A nerve growth factor (NGF)-sensitive S6 kinase was purified by alkaline lysis of PC12 cells. The activity in lysates from NGF-treated cells was 10-20-fold higher than that from controls. Half-maximal stimulation of the S6 kinase by NGF treatment occurred in approximately 5 min, and the activity returned almost to basal levels by 2 h. A rapid purification method was devised in which crude extract was applied directly to a PBE 94 column after buffer exchange on a PD-10 column (Sephadex G-25 M). The activated S6 kinase was purified to at least 673-fold with a recovery of approximately 70%.

The S6 kinase has an apparent molecular weight of 45,000 and is highly specific for S6. It is not inhibited by the specific inhibitor of cAMP-dependent protein kinases, or by chlorpromazine or sodium vanadate, nor is it activated by Ca²⁺/calmodulin. It was inhibited by EGTA, β-glycerophosphate, or NaF. Phosphorylation occurred solely on serine residues.

The S6 kinase activity from control cells and from NGF-treated cells eluted at pH 5.69 and 5.58, respectively, during PBE 94 column chromatography. Pre-treatment of crude extract from NGF-stimulated cells with alkaline phosphatase resulted in an elution of the enzyme at the position of S6 kinase from control cells and a concomitant decrease in activity. These results indicate that phosphorylation is involved in the mechanism of S6 kinase activation.

Nerve growth factor (NGF) is a polypeptide required for the survival and development of sympathetic and sensory neurons (1–3). The molecular mechanism(s) by which it acts are not known, but there is evidence that changes in intracellular phosphorylation are involved (4–10). Among the phosphorylations influenced by nerve growth factor is the phosphorylation of the ribosomal protein S6 (5).

There have been several previous reports of activated S6 kinases in cell-free preparations from various stimulated cells. An insulin-sensitive S6 kinase has been seen in cell-free extracts of 3T3-L1 cells, and its characteristics suggested that it is similar to casein kinase I (11); it has been purified recently to which kinase is involved in the nerve growth factor-stimulated phosphorylation of S6.

The mechanism by which S6 kinase is activated is not known. Novak-Hofer and Thomas (14, 28) have studied the EGF-mediated activation of an S6 kinase in Swiss 3T3 cells and suggested that phosphorylation is involved in S6 kinase activation. Tabarini et al. (12) have suggested that the insulin-stimulated S6 kinase from 3T3-L1 cells is activated by phosphorylation by tyrosine protein kinase or protein kinase C. Blenis and Erikson (29) studied a transformation-sensitive S6 kinase seemingly regulated by the action of tyrosine kinase or protein kinase C. Erikson and Maller (30) also suggested that S6 kinase is regulated by protein tyrosine kinases. These reports are consistent in that they indicate that phosphorylation of S6 kinase is involved in its activation.

In a previous paper (31) we described a soluble S6 kinase from PC12 cells, the action of which is stimulated by prior treatment of the cells with nerve growth factor. In this study, we have purified this S6 kinase, explored its properties, and tried to understand the mechanism by which it is activated.

**EXPERIMENTAL PROCEDURES**

**Culture Conditions**

PC12 cells were cultured as monolayers in 150-cm² culture flasks in Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum, 7% horse serum, and 100 µg of streptomycin and 100 units of penicillin/mL. They were kept at 37 °C in an atmosphere containing 5% CO₂. The cells were split in a 1:4 or 1:1 ratio each week, and the medium was changed once during the week. The cells were treated in the culture flasks with one or more of the following factors, usually for 60 min: NGF (50 ng/mL), 5'-N-ethylcarboxamidodeadenosine (NECA) (1 X 10⁻⁴ M), dibutyryl cAMP (5 X 10⁻⁴ M). Control cultures were kept under similar conditions. After treatment, the cells were collected and washed by centrifugation (1100 X g, 5 min) as described previously (10).

**Preparation of Cell-free Extracts**

A lysis buffer (20 mM sodium borate, pH 10.2, containing 0.2 mM EDTA, 0.1 mM Na₂MoO₄, and 1 mM phenylmethylsulfonfyl fluoride) was added to the washed cell pellets and the mixture agitated on a Vortex mixer for 5 s to lyse the cells. The volume of lysis buffer used was 0.5 ml for cells from one flask. Since phenylmethylsulfonfyl fluoride is labile in aqueous solution, it was added to the lysis buffer.
from a stock solution made in n-propyl alcohol (1/100 dilution) just before the lysis buffer was added to the cells. The lysate was immediately neutralized with 1 M MOPS, pH 7.0, containing 100 mM MgCl₂ and 10 mM dithiothreitol to give pH 7.3 (10% (v/v) volume was used for neutralization). The preparations were centrifuged at 2 °C for 2.5 h at 125,000 × g. The supernatants typically contained about 0.5 mg of protein/ml. Protein concentration was determined by the dye-binding method of Bradford (32) using Bio-Rad reagents.

**Preparation of 40 S Ribosomal Subunits from Rat Liver**

Ribosomal 40 S subunits were prepared from livers of adult Sprague-Dawley rats according to the method of Thomas et al. (33). They were frozen and stored at −70 °C in buffer C (33) containing 50% glycerol.

