

Partial purification and characterization of a macromolecule which enhances fluoride activation of adenylate cyclase

(adenosine 3',5'-cyclic monophosphate/guanosine triphosphate/synaptic membranes/heat-stable modulator)

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ABSTRACT Fluoride activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] is significantly enhanced (2 to 5 times) by a protein factor isolated from rat brain. The fluoride-dependent adenylate cyclase stimulator (FCS) is nondialyzable, trypsin-labile, and stable at 90°C for 10 min. FCS stimulates adenylate cyclase activity only in the presence of NaF (2–25 mM) and this effect is independent of added GTP, 5'-guanylylimidodiphosphate, or calcium. FCS has been purified roughly 3000-fold from a 12,000 × *g* supernatant fraction of rat brain homogenate. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis and sucrose density gradient sedimentation suggest that FCS is a monomer with an apparent M_r of 59,000. Isoelectric focusing indicates FCS has a *pI* of 8.9. FCS from rat brain stimulates fluoride-activated adenylate cyclase from a variety of cell types, and FCS can also be isolated from rat liver. The effects of FCS are not reversed by washing membranes when the membranes and FCS are preincubated with NaF. The K_m of adenylate cyclase for ATP and the fluoride concentration causing half-maximal activation are unchanged by FCS; however, FCS increases the V_{max} by 2.5-fold. FCS may act to increase the catalytic efficiency of fluoride-activated complexes of the GTP-binding unit with adenylate cyclase or to enhance the formation of additional active complexes.

In 1958, Rall and Sutherland described a 4- to 10-fold activation of dog liver adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] by fluoride (1). Fluoride was originally added to prevent inactivation of glycogen phosphorylase and to inhibit the hydrolysis of ATP. However, it soon became clear that 1–25 mM fluoride ion specifically activates adenylate cyclase in a wide variety of tissues (2). Chloride and bromide also will stimulate cyclase, but only at concentrations >200 mM (3). In addition to activating adenylate cyclase, fluoride at concentrations between 2 and 25 mM is known to activate only one other enzyme—a light- and GTP-dependent cyclic GMP phosphodiesterase [PDE; 3':5'-cyclic-nucleotide 5'-nucleotidohydrolyase (EC 3.1.4.17)] found in vertebrate photoreceptors (4).

The activation of adenylate cyclase by fluoride is time- and temperature-dependent and is not reversed by simple washing when magnesium is present during preincubation (5). Prior activation of adenylate cyclase by cholera toxin proportionally diminishes the efficacy of fluoride (and vice versa) (6). Studies with detergent-solubilized adenylate cyclase preparations indicate that fluoride-dependent activation does not require the presence of hormone receptor (7). However, fluoride has been reported to inhibit the response to glucagon and epinephrine in adipocytes (8) although enhancing calcitonin response in renal cortex (9). Recent evidence with mutant cell lines (10) and detergent-solubilized turkey erythrocyte membrane preparations (11) indicates that a GTP-binding component participates in adenylate cyclase activation by fluoride. Although usually regarded as a phenomenon observed *in vitro*, recent evidence

indicates that dietary fluoride can increase adenosine 3',5'-cyclic monophosphate (cAMP) accumulation and basal adenylate cyclase activity in liver cells (12).

Recent studies in this laboratory have emphasized the striking similarities between light-activated photoreceptor PDE and the hormone-activated adenylate cyclase systems (13). In the photoreceptor system, a number of soluble proteins play an active role in the regulation of PDE activity. An interesting loss of hormone sensitivity appears as a concomitance of the transition between intact cell systems and particulate or subcellular membrane preparations from most regions of mammalian brain (14). In this context we wished to evaluate the possibility that soluble proteins, discarded during the preparation of synaptosomal particles, might participate (in analogy to the photoreceptor system) in the regulation of adenylate cyclase activity.

A variety of protein factors have been reported to stimulate adenylate cyclase. These include: the heat-stable calcium-binding protein, calmodulin (M_r about 20,000), that stimulates both adenylate cyclase and phosphodiesterase in the presence of 0.01–0.5 mM calcium (15); a soluble 1200-dalton rat brain peptide that activates rat brain synaptosomal adenylate cyclase (16); and certain, as yet uncharacterized, liver cytosolic factors that activate hepatic adenylate cyclase in conjunction with guanylyl nucleotides (17, 18).

