiabetics and type 1 diabetics (Table 1). This difference is statistically significant at $P < .001$, although our findings do not indicate that these large insertional elements are sufficient for, nor necessarily associated with, development of diabetes. While this work was being done, similar length polymorphism in the 5′-flanking region of the human insulin gene was reported in 8 of 15 individuals studied by Bell *et al.* (*6*). Insertions of 100 to 2600 bp were noted, which were not restricted to diabetics (*21*). In view of the known complexity of the genetic factors contributing to type 2 diabetes (*1*), it is not surprising that our data do not show a simple relation between the presence of insertions and disease. However, our results support a contribution to the etiology of diabetes from insertions 5′ to the insulin gene.

PETER ROTWEIN, ROSE CHYN
JOHN CHIRGWIN
Departments of Medicine and
Anatomy, Washington University,
St. Louis, Missouri 63110
BARBARA CORDELL
HOWARD M. GOODMAN
Howard Hughes Medical Institute
Laboratory, Department of
Biochemistry and Biophysics,
University of California,
San Francisco 94143
M. ALAN PERMUTT
Department of Medicine,
Washington University

References and Notes

1. J. I. Rotter and D. L. Rimoin, *Am. J. Med.* **70**, 116 (1981).
2. National Diabetes Data Group International Workgroup, *Diabetes* **28**, 1039 (1979).
3. S. S. Fajans, in *Endocrinology*, L. J. DeGroot, Ed. (Grune & Stratton, New York, 1979), vol. 2, p. 1007.
4. O. G. Kolterman, J. Insel, M. Saekow, J. M. Olefsky, *J. Clin. Invest.* **65**, 1272 (1980).
5. D. J. Weatherall and J. B. Clegg, *Cell* **16**, 467 (1979).
6. G. I. Bell, R. L. Pictet, W. J. Rutter, B. Cordell, E. Tischer, H. M. Goodman, *Nature (London)* **284**, 26 (1980); G. I. Bell, R. Pictet, W. J. Rutter, *Nucleic Acids Res.* **8**, 4001 (1980); A. Ullrich, T. J. Dull, A. Gray, J. Brosius, I. Sures, *Science* **209**, 612 (1980); R. V. Lebo, L. C. Yu, B. Cordell, M.-C. Chalving, J. C. Chong, A. V. Carrano, H. M. Goodman, Y. W. Kan, in preparation.
7. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
8. Y. W. Kan and A. M. Dozy, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5631 (1978); J. M. Taylor *et al.*, *Nature (London)* **251**, 392 (1974).
9. A. Boyum, *Scand. J. Clin. Lab. Invest. Suppl.* **97**, 6 (1968).
10. B. Cordell *et al.*, *Cell* **18**, 533 (1979).
11. We thank Gary Temple in Y. W. Kan's laboratory for furnishing a detailed protocol of the procedure.
12. G. M. Wahl, M. Stern, G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3683 (1979).
13. P. W. J. Rigby, M. Dieckmann, C. Rhodes, P. Berg, *J. Mol. Biol.* **113**, 237 (1977).
14. H. Harris and D. A. Hopkinson, *Hum. Genet.* **36**, 9 (1972).
15. A. J. Jeffreys, *Cell* **18**, 1 (1979).
16. S. C. M. Kwok, S. J. Chan, A. H. Rubenstein, R. Poucher, D. F. Steiner, *Biochem. Biophys. Res. Commun.* **98**, 844 (1981); B. D. Given *et al.*, *N. Engl. J. Med.* **302**, 129 (1980); H. Tager *et al.*, *Nature (London)* **281**, 122 (1979); K. H. Gabbay, R. M. Wolff, J. Wolff, M. Mako, A. H. Rubenstein, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2881 (1979); Y. Kanazawa, M. Hoyashi, M. Ikeuchi, M. Kasuga, Y. Oka, H. Sato, K. Kiramatsu, K. Kosaka, in *Proinsulin, Insulin, C-Peptide*, B. Shigeaki, T. Kaneko, N. Yanaihara, Eds. (Excerpta Medica, Amsterdam, 1979), p. 262.
17. A. R. Wyman and R. White, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6754 (1980); J. N. Strathern, J. B. Hicks, A. J. S. Klar, K. Nasmyth, *J. Supramol. Struct. Cell. Biochem. Suppl.* **5**, 399 (1980).
18. M. P. Calos and J. H. Miller, *Cell* **20**, 579 (1980); R. McKay, *Nature (London)* **287**, 188 (1980).
19. J. Corden, B. Wasylyk, A. Buchwalder, P. Sassone-Corsi, C. Kedinger, P. Chambon, *Science* **209**, 1406 (1980); R. Grosschedl and M. L. Birnstiel, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1432 (1980); L. H. T. Van der Ploeg, A. Konings, M. Oort, D. Roos, L. Bernini, R. A. Flavell, *Nature (London)* **283**, 637 (1980).
20. C. Wu and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1577 (1981).
21. G. Bell, personal communication.
22. We thank the Washington University Diabetes Research and Training Center and Drs. William C. Knowler and Barbara Howard for blood samples used in DNA preparation. Supported by NIH grant AM-16724, National Research Service Award AM-07120 (to P.R.), PHS career development award AM-00033 (to M.A.P.), and NIH grant CA-14026 (to H.M.G.).