**Assay for S6 Kinase Activity**

Rat liver 40 S ribosomal subunits were used as substrate for measuring S6 kinase activity. The assay mixtures contained a total volume of 100 μl using a reaction mixture containing 50 mM MOPS, pH 7.2, 10 mM MgCl₂, 1 mM dithiothreitol, 60 μM ATP, 2 μCi of [γ-32P]ATP, 0.34 A₅₀₀ unit of 40 S ribosomal subunits, and an S6 kinase preparation. The reaction was started by the addition of 40 S ribosomal subunits or a mixture of ATP and [γ-32P]ATP and the incubation continued for 30 min at 30 °C. The reaction was stopped by the addition of 100 μl of 0.5 M dodecyl sulfate sample buffer containing 125 mM Tris-HCl buffer, pH 6.7, 2% SDS, 20% glycerol, and 5% β-mercaptoethanol. The samples were boiled for 3 min and analyzed by SDS-gel electrophoresis on 10% polyacrylamide gels according to the method of Laemmli (34). The gels were stained with Coomassie Brilliant Blue, and autoradiograms were prepared by exposing the dried gels to Kodak XAR film at −70 °C using an intensifying screen (Du Pont Cronex Lightning Plus XA).

**Purification of S6 Kinase from PC12 Cells**

All procedures were carried out at 0-5 °C; the samples were never frozen. At each step of purification, the activity was assayed by the phosphorylation of 40 S ribosomal proteins from rat liver.

**Method 1—Cell-free extracts were prepared from cells in 50 flasks (13.8 mg of protein) as described above in the absence or presence, during the preparation, of 80 mM glycerophosphate and 20 mM EGTA. The extracts from control and from NGF-treated cells were filtered and applied to a PBE 94 column as described under "Method 1." All procedures were done quickly as possible.**

**Two-dimensional Polyacrylamide Gel Electrophoresis**

40 S ribosomal subunits from rat liver were incubated for 2 h with [γ-32P]ATP and the purified S6 kinase in the assay mixture described above. The reaction was terminated by the addition of 950 μg of unlabeled 40 S ribosomal subunits, 0.1 volume of 1 M MgCl₂, and 2 volumes of glacial acetic acid. The ribosomal proteins were extracted, precipitated with acetone cooled to −20 °C, and subjected to two-dimensional electrophoresis as described by Thomas et al. (35) except that the first-dimension electrophoresis was carried out with a slab gel instead of a glass tube for 30 min at 70 °V and then for 17 h at 180 V. The gel was cut with a thin knife blade to give a strip (1.5 mm × 12 cm × 6 mm) for the second-dimension gel electrophoresis. Gels were stained with Coomassie Blue, dried, and autoradiographed as described above.

**Phosphoamino Acid Analysis**

The analysis of amino acids phosphorylated in S6 by S6 kinase was performed by thin-layer cellulose electrophoresis as previously described (4) except that the electrophoresis buffer had a pH of 3.5 (acetic acid/pyridine/water, 50:5:945) (36) and the electrophoresis itself was carried out at 1000 V for 45 min.

**Treatment with Alkaline Phosphatase**

Alkaline phosphatase treatment was carried out for 30 min at 30 °C. One-half unit (1 unit is the activity that hydrolyzes 1 μmol of 4-nitrophenylphosphate in 1 min at 37 °C in 1 ml diethanolamine buffer, 10 mM 4-nitrophenylphosphate, 0.25 mM MgCl₂, pH 9.8) of calf intestinal alkaline phosphatase was added (0.01 volume) to 2.5 ml of the crude extract (1.1 mg of protein). The crude extract was prepared from 4 flasks by alkaline lysis and passage through PD-10 columns eluted with 25 mM imidazole HCl buffer, pH 7.0, as described above. The activity of alkaline phosphatase under the conditions adopted here (pH 7.0, 30 °C) was only 1-2% of that determined under the standard conditions (pH 9.8, 37 °C). Control reactions were conducted in a similar manner, but without alkaline phosphatase.

**Pretreatment of Cell-free Extract with cAMP-dependent Protein Kinase**

Cyclic AMP-dependent protein kinase was added for 10 min at 30 °C in 0.1 ml containing 50 mM MOPS buffer, pH 7.0, 10 mM MgCl₂, 1 mM dithiothreitol, 0.06 ml of extract (5.0 μg of protein), 5 units of CAMP-dependent protein kinase, and 10 μM ATP. The pretreatments were terminated by the addition of the inhibitor of CAMP-dependent protein kinase at concentrations sufficient to inhibit the transfer of 10 pmol of 32P to histone (Type V-S) by CAMP-dependent protein kinase. The 40 S ribosomal subunits were added and S6 kinase activity of this treated preparation measured. The cAMP-dependent kinase was reconstituted in deionized water containing 50 mg/ml dithiothreitol 10 min prior to use. Control reactions were conducted in a similar manner but with 50 mg/ml dithiothreitol without CAMP-dependent protein kinase.