We report here the partial purification and characterization of a soluble, heat-stable, calcium-independent, trypsin-labile macromolecule from rat brain that enhances the fluoride-induced activation of adenylate cyclase. This fluoride-dependent adenylate cyclase stimulator (FCS) exerts effects on particulate adenylate cyclase isolated from a variety of tissues.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley weanling (21 days old, 40–60 g) rats were used for the preparation of synaptosomes and other rat tissues. *Antheraea pernyi* (Chinese oak silk moth) were maintained in diapause until used and were a gift from Spencer J. Berry.

Preparation of Synaptic Membrane Fractions. Crude synaptic membranes were prepared by a modification of the method of Gray and Whittaker (19). Rats were killed by cervical dislocation, and the brains were quickly removed into ice-cold ringers. Cerebral cortices were separated and homogenized in 5 ml of standard medium [0.32 M sucrose/50 mM Tris maleate, pH 7.8/5 mM MgSO₄/1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)/1 mM dithiothreitol] per brain. The homogenizer was rinsed with

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Abbreviations: PDE, 3':5'-cyclic-nucleotide phosphodiesterase [3':5'-cyclic-nucleotide 5'-nucleotidohydrolyase (EC 3.1.4.17)]; Gpp[NH]p, 5'-guanylylimidodiphosphate; NaDodSO₄, sodium dodecyl sulfate; FCS, fluoride-dependent adenylate cyclase stimulator; cAMP, adenosine 3',5'-cyclic monophosphate (cyclic AMP); EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

an equal volume of standard medium, and the washings were combined with the homogenate and centrifuged at $600 \times g$ (5 min, 4°C). The supernatant fraction S_1 was collected and re-centrifuged at $10,000 \times g$ (20 min, 4°C) and the $10,000 \times g$ supernatant fraction S_2 and those of the subsequent wash steps were saved for the preparation of FCS. The $10,000 \times g$ pellet P_2 was resuspended in standard medium (10 ml per brain) and recentrifuged at $10,000 \times g$ for 20 min. The resulting pellet was resuspended in 5 ml of 10 mM Tris maleate, pH 7.5/1 mM EDTA/1 mM dithiothreitol per brain and recentrifuged. This was followed by an additional centrifugation with standard medium. The synaptic membrane-enriched pellet was resuspended in standard medium to achieve a protein concentration of 1–2 mg/ml and used for assay of adenylate cyclase. This preparation of synaptic membranes can be stored under liquid nitrogen for 30 days without loss of activity.

Rat hypothalamic membrane vesicles are prepared by the above method. Rat renal medulla membrane particles (20), rat liver membrane particles (21), myocardial membrane particles (22), and silk moth brain membrane particles (23) were prepared as described.

Adenylate cyclase was assayed with each type of particle suspended in the standard buffer used for its preparation. Unless otherwise noted, membranes (≈ 1 mg of protein per ml; 125 μg per assay tube) \pm fluoride \pm FCS were preincubated at 30°C for 15 min in 20 mM Tris maleate (pH 7.8; brain tissues) or Tris-HCl (pH 7.4; other tissues) containing 5 mM MgSO_4 and 1 mM dithiothreitol. After preincubation, the particles were placed on ice for 10 min. The adenylate cyclase assay (10 min, 30°C) was started by the addition of 0.5 mM ATP/0.5 mM isobutylmethylxanthine and 0.6 M phosphoenolpyruvate/pyruvate kinase (0.5 mg/ml). The reaction was stopped by boiling (4 min). The reaction volume was 50 μl and reaction rates were linear for >15 min. In the presence of the ATP-regenerating system, ATP concentrations (0.05–2 mM) remained constant for >15 min as measured by the luciferase assay (24), and this was not altered by the addition of FCS or 10 mM NaF. cAMP was assayed by a modification of the method of Brown *et al.* (25) by using a rabbit muscle cAMP binding protein. Values (pmol of cAMP per mg of protein per min) were corrected by subtraction of a boiled membrane blank. Recovery of cAMP added to a boiled membrane blank was $>98\%$. PDE activity was assayed as described (26).