8 May 1981; revised 24 June 1981

Growth Inhibition by Adenosine 3′,5′-Monophosphate Derivatives Does Not Require 3′,5′ Phosphodiester Linkage

Abstract. *Analogs of adenosine 3′,5′-monophosphate (cyclic AMP) inhibit the growth of cultured cell lines. The effects of 8-bromo- and N6-butyryl-substituted analogs of cyclic and noncyclic AMP on six cell lines were examined and were equally inhibitory. Variant cell lines with altered cyclic AMP-dependent protein kinase were more resistant to both cyclic and noncyclic nucleotides. We conclude that growth inhibition by analogs of cyclic AMP (i) does not require a 3′,5′ phosphodiester bond and (ii) may be mediated by a pathway involving endogenous cyclic AMP.*

Ryan and Heidrick (*1*) and Burk (*2*) reported that theophylline, a cyclic nucleotide phosphodiesterase inhibitor, and adenosine 3′,5′-monophosphate (cyclic AMP) inhibited the growth of BHK and HeLa cells, respectively. Subsequent studies with a wide variety of cultured cells demonstrated growth inhibition by cyclic AMP derivatives (*3, 4*). Such studies may indicate a role for cyclic AMP as a negative regulator of cell proliferation; however, growth stimulatory actions of cyclic nucleotides have also been reported (*5, 6*). In addition, studies with adenylate cyclase–deficient or protein kinase–deficient cell lines suggest a nonobligatory role for the nucleotide (*7*). Studies of the effects of cyclic AMP derivatives on growth are difficult to interpret because of the well-recognized biological potency of catabolites derived from the nucleotides in culture systems.

In previous studies (*8*) with cultured pituitary cells we reported that bromo and butyryl analogs of cyclic AMP inhibited growth by two distinguishable mechanisms. At low concentrations, adenosine derivatives were generated that resulted in cytotoxicity in adenosine-sensitive cells because of pyrimidine starvation. At higher concentrations in adenosine-resistant, adenosine kinase–deficient cell lines, both cyclic (3′,5′) and noncyclic (5′) AMP derivatives were growth inhibitory. In the studies reported here, we demonstrate that growth inhibition by cyclic AMP derivatives in a wide variety of cell lines does not require the 3′,5′ phosphodiester linkage. These results indicate that long-term effects of exogenous cyclic AMP derivatives are exerted by an unanticipated mechanism. However, additional studies also indicate that nucleotide-induced growth inhibition may involve both adenylate cyclase and cyclic AMP–dependent protein kinase.

As shown in Fig. 1, both 8-bromo and N6-monobutyryl cyclic AMP inhibited the growth of all cell lines examined. In each case, however, the corresponding 5′ AMP derivatives were equally potent. In several cell lines (L, CHO, and GH_1), bromo and butyryl derivatives (cyclic and noncyclic) were inhibitory over the same concentration range. In contrast, with BHK, BALB/c, and Y1 cells, 8-bromo nucleotides were more inhibitory than N6-monobutyryl nucleotides. Equipotency of cyclic and noncyclic derivatives was also observed with mono-, di-, and tributyryl nucleotides with 3T3 cells (not shown).