**Pretreatment of Cell-free Extract with Protein Kinase C**

Protein kinase C was added for 3 min at 30 °C in 0.1 ml containing 20 mM Tris-HCl buffer, pH 7.5, 50 mM 2-mercaptoethanol, 5 mM phenylisothioureasulfonyl fluoride, 5 mM magnesium acetate, 0.5 mM CaCl₂, 12.5 μg/ml phosphatidylserine, 1.25 μg/ml diolein, 0.05 ml of extract (5.0 μg of protein), 1.5 units of protein kinase C, and 10 μM ATP according to Takai et al. (37). Control reactions were conducted in a similar manner without kinase C. The pretreatments were terminated by the addition of 0.5 mM ethylene glycol betaine (58). The 40 S ribosomal subunits were added and S6 kinase activity of this treated preparation measured.

**Materials**

NGF was purified by the method of Bocchini and Angeletti (39). NECA was a gift of Dr. Dean Londos, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD. [γ-32P]ATP was purchased from New England Nuclear. Purified S6 was kindly provided by Drs. Alan Lin and Ira Wool, University of Chicago. Protein kinase C purified from rat brain was a gift from Dr. Kuo-Ping Huang, National Institute of Child Health and Human Development, Be-
RESULTS

NGF-sensitive S6 Kinase—Cell-free lysates were prepared by an alkaline lysis method which has been used to disrupt cells gently for the preparation of plasma membranes (40), as described under "Experimental Procedures." Fig. 1A (lanes 1-5) shows the results obtained when S6 kinase activity in a lysate from control cells was compared to the S6 kinase activity in a lysate from NGF-treated cells. Increased phosphorylation of S6 ($M_\text{r}$ = 31,000) was consistently detected and was generally 10-20-fold greater in the cell lysates in the presence or absence of 40 S ribosomal subunits; no increase in the phosphorylation of any other protein was observed. This observed difference in specific activity was maintained even after S6 kinase activity from control or NGF-treated cells was purified by heparin-Sepharose CL-6B, gel filtration chromatography, and PBE 94 column chromatography and PBE 94 column chromatography (Fig. 1B, lanes 1 and 2) as described below.

**Purification of S6 Kinase**—S6 kinase was purified extensively from control and from NGF-treated PC12 cells. Soluble cell-free extracts, prepared as described under "Experimental Procedures," were subjected to heparin-Sepharose CL-6B column chromatography and elution with a linear gradient of 0-0.75 M NaCl. Two peaks of S6 kinase activity, designated I and II in their order of appearance from the column, were eluted by 30 and 130 mM NaCl (Fig. 2, A-D). Peak I is higher in extracts from NGF-treated cells; peak II is not. When the alkaline lysis of cells was carried out in the presence of 80 mM β-glycerophosphate and 20 mM EGTA, additions said to be required during the preparation of cell extracts in order to obtain fully active S6 kinase(s) in growth factor-stimulated Swiss 3T3 cells (14), the activity in peak I virtually disappeared (Fig. 2, E and F); the activity in peak II, it should be noted, was significantly stimulated. The further purification of the NGF-sensitive S6 kinase in peak I, then, was done in the absence of β-glycerophosphate or EGTA; in all subsequent steps this activity fractioned as a single peak. Peak I from the heparin-Sepharose CL-6B column was chromatographed on Sephadex G-200 (Fig. 3). The S6 kinase activity eluted with an apparent molecular weight of 45,000. The final step of purification was chromatography on a PBE 94 chromatofocusing column and elution with a pH gradient of 7-4 using Polybuffer 74. The amount of protein present at this point was too low to obtain a protein profile by the Bradford method (52). The S6 kinase activities from control cells and from NGF-treated cells were eluted at pH 5.69 and 5.58, respectively (Fig. 4). The S6 kinase activities from control cells and from NGF-treated cells was maintained throughout the purification.

It was impossible to add 40 S subunits at a concentration sufficient to saturate the enzyme, and thus, the specific activity values obtained may underestimate the activity. In any case, the final enzyme preparation from NGF-treated PC12 cells was purified approximately >15-fold from the crude extract with a very low recovery. The enzyme was very unstable; a great deal of activity was lost during the purification procedure, and the purified enzyme lost its residual activity completely within 15 h. The addition of dithiothreitol (1 mM) and Mg$^{2+}$ (10 mM) did not restore the lost activity and was not effective in preserving activity. Storage at $-80^\circ$ C in the presence of 50% glycerol also did not prevent inactivation. The peak in Fig. 4A was pooled, concentrated, electrophoresed on a 10% SDS gel, and silver stained by the procedure of Oakley et al. (41), but no protein band was detected.