Purification of FCS. Pooled rat cerebral cortex supernatant fractions (S_1 or S_2) were incubated at 90°C for 10 min and immediately cooled on ice. After centrifugation ($15,000 \times g$, 30 min, 4°C), the supernatant fractions were pooled. Finely powdered $(\text{NH}_4)_2\text{SO}_4$ was gradually added to the stirred ice-cold supernatant material to a concentration of 60% (wt/vol). The resulting solution was centrifuged ($40,000 \times g$, 60 min, 4°C), and the supernatant material was collected and dialyzed overnight against 10 mM Tris-HCl (pH 7.8) with 1.5 mM 2-mercaptoethanol. The dialysate (≈ 20 ml per brain) was lyophilized, resuspended in 0.5 ml of H_2O per brain, and centrifuged at $105,000 \times g$ for 2 hr. The resulting supernatant fraction DS_3 was concentrated and washed with an Amicon CF 50 filter cone.

Isoelectric Focusing. FCS was subjected to isoelectric focusing in an ISCO density gradient apparatus by using 22 ml of a 5–50% (vol/vol) glycerol gradient containing 4% (vol/vol) Ampholines (two parts pH 3–10 to one part pH 8–10.5). The anodic buffer was 0.25% phosphoric acid/5% glycerol and the cathodic buffer was 0.5% NaOH/50% glycerol. The sample (200–500 μg of protein) was dissolved in 25% glycerol, and 1 ml was applied to the center of the 22-ml gradient after 24 hr at prefocusing at 1000 V. Samples were focused for 48 hr at

Table 1. Effect of dialyzed supernatant fraction DS_3 on adenylate cyclase activity in cerebral cortex synaptosomal membranes

Addition	Adenylate cyclase activity*	
	Control	With DS_3
None (basal)	90 \pm 7.2	102 \pm 9.1
Isoproterenol (10^{-5} M)	84 \pm 7	106 \pm 8.5
GTP (10^{-4} M)	92 \pm 8.1	90 \pm 8.2
Gpp[NH]p (10^{-5} M)	228 \pm 15	192 \pm 15
NaF (10 mM)	240 \pm 12	576 \pm 16
NaF (10 mM) with heated DS_3 (90°C for 10 min)		562 \pm 11
NaF (10 mM) with trypsin-treated DS_3 †		244 \pm 12
NaF (10 mM) with 1 M acetic acid-treated DS_3 ‡		236 \pm 10
NaF (10 mM) with Amicon CF50-retained DS_3		568 \pm 12

* Values are expressed as pmol of cAMP produced per mg of protein per min \pm SEM and are means of triplicate determinations. The data shown are from one of three or more comparable experiments. Dialyzed supernatant fraction DS_3 was added at a protein concentration of 0.04 mg/ml.

† Trypsin digestion (1 mg/ml at 30°C for 10 min) was stopped by heating for 3 min at 100°C ; this additional heating did not significantly effect a non-trypsin-treated control.

‡ DS_3 was exposed to 1 M acetic acid for 30 min at 4°C . Acid was removed by washing in Amicon CF50 cone.

1000 V, and 1-ml fractions were dialyzed, lyophilized, resuspended in one-tenth of the original volume, and assayed for FCS activity.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out using a 4–30% (wt/vol) polyacrylamide (Pharmacia) gradient gel or an 8.5% polyacrylamide tube gel with a 3.5% (wt/vol) stacking gel (27). Gels were cut longitudinally and one-half was stained with Coomassie blue. The other half was cut into 1-mm slices, which were extracted overnight in 50 μl of 20 mM Tris-HCl, pH 7.5/5 mM MgSO_4 /1 mM dithiothreitol and assayed in the presence of the gel for FCS activity. Na-DodSO₄/polyacrylamide gel electrophoresis for molecular weight determination was performed in 7.5% polyacrylamide gels (with a 3% stacking gel) by using the discontinuous buffer

Table 2. Partial purification of FCS from rat brain

Purification steps	Total activity units* $\times 10^2$	Percentage of original activity†	Total protein, mg	Specific activity, units/mg*	Purification factor
105,000 $\times g$ dialyzed supernatant (DS_3)	380	100	865	44	1
Boiling 10 min	473	124‡	120	394	9
Ammonium sulfate fractionation	353	93	11.0	3.2×10^3	73
Isoelectric focusing	26.6	7	0.46	5.8×10^4	1326
Gradient gel electrophoresis	5.5	1.5	0.046	1.2×10^5	2727

* One unit of FCS activity represents a 50% increase in NaF-activated adenylate cyclase activity.