Noncyclic and cyclic AMP derivatives are extensively converted to adenosine derivatives during cell culture. Adenosine is cytotoxic to cultured cells as the result of inhibition of pyrimidine nucleotide biosynthesis (*9*). Growth inhibition reported in these studies cannot be mediated by such a pathway since uridine failed to prevent growth inhibition. Furthermore, similar growth inhibition by nucleotides is observed with adenosine-resistant, adenosine kinase–deficient cell lines (GH_1 and Y1; Fig. 1) (*10*).

The phosphodiesterase inhibitor the-

0036-8075/81/0904-1120$01.00/0

ophylline was itself growth inhibitory at concentrations used by others (*11*) and failed to potentiate effects of dibutyryl cyclic AMP. Extensive (~ 75 percent) degradation of monobutyryl cyclic AMP by exogenously added phosphodiesterase failed to influence growth inhibitory actions of the nucleotide (not shown). These results supported the conclusion that the 3′,5′ phosphodiester linkage was not essential for growth inhibition.

Several cell lines reported to have altered cyclic AMP–dependent protein kinase activity have been isolated as variants resistant to the growth inhibitory effects of 8-bromo cyclic AMP. As shown in Fig. 2, variant Y1 adrenal cells isolated by Rae *et al.* (*12*) and CHO variants isolated by Gottesman *et al.* (*13*) exhibited increased resistance to 8-bromo cyclic AMP or N6-monobutyryl cyclic AMP. Kinase variants also showed cross-resistance to corresponding 5′ AMP derivatives (Fig. 2). Cross-resistance of 8-bromo cyclic AMP–resistant, adenylate cyclase–deficient GH_1 cells to 8-bromo AMP has also recently been demonstrated (*8*).

Although numerous investigators have used exogenous cyclic AMP derivatives in culture systems, little is known about the metabolism and fate of these compounds. Extensive degradation of these compounds is known to occur in long-term cultures. Efforts to demonstrate direct transport of intact nucleotides into cultured cells have been limited and frequently unsuccessful (*4*). In spite of these uncertainties, it is widely assumed that high concentrations of externally applied nucleotides somehow mimic endogenous cyclic AMP.

In the studies reported here, derivatives of noncyclic AMP were as potent as cyclic AMP derivatives in inhibiting growth. It is possible that the similarity of the dose inhibition curves for the AMP and cyclic AMP derivatives was purely fortuitous. We regard this as unlikely since dose-for-dose equivalence was observed in six different cell lines in the three types of culture media with two types of nucleotide analogs. Equipotency of AMP and cyclic AMP derivatives may result from growth inhibition mediated by a common pathway distinct from adenosine-induced pyrimidine starvation. Our results suggest that such a pathway is related to the metabolism or action of endogenous cyclic AMP. Cell lines selected for resistance to 8-bromo cyclic AMP and characterized as having altered cyclic AMP–dependent protein kinase (*12, 13*) [or adenylate cyclase (*8*)] exhibited cross-resistance to AMP derivatives. Several modes of action of nucleotides relevant to endogenous cyclic AMP are possible and deserve further attention: (i) activation of adenylate cyclase by adenosine derivatives (*14*); (ii) conversion of nucleotide metabolites to cyclic AMP derivatives intracellularly