In order to obtain an active purified preparation of S6 kinase, a rapid and improved purification method was developed (Purification Method 2). To provide a shorter handling time, generally, four cultured flasks were used to prepare one sample as described under "Experimental Procedures." For this improved method, the cell lysis was passed through a PD-10 column to exchange the buffer for that used in the subsequent PBE 94 column. In this one-step purification, the
A CONTROL

B NGF-TREATED

C CONTROL (EGTA AND β-GLYCEROPHOSPHATE)

D NGF-TREATED (EGTA AND β-Glycerophosphate)

E CONTROL

F NGF-TREATED

Fig. 2. Chromatography of S6 kinase activity on heparin-Sepharose. Cell-free extracts (13.8 mg of protein) from control or NGF-treated (50 ng/ml, 60 min) cells were prepared as described under "Experimental Procedures" in the absence or presence of β-glycerophosphate and EGTA, filtered through Millex-GS (0.22 μm, Millipore) filters, and applied to heparin-Sepharose CL-6B columns (12.5 × 0.9 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0. The column was washed with 1 bed volume of the same buffer at a flow rate of 0.52 ml/min and was eluted at the same flow rate with a linear gradient made up of 70 ml each of 0 and 0.75 M NaCl in the same buffer. Fractions of 2.1 ml were collected, and portions (10 μl) of each fraction were incubated with [γ-32P]ATP for 30 min at 30 °C according to the routine assay method. Samples were subjected to SDS-gel electrophoresis and autoradiography. A and B, elution profiles of S6 kinase activity from extracts prepared in the absence of β-glycerophosphate and EGTA from control cells (A) and from NGF-treated cells (B) determined by liquid scintillation spectroscopy of the 32P incorporated into the gel band corresponding to S6. The elution profiles of protein (---) were measured at 280 nm. C–F, autoradiograms from 10% SDS-polyacrylamide gels of the phosphorylation of each fraction from heparin-Sepharose CL-6B column chromatography. Cell-free extracts were prepared in the absence (C, D) or presence (E, F) of 80 mM β-glycerophosphate and 20 mM EGTA from control cells (C, E) and NGF-treated cells (D, F). Arrows indicate the position of ribosomal protein S6.

activity of S6 kinase was eluted at the same position as in Purification Method 1 as a single peak that was well resolved from the bulk of the protein in the preparation (Fig. 5). The S6 kinase was purified at least 673-fold (specific activity, >9500 pmol/min/mg of protein) with a recovery of activity of approximately 70%. Furthermore, the S6 kinase activity from NGF-treated cells was again eluted at a more acidic pH than was that from control cells. The specific activity of the S6 kinase in preparations from NGF-treated cells is 3-10-fold higher than that from control cells. This improved one-step purification method (Purification Method 2) was used to prepare enzyme for the following experiments.

Effect of Protein Concentration and Incubation Time on S6 Phosphorylation in Vitro—In order to choose optimal conditions under which to measure S6 kinase activity in vitro, the effect of an increasing concentration of an enzyme purified
by Purification Method 2 from cells treated for 60 min with NGF was examined. The results in Fig. 6A show that the amount of \(^{32}\)P incorporated into S6 in 30 min increases linearly up to the strength of an undiluted preparation (the protein concentration is less than 13 ng/assay) from a PBE column peak. The reaction is linear for almost 60 min (Fig. 6B). Therefore, in experiments described below, assays were carried out for 30 min with undiluted enzyme from the PBE column peak.

**Substrate Specificity**—The S6 kinase preparations obtained by both Purification Methods 1 and 2 were tested for their ability to catalyze the phosphorylation of S6, casein, phosphotyrosine, histones H4, H2B, and H1, mixed histone, and myelin basic protein. Of the substrates tested, the NGF-sensitive S6 kinase exhibited activity only toward S6 (Table I). The catalytic subunit of cAMP-dependent protein kinase also phosphorylates S6 strongly (specific activity, 11 nmol/min/mg of protein) under the assay conditions used in these experiments. However, in contrast, the cAMP-dependent protein kinase also catalyzed the phosphorylation of casein, phosphotyrosine, and histone H1 at least as well as did S6; its activity was inhibited by the protein inhibitor of cAMP-dependent protein kinase (data not shown).

**Properties of the Purified Enzyme**—The purified enzyme was found to be inhibited by several substances. As shown in Table II, EGTA, \(\beta\)-glycerophosphate, and NaF were potent inhibitors. The enzyme was also inhibited slightly by calmodulin. No effect was observed with chlorpromazine, sodium vanadate, or Ca\(^{2+}\). These results coincide with those obtained with crude extract (31). In addition, the inability of the protein inhibitor of cAMP-dependent protein kinase to block phosphorylation by the S6 kinase provides further evidence that this preparation is free of catalytic subunits of cAMP-dependent protein kinase.

**Phosphorylation of S6 in Vitro**—In order to confirm that the phosphorylated protein \(M_0 = 31,000\) shown in Fig. 1 was indeed S6 and to determine the distribution of radiolabel among the derivatives, 40 S ribosomal subunits were phosphorylated in vitro, extracted, analyzed by two-dimensional gel electrophoresis, and the gel subjected to autoradiography (Fig. 7). By superimposing the autoradiogram and the stained gel (Fig. 7A), phosphorylation by S6 kinase purified from NGF-treated cells can be seen as an increase in the radioactivity associated with S6 and corresponding shift in the mobility of phosphorylated S6 during two-dimensional electrophoresis (35); we can estimate that 4–5 phosphate groups were incorporated per S6 molecule, as shown by the spots designated d and e in Fig. 7B. With purified enzyme from control cells, only minor phosphorylation of S6 was observed (Fig. 7C). Phosphorylation of other ribosomal proteins was negligible. The phosphorylation of S6 in vitro occurred solely on serine residues (Fig. 8).