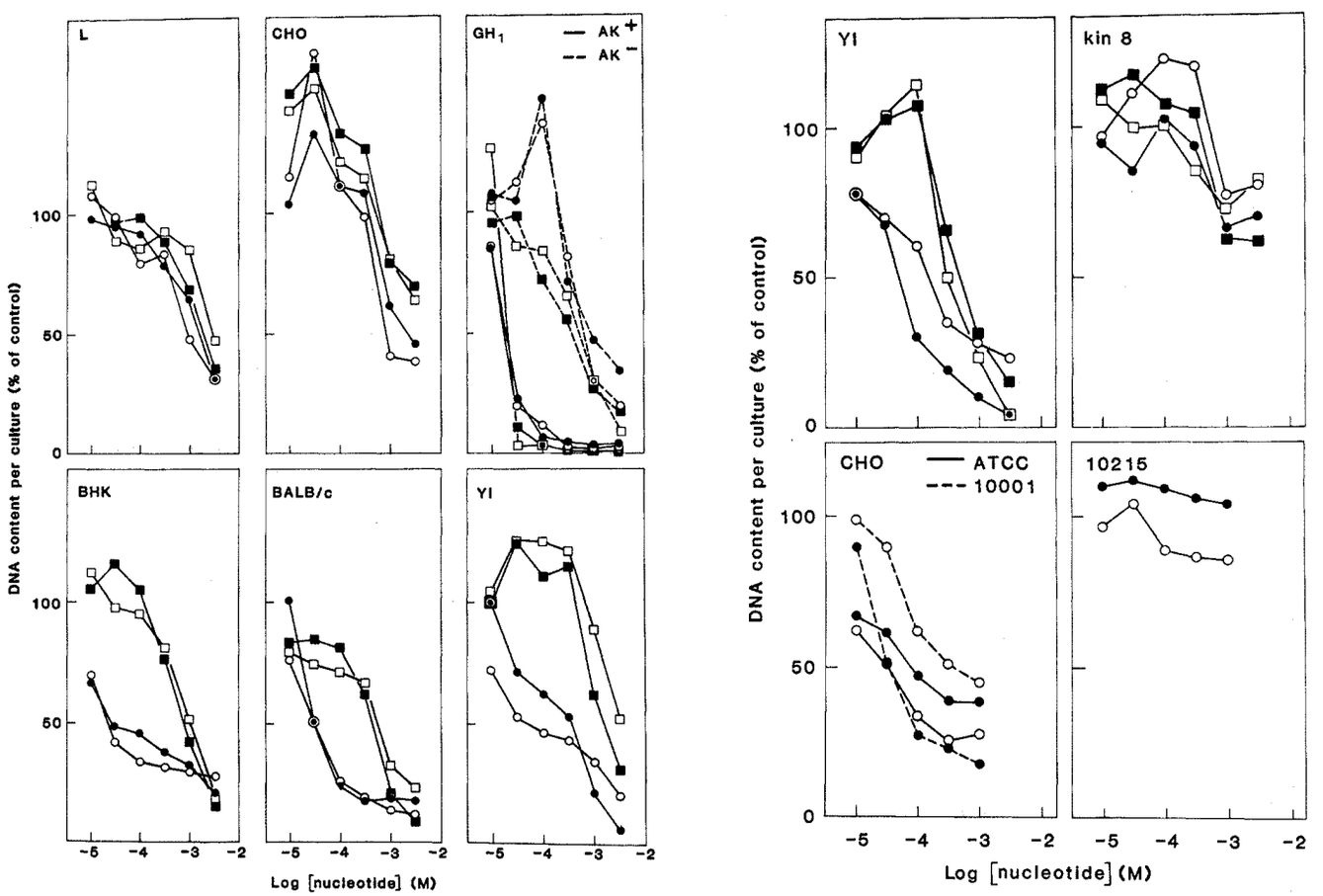

Fig. 1 (left). Growth inhibition of cultured cells by 8-bromo and N6-monobutyryl derivatives of cyclic and noncyclic AMP. Cells were inoculated into 1.6-cm wells of Linbro cluster trays (3×10^4 cells per milliliter). Sixteen hours after the cells were plated, nucleotides at the indicated concentrations were added: ●, 8-bromo cyclic AMP; ○, 8-bromo AMP; ■, N6-monobutyryl cyclic AMP; and □, N6-monobutyryl AMP. After incubation at 37°C with 5 percent CO_2, the cultures were terminated by aspirating the medium and scraping the cells into water. The cells were then disrupted by sonication. The DNA content of the cells was determined with the fluorescent dye 4′,6-diamidino-2-phenylindole according to methods similar to those of Brunk *et al.* (*18*). The sources of the cells, culture media used, and duration of growth were as described in (*19*). Fig. 2 (right). Growth inhibition of Y1 adrenal and CHO wild-type and kinase variants by cyclic and noncyclic AMP derivatives. Details of the culture methods, DNA determination, and description of symbols are given in the legend to Fig. 1 and (*19*). Wild type Y1 and variant kin 8 were provided by B. Schimmer, University of Toronto. The CHO wild type (10001) and kinase variant (10215) were obtained from M. Gottesman, National Institutes of Health.

(*15*); (iii) inhibition of cyclic nucleotide phosphodiesterases by metabolites (*16*); and (iv) conversion of adenosine-related metabolites to *S*-adenosyl homocysteine derivatives with consequent alteration of phosphodiesterase or adenylate cyclase (*17*). Alternative (i) is particularly attractive since it explains the basis for successful isolation of adenylate cyclase–deficient variants of Y1 adrenal (*12*) and GH pituitary cells (*8*) as 8-bromo cyclic AMP–resistant.