**Time Course of Nerve Growth Factor Effect**—To determine the time course of the NGF effect, cells were harvested at various times after NGF treatment, and cell-free extracts were prepared and purified by PBE 94 column chromatography (Purification Method 2) as described under "Experimental Procedures." The active fractions were pooled, and the pooled activity was measured. As shown in Fig. 9, half-maximal \(^{32}\)P incorporation into ribosomal S6 protein occurred in approximately 5 min. After 1 h of exposure to NGF, the S6 kinase activity was beginning to decrease and by 2 h had almost returned to the basal level.

**Effect of Pretreatment with Alkaline Phosphatase**—It is reasonable to suggest that the effects of NGF on S6 kinase shown in Figs. 4 and 5, that is, a higher specific activity and a shift of the elution position to a more acidic pH on the chromatofocusing PBE column, are brought about by phosphorylation of the kinase. To explore this possibility, the effect of pretreatment of the S6 kinase preparation with alkaline phosphatase was examined according to the method described under "Experimental Procedures." As shown in Fig. 10, pretreatment of cell-free extracts from NGF-treated cells with alkaline phosphatase causes a decrease in activity and a shift in the elution of the kinase to the position of S6 kinase from control cells. On the other hand, the S6 kinase in extracts from control cells showed no change in activity or subsequent elution pattern on the PBE 94 column due to pretreatment with alkaline phosphatase. These results strongly suggest that phosphorylation is involved in the activation of S6 kinase from PC12 cells by NGF. This experiment has been repeated five times with qualitatively similar results. When the S6 kinase purified by Method 1 under "Experimental Procedures" was used, qualitatively similar results were also obtained (data not shown).

**Effect of Dibutyryl cAMP, NECA, and Nerve Growth Factor on S6 Kinase Activity in PC12 Cells**—In a previous paper (31) we showed that treatment of intact PC12 cells with dibutyryl cAMP or NECA stimulated the subsequent cell-free phosphorylation of S6. It was suggested that although S6 kinase is not cAMP-dependent protein kinase per se, the action of a cAMP-dependent protein kinase is involved in S6 kinase activation. Figs. 11C and 12C show the elution patterns of S6 kinase from extracts prepared from the cells treated with dibutyryl cAMP or NECA on PBE 94 column chromatography. Each fraction was assayed in the presence of the inhibitor of cAMP-dependent protein kinase to block the direct action of cAMP-dependent protein kinase on S6. The elution patterns were indistinguishable from that produced by the treatment of PC12 cells by NGF alone (Figs. 11B and 12B). That is, the specific activity of S6 kinase is much higher in preparations.
from NGF-treated cells, and the elution position of the enzyme was shifted to a more acidic pH on the PBE column as compared to that of control cells (Figs. 11A and 12A). Furthermore, the extents of stimulation of phosphorylation achieved by the individual ligands were compared with those seen when the ligands were added in combination. The results of these experiments are shown in Figs. 11D and 12D. NGF and dibutyryl cAMP were not additive in the phosphorylation of S6 (Fig. 11D). The combination of NGF and NECA also was not additive (Fig. 12D), pointing to cAMP as a convergence point in the actions of NGF and NECA on S6 kinase stimulation. These results suggest that the enhanced phosphorylation of S6 in cells treated with NGF or cAMP occurs through a common mechanism. These experiments have been repeated three times with qualitatively similar results.

The Effect of Pretreatment with cAMP-dependent Protein Kinase or Protein Kinase C—Experiments were designed to see if an involvement of cAMP-dependent protein kinase or protein kinase C in the NGF-dependent activation of S6 kinase could be demonstrated directly. Cell-free extracts prepared by alkaline lysis from control or from NGF-treated cells were pretreated with the catalytic subunit of cAMP-dependent protein kinase or with protein kinase C as described under “Experimental Procedures.” The pretreatments were terminated by the addition of the inhibitor of cAMP-dependent kinase or chlorpromazine, respectively. Then 40 S ribosomal subunits were added and the S6 activity of the treated preparation measured. As shown in Fig. 13, pretreatment of cell-free extracts from control cells with the catalytic subunit of cAMP-dependent protein kinase caused an increase in S6 kinase activity (Fig. 13A, lanes 1 and 2), but it also elicits an increase in extracts from NGF-treated cells (Fig. 13A, lanes 3 and 4). More detailed experiments were carried out using PBE 94 column chromatography in order to clarify the involvement of cAMP-dependent protein kinase in S6 kinase activation (Fig. 14, A–D). In this experiment, the assay of S6 kinase was carried out in the presence of the inhibitor of cAMP-dependent protein kinase to avoid the direct involvement of cAMP-
according to the routine assay method. Samples were subjected to of each fraction were incubated with \([\gamma-P]ATP\) for 30 min at 30 °C. The elution profiles of protein kinase in S6 phosphorylation. Under these conditions, no activity of the catalytic subunits of cAMP-dependent protein kinase in the direct phosphorylation of S6 was observed (Fig. 14E). As shown in Fig. 14F, pretreatment of cell-free extracts from control cells with the catalytic subunit of cAMP-dependent protein kinase produces an increase in S6 kinase activity and causes it to elute at a more acidic pH region, but the elution profile of S6 kinase was not the same as that of S6 kinase from NGF-treated cells. Pretreatment of cell-free extracts from NGF-treated cells with the catalytic subunit of cAMP-dependent protein kinase elicited a comparable activation and shift of elution peak of S6 kinase on the PBE 94 column. But two peaks of S6 kinase activity were observed in the elution profile of pretreated extracts. One coincides with the activated S6 kinase from NGF-treated cells, and the other is eluted at an even more acidic region. This experiment has been repeated five times with qualitatively similar results. When S6 kinase prepared by Purification Method 1 under “Experimental Procedures” was used, qualitatively similar results were obtained (data not shown). Protein kinase C treatment had no effect on the activity of S6 kinase under the present conditions (Fig. 13B).

**DISCUSSION**

The results presented here describe a procedure for purifying an S6 kinase from PC12 cells highly specific for S6, the major phosphorylated protein in 40 S ribosomal subunits. The homogeneity of the purified enzyme could not be judged by SDS-polyacrylamide gel electrophoresis and silver staining because of the low amount of enzyme available. Furthermore, the NGF-sensitive S6 kinase is very unstable and loses most

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**Fig. 5. Chromatofocusing of cell-free extracts from PC12 cells on PBE 94.** Crude extracts (1.12 mg of protein from 4 flasks of cells) were prepared as described under “Experimental Procedures” from control cells or NGF-treated (50 ng/ml, 60 min) cells, filtered through Millex-GS (0.22 μm, Millipore) filters, and applied to PD-10 columns (Pharmacia) equilibrated with 25 mM imidazole HCl buffer, pH 7.0. The columns were eluted with the same buffer and fractions of 20 drops each (~0.7 ml) collected. The absorption of each fraction was read at 280 nm, and the peak fractions (4–6) were pooled and applied to PBE 94 columns (12.5 × 0.9 cm) and analyzed as described in the legend to Fig. 4. Fractions of 1.0 ml were collected, and portions of each fraction were incubated with \([\gamma-P]ATP\) for 30 min at 30 °C according to the routine assay method. Samples were subjected to SDS-gel electrophoresis and autoradiography. A, the elution profiles of S6 kinase activity from control cells (C) and from NGF-treated cells (D) were determined by liquid scintillation spectroscopy of the \(^{32}P\) incorporated into S6. The elution profiles of protein activity (—) were measured at 280 nm. The gradient of pH was measured by pH meter (—–). Autoradiogram from 10% SDS-polyacrylamide gels of extracts from control cells (B) and NGF-treated cells (C) of the phosphorylation of each fraction of PBE 94 column chromatography. Arrows indicate the position of ribosomal protein S6. D, the corresponding densitometer tracing of the autoradiograms shown in B and C. The fraction numbers are shown at the bottom.

**Fig. 6. S6 phosphorylation by purified S6 kinase from NGF-treated PC12 cells.** A, effect of increasing concentration of purified protein; B, time course of the reaction. 40 S ribosomal subunits (0.34 A₅₅₀ units) from rat liver were incubated with \([\gamma-P]ATP\) and the purified S6 kinase from NGF-treated PC12 cells (50 ng/ml, 60 min), and \(^{32}P\) incorporated into S6 was measured as described under “Experimental Procedures.” In A, increasing concentrations of the purified protein were incubated for 30 min at 30 °C. The relative concentration 1 indicates undiluted samples from the highest active fraction from PBE 94 column chromatography (Fig. 5A, less than 13 ng of protein/assay). In B, aliquots of the undiluted fraction from A (relative concentration, 1) were incubated for increasing times at 30 °C.
acrylamide and excision of the S6 band. Portions (10 μl, less than 13 ng of protein/assay) of fractions from the PBE 94 column were assayed with the indicated substrate. Two different enzyme preparations were used: incubations in Experiment 1 contained the enzyme prepared from NGF-treated PC12 cells by Purification Method 2 (Fig. 5A), while incubations in Experiment 2 contained the enzyme prepared from NGF-treated PC12 cells by Purification Method 2 (Fig. 5A).

**Table I**

| Substrate                  | Concentration | ^32P incorporated into substrate | Experiment 1 | Experiment 2 |
|----------------------------|---------------|---------------------------------|---------------|---------------|
| 40 S ribosomal subunits    | 0.50 mg/ml    | 413 (100)                       | 8235 (100)    |               |
| Casein                     | 1.14 mg/ml    | 7 (2)                           | 2 (0)         |               |
| Phosvitin                  | 0.57 mg/ml    | 8 (2)                           | 10 (0)        |               |
| Histone VI-S (fα)          | 0.28 mg/ml    | 0 (0)                           | 13 (0)        |               |
| Histone VIII-S (fα)        | 0.28 mg/ml    | 10 (2)                          | 7 (0)         |               |
| Histone H2B                | 0.28 mg/ml    | 0 (0)                           | 8 (0)         |               |
| Mixed histone              | 0.57 mg/ml    | 7 (2)                           | 0 (0)         |               |
| Histone H1                 | 0.25 mg/ml    | 3 (1)                           | 4 (0)         |               |
| Myelin basic protein (rabbit) | 0.28 mg/ml | 0 (0)                           | 0 (0)         |               |
| Myelin basic protein (porcine) | 0.28 mg/ml | 0 (0)                           | 0 (0)         |               |