We cannot entirely exclude the possibility that AMP and cyclic AMP derivatives may act by a common pathway unrelated to cylcic AMP action. Variants that are 8-bromo cyclic AMP–resistant and protein kinase– and adenylate cyclase–deficient might have a more primary lesion in a cyclic AMP–independent pathway [such as methylation; see (*17*)]. However, our results do indicate that cyclic AMP derivatives inhibit growth by a novel pathway that does not require an intact 3′,5′ phosphodiester linkage.

THOMAS F. J. MARTIN
JUDITH A. KOWALCHYK
Department of Zoology, University of Wisconsin, Madison 53706

References and Notes

1. W. L. Ryan and M. L. Heidrick, *Science* **162**, 1484 (1968).
2. R. R. Burk, *Nature (London)* **219**, 1272 (1968).
3. I. H. Pastan, G. S. Johnson, W. B. Anderson, *Annu. Rev. Biochem.* **44**, 491 (1975).
4. F. J. Chlapowski, L. A. Kelly, W. B. Butcher, *Adv. Cyclic Nucleotide Res.* **6**, 245 (1975).
5. A. L. Boynton, J. F. Whitfield, R. J. Isaacs, R. G. Tremblay, *Life Sci.* **22**, 703 (1978).
6. J. M. Pawelek, *J. Cell. Physiol.* **98**, 619 (1979).
7. P. Coffino, J. W. Gray, G. M. Tomkins, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 878 (1975).
8. T. F. J. Martin and S. A. Ronning, *J. Cell. Physiol.*, in press.
9. L. J. Gudas, A. Cohen, B. Ullman, D. W. Martin, Jr., *Somat. Cell Genet.* **4**, 201 (1978).
10. We found that adenosine-sensitive cultures are ordinarily protected from adenosine-mediated pyrimidine starvation by the ample adenosine deaminase in calf serum. 2-Deoxycoformycin, an adenosine deaminase inhibitor, sensitized CHO and 3T3 cells to nucleotide inhibition (reversible by pyrimidines). In contrast, there was no effect of 2-deoxycoformycin on cultures in horse serum. Horse serum lacks adenosine deaminase and cultures in horse serum were sensitive to low concentrations of nucleotides (reversible with pyrimidines) (see GH_1-AK^+ in Fig. 1) unless they were adenosine-resistant, adenosine kinase–deficient (Y1 and GH_1-AK^-). Loss of adenosine kinase confers resistance to low concentrations of both cyclic and noncyclic nucleotides on cells cultured in horse serum. (see GH_1 in Fig. 1) (*8*).
11. G. S. Johnson and I. Pastan, *J. Natl. Cancer Inst.* **48**, 1377 (1972).
12. P. A. Rae *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1896 (1979).
13. M. M. Gottesmann, A. Le Cam, M. Bukowski, I. Pastan, *Somat. Cell Genet.* **6**, 45 (1980).
14. C. Londos, D. M. F. Cooper, J. Wolff, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2551 (1980).
15. T. P. Zimmerman, *Biochem. Pharmacol.* **28**, 2533 (1979).
16. C. M. Liang, Y. P. Liu, B. A. Chabner, *ibid.* **29**, 277 (1980).
17. T. P. Zimmerman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5693 (1980).
18. C. F. Brunk, K. C. Jones, T. W. James, *Anal. Biochem.* **92**, 497 (1979).
19. The sources of the cells, culture media used, and duration of growth were as follows: L929, American Type Culture Collection (ATCC), Dulbecco's minimum essential medium (DMEM) plus 10 percent calf serum (CS), 4 days; CHO-Kl, C. Raetz, University of Wisconsin, F10 plus 10 percent fetal calf serum (FCS), 4 days; GH_1, AK^+ from ATCC, AK^- isolated as described (*8*), MEM plus 7.5 percent serum (horse and fetal calf, in a ratio of 6:1), 5 days; BHK-21, ATCC, DMEM plus 10 percent CS, 4 days; BALB/c, I. Pastan, National Institutes of Health, DMEM plus 10 percent CS, 6 days; Y1 mouse adrenal, ATCC, F10 plus 15 percent serum (horse and fetal calf, 6:1), 5 days. The 8-bromo cyclic AMP and 8-bromo AMP were from Sigma; N6-monobutyryl cyclic AMP and N6-monobutyryl AMP were synthesized as described (*20*).
20. T. Posternak and G. Weiman, *Methods Enzymol.* **38C**, 399 (1974).
21. We thank J. Douros, National Cancer Institute, for providing the 2-deoxycoformycin. This research was funded by the National Institute of Arthritis, Metabolism and Digestive Diseases under grant AM 25861 and by the American Diabetes Association.