**Table II**

Effect of various agents on nerve growth factor-sensitive S6 kinase from PC12 cells

Kinase reactions were carried out for 30 min at 30 °C as described under "Experimental Procedures" with the indicated additions. The radioactivity in S6 was quantified by gel electrophoresis and excision of the S6 band. Portions (10 μl, less than 13 ng of protein/assay) of fractions from the PBE 94 column were assayed in the presence of each drug at the indicated final concentration. Two different enzyme preparations were used; incubations in Experiment 1 contained the enzyme prepared from NGF-treated PC12 cells by Purification Method 1 (Fig. 4A), while incubations in Experiment 2 contained the enzyme prepared from NGF-treated PC12 cells by Purification Method 2 (Fig. 5A).

**Figure 7.** Two-dimensional gel analysis of 40 S ribosomal proteins phosphorylated by S6 kinase from PC12 cells. 40 S ribosomal subunits (0.34 A260 units) from rat liver were incubated with [γ-^32P]ATP and the purified S6 kinase (10 μl of active fraction shown in Fig. 5A) as described under "Experimental Procedures" except that the reaction was carried out for 2 h. The reaction was terminated by the addition of 950 μg of unlabeled 40 S ribosomal subunits, 0.1 volume of 1 M MgCl2, and 2 volumes of glacial acetic acid. The ribosomal proteins were extracted, precipitated with −20 °C acetone, and subjected to two-dimensional gel electrophoresis as described under "Experimental Procedures." A, the Coomassie Blue-stained gel (S6 kinase from NGF-stimulated cells). B and C, the corresponding autoradiograms of the region containing ribosomal protein S6 phosphorylated by the purified S6 kinase from NGF-stimulated cells (B) and control cells (C). In B, d and e represent the positions of S6 phosphorylated with 4 and 5 mol of phosphate/mol of S6, respectively. The arrows indicate the position of unphosphorylated S6.

of NGF-sensitive S6 kinase. Indeed, the purified S6 kinase from PC12 cells is also quite sensitive to these compounds.

It is important to compare the properties of this S6 kinase with the properties of other S6 kinases described by various investigators and especially to the properties of those shown to be stimulated by treatment of the cells with various peptide effectors. A significant feature of the present enzyme is its high degree of specificity for S6 relative to other substrates frequently used for protein kinases in *vitro*. The enzyme did not phosphorylate casein, phosvitin, various histones, or myelin basic protein. The S6 kinase is clearly distinct from cAMP-dependent protein kinase because it is unaffected by the specific inhibitor of cAMP-dependent protein kinases and because cAMP-dependent protein kinase can phosphorylate a number of the substrates described above. The S6 kinase studied here is not inhibited by chlorpromazine, a potent kinase C inhibitor (38). Ca2+/calmodulin does not stimulate the S6 kinase activity. Thus, the properties of the NGF-sensitive S6 kinase from PC12 cells also distinguish it from such well-defined kinases as protein kinase C, casein kinase,
and calmodulin-dependent protein kinase.

Lubben and Traugb (22) have reported that a partially purified preparation of protease-activated kinase II is an S6 protein kinase. The protease-activated kinase II proenzyme migrates on gel filtration with a $M_r = 80,000$, and the protease-activated species has a $M_r = 45,000–55,000$ (22). But, unlike the protease-activated species, the S6 kinase from PC12 cells does not phosphorylate histone H1, and our previous studies do not support a protease activation mechanism for the S6 kinase from PC12 (31). Erikson and Maller (30) reported the purification of S6-specific kinase from Xenopus eggs, but its molecular weight was found to be 92,000. Based on substrate specificity and molecular size alone, the NGF-sensitive S6 kinase is most similar to an insulin-stimulated S6 kinase from 3T3 cells; the latter has $M_r = 50,000-60,000$ (12).

A glia-derived neurite-promoting factor with protease inhibitory activity (42) has properties quite similar to those of...
this S6-specific kinase from PC12 cells; these include molecular size (Mr = 43,000 for the factor) and chromatographic behavior on both heparin-Sepharose CL-4B and Affi-Gel blue columns (data not shown). But neither uronokinase nor plasminogen activator-dependent caseinolysis was inhibited by the S6-specific kinase from PC12 cells (data not shown).

It is also important to know how S6 kinase is activated by prior treatment of the cells with nerve growth factor. Novak-Hofer and Thomas (14, 28) have studied the EGF-mediated activation of an S6 kinase in Swiss mouse 3T3 cells and have suggested the possibility that modification of the enzyme by phosphorylation might be sufficient to inhibit the transfer of 10 pmol of 32P to histone (Type V-S)/min by cAMP-dependent protein kinase inhibitor which is sufficient to inhibit the activation of an S6 kinase in Swiss mouse 3T3 cells and have suggested that phosphorylation is involved in the mechanism of S6 kinase activation. The data presented here support these previous hypotheses. These results include the observations of Erikson (29) and Maller (30) that S6-specific kinases in rat liver and Xenopus oocytes and also suggested that the S6 kinase is capable of being regulated by protein tyrosine kinases, either directly by tyrosine phosphorylation or indirectly through the action of one or more intermediates between protein tyrosine kinases and protein serine kinases. Thus, several previous reports have suggested that phosphorylation is involved in the mechanism of S6 kinase activation. The data presented here support these previous hypotheses. These results include the obser-
Fig. 14. Effect of pretreatment with cAMP-dependent protein kinase on elution profile of S6 kinase activity from the PBE 94 column chromatography. Soluble cell-free extracts (1.1 mg of protein) were prepared from 4 flasks of control and 4 flasks of NGF-treated cells, passed through PD-10 columns, pretreated with cAMP-dependent protein kinase, and applied to PBE94 columns as described under "Experimental Procedures." 40 S ribosomal subunits (0.34 A260 units) from rat liver were incubated with [γ-32P]ATP, and portions (10 μl) of each fraction were assayed in the presence of cAMP-dependent protein kinase inhibitor for 30 min at 30 °C according to the routine assay method. The reactions were stopped with SDS sample buffer and subjected to SDS-gel electrophoresis and autoradiography. A, untreated extract from control cells; B, untreated extract from NGF-treated cells; C, extract from control cells pretreated with cAMP-dependent protein kinase (71 units/ml); D, extract from NGF-treated cells pretreated with cAMP-dependent protein kinase (71 units/ml); and E, same as C and D, but without the addition of S6 kinase fractions. Arrows indicate the position of ribosomal protein S6. F, densitometer tracing of autoradiograms shown in A–E. The fraction numbers are shown at the bottom.

Observations that 1) activated S6 kinase from NGF-treated cells is eluted at a more acidic pH from a chromatofocusing column than the enzyme from control cells and 2) pretreatment of activated S6 kinase with alkaline phosphatase causes a deactivation and a shift of the elution position to that of S6 kinase from control cells. In this regard it is reasonable to mention that in some experiments alkaline phosphatase treatment of enzyme from NGF-treated cells produced a kinase that...
seemed somewhat less stable than comparably treated enzyme from controls. It may be that a simple phosphorylation-dephosphorylation mechanism is not sufficient to explain these transitions; some activity-influencing conformational changes may accompany the phosphorylation.

If S6 kinase from PC12 cells is activated by phosphorylation, it is of interest to know what kinase is responsible for the activation. We have reported recently (31) that treatment of intact PC12 cells with dibutyryl cAMP or NECA also increases the subsequent cell-free phosphorylation of S6 in vitro, suggesting that NGF may stimulate protein phosphorylation through an elevation of the intracellular cAMP level. This suggestion is supported by the present data that show that stimulation of protein phosphorylation elicited by saturating amounts of both NGF and NECA (or dibutyryl cAMP) is not additive. These results are consistent with previous data indicating that NGF raises intracellular cAMP levels and that cAMP can mimic some of the effects of NGF on the cells (43) and also support the results of Halegoua and Patrick (5) indicating that NGF, cholera toxin, and cAMP stimulate the phosphorylation of some proteins (such as S6) through a common mechanism.

The simplest interpretation of our results would be a model in which NGF binds to receptors which then activate adenylyl cyclase to elevate intracellular cAMP levels. cAMP-dependent protein kinase would subsequently be activated to phosphorylate its substrates, among them S6 kinase. To gain support for this model, we asked whether or not cAMP-dependent protein kinase directly phosphorylates and activates S6 kinase in vitro. Our data indicate that pretreatment of cell-free extracts from control cells with cAMP-dependent protein kinase results in an activation of S6 kinase activity, but the elution profile of the pretreated enzyme on PBE 94 column was not the same as that of activated kinase from NGF-treated cells. Furthermore, the pretreatment also elicits some activation of S6 in extracts from NGF-treated cells. To interpret the effect of cAMP-dependent protein kinase, there are two possibilities. 1) cAMP-dependent protein kinase does cause a phosphorylation-activation of S6 kinase in vivo but catalyzes a further incorporation of phosphate in vitro to produce a hyper-phosphorylated enzyme. In this case, if we choose more appropriate conditions, such as incubation time and enzyme concentration, we may be able to get an elution pattern on PBE 94 column comparable to that of activated S6 kinase from NGF-treated cells. 2) Pretreatment with cAMP-dependent protein kinase is artificial and does not reflect events in vivo.

Treatment of cell extracts from control or NGF-treated cells with protein kinase C had no effect on the activation of S6 kinase under the present conditions. This result is consistent with the fact that 12-O-tetradecanoylphorbol 13-acetate treatment of intact PC12 did not elicit an increase of S6 kinase activity (31). It should be noted that NGF activates a different pathway in S6 phosphorylation in PC12 cells than that of Nsp100 phosphorylation, in which protein kinase C is involved (44).

In conclusion, we can say from the results presented here that NGF causes an activation of cAMP-dependent protein kinase following elevation of intracellular cAMP level, and this cAMP-dependent protein kinase directly or indirectly causes the phosphorylation-activation of S6 kinase in PC12 cells. To confirm these results more experiments, including 1) an evaluation of which amino acid residue in S6 kinase is involved in its activation and 2) an inquiry into the relationship between the degree of phosphorylation and the degree of activation of S6 kinase are needed.

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