24 March 1981; revised 26 May 1981

Somatomedin B: Mitogenic Activity Derived from Contaminant Epidermal Growth Factor

Abstract. *The mitogenic effect of somatomedin B on human cultured glial cells was neutralized by the addition of antibodies to mouse epidermal growth factor. Somatomedin B contained epidermal growth factor–like activity, competing for binding to the epidermal growth factor receptor. It is concluded that contaminating epidermal growth factor may explain the entire mitogenic activity of somatomedin B.*

It was previously demonstrated in this laboratory that a polypeptide fraction derived from a somatomedin-enriched extract of Cohn fraction IV from human plasma had mitogenic effects on human quiescent glial cells in vitro (*1*). Charge and size fractionation indicated that the glial-cell stimulating activity was distinct from the activity promoting incorporation of $^{35}SO_4$ into cartilage chondroitin sulfate, present in the same preparation. These two activities were denoted somatomedin B (SM-B) and A (SM-A), respectively (*2*). No apparent relation seems to exist between SM-A and B (*3*) and, in fact, it has not been unequivocally demonstrated that SM-B conforms to the definition of a true somatomedin (*4*). SM-B has been purified to apparent homogeneity and its amino acid sequence has been determined (*5*). Highly purified preparations of SM-B show remarkably low specific mitogenic activity; in cultures of serum-deprived glial cells about 10 μg of SM-B per milliliter are required to produce an increase in DNA synthesis equivalent to that induced by 1 percent human serum (*6*). Authentic growth factors such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) are regularly three to four orders of magnitude more potent in the same system (*7, 8*). Trace contaminants (< 0.1 percent) of such factors may therefore have contributed to the observed biological effects, as suggested previously (*9*). Since EGF has physicochemical properties similar to those of SM-B it might appear as a contaminant in preparations of the latter. Experiments were therefore undertaken to investigate the involvement of EGF in the mitogenic activity of SM-B. It is demonstrated that most or all of the stimulatory effect of SM-B on glial cells may be ascribed to contaminant EGF.

Rabbit antibodies to pure mouse EGF (mEGF) were prepared as described (*10*). Exposure of SM-B to these antibodies, followed by absorption of the antigen-antibody complexes to Protein A Sepharose (*11*), markedly reduced the mitogenic activity of the resulting solu-

Table 1. Mitogenic activity of SM-B, hEGF, and PDGF after exposure to rabbit antibodies to mEGF. Fifty microliters of SM-B (*6*) (2 mg/ml), partially purified hEGF (*12*) (1 μg/ml), or partially purified PDGF (*8*) (1 μg/ml) in buffer containing 0.15*M* NaCl and 0.01*M* phosphate, *p*H 7.0, was mixed either with antibodies to mEGF (250 μg in 50 μl of the same buffer) prepared by passage of the rabbit immune serum through a column of Protein A Sepharose (*11*) or with plain buffer. After incubation at 22°C for 1 hour and at 4°C overnight, 100 μl of Protein A Sepharose was added. Tubes were kept for 1 hour at 22°C with occasional gentle mixing and then centrifuged. Portions of supernatants were assayed for multiplication stimulating activity, determined by the increase in [^{3}H]thymidine incorporation into DNA of serum-deprived glial cells as described (*8*), at a final concentration of 2 percent (by volume). The biological activity is recorded as the percentage of that given by 1 percent serum; in the presence of 1 percent human serum 60 to 70 percent of the cells were stimulated, as measured by autoradiography.

Growth factor preparation	Addition of antibodies to mEGF	Stimulatory activity (%)
SM-B	–	95
SM-B	+	29
hEGF	–	98
hEGF	+	40
PDGF	–	118
PDGF	+	119
None	–	0
None	+	34
Human serum (1 percent)	–	100

0036-8075/81/0904-1122$01.00/0