† Percentage of activity found in the dialyzed supernatant (DS_3).

‡ The increase in activity after boiling is, in part, due to the inactivation of PDE present in the starting material.

Table 3. FCS effect on adenylate cyclase activity in membrane preparations from various tissues

Additions	Adenylate cyclase activity*					
	Cerebral cortex	Hypothalamus	Silk moth brain	Liver	Renal medulla	Heart muscle
None (basal)	85 ± 6	51 ± 2	3.7 ± 0.4	3.9 ± 0.4	8.4 ± 0.8	16.7 ± 1
NaF	279 ± 14	77 ± 2	28 ± 2	39 ± 2.5	58 ± 1.5	30.4 ± 0.7
FCS	93 ± 5	50 ± 3	3.4 ± 0.4	2.5 ± 0.4	7.6 ± 0.7	15.8 ± 0.7
NaF with FCS	618 ± 19	174 ± 4	61.6 ± 4	112 ± 6	186 ± 7	71.7 ± 6

* Values are pmol of cAMP per mg of protein per min ± SEM for triplicate determinations. Data shown are from one of two or more similar experiments. FCS (0.06 μ g) purified through the isoelectric-focusing step was added where noted.

system of Laemmli (28). Samples and standards were reduced and alkylated immediately prior to electrophoresis (29).

Sucrose Density Centrifugation. FCS was centrifuged (18 hr) in 3.8 ml of a 5–20% (wt/wt) sucrose gradient (50,000 rpm, Beckman SW 56 rotor), and 0.2-ml fractions were assayed for FCS activity. Standards included: catalase, yeast alcohol dehydrogenase, cytochrome *c* (30), bovine serum albumin and ovalbumin. Proteins were determined by the method of Bradford (31).

RESULTS

Initial Findings: Activity and Stability of FCS. In the preparation of rat cerebral cortex synaptic membranes, three 10,000 \times *g* supernatant fractions generated in the washing steps of fraction S₂ were ordinarily discarded. When the S₂ 10,000 \times *g* supernatant fraction was recentrifuged (105,000 \times *g*, 1 hr, 0°C), dialyzed (DS₃), and added back to membranes, it did not significantly affect basal or GTP- or 5'-guanylimidodiphosphate (Gpp[NH]p)-stimulated adenylate cyclase activities. Isoproterenol, epinephrine, norepinephrine, serotonin, dopamine, and histamine did not activate this synaptosome-enriched adenylate cyclase preparation, in agreement with the observations of others (14). DS₃ failed to enhance synaptosomal adenylate cyclase responsiveness to any of these hormones. However, in the presence of 10 mM NaF and DS₃, we observed a striking enhancement of adenylate cyclase activity over that produced by fluoride alone (Table 1). FCS activity was non-dialyzable and stable at 90°C for 10 min. FCS was inactivated by 1 M acetic acid and was trypsin-labile. FCS activity was retained by an Amicon CF 50 filter cone (Table 1).

Effects of FCS on the Kinetics of Adenylate Cyclase Activation. Fluoride-dependent, half-maximal activation of adenylate cyclase in the presence or absence of FCS was observed at 25 mM NaF. Maximal activation of adenylate cyclase in the presence or absence of FCS was observed at 25 mM NaF.

Fluoride activation of adenylate cyclase increased the *V*_{max} with little change in the apparent *K*_m for ATP (2). Extensively purified FCS (Table 2, step 5) in the presence of NaF did not change the apparent *K*_m for ATP (0.3 mM by Lineweaver Burke, or Hofstee analysis) and increased the *V*_{max} 2.5-fold.

Using the extensively purified FCS, we observed half-maximal effects on the fluoride-dependent adenylate cyclase activation at a protein concentration of about 3 ng/ml. Saturation of this effect was seen at a protein concentration of 30 ng/ml, at which FCS and fluoride produced an activation 3 times that observed with fluoride alone.

Reversibility of FCS Activity. The reversibility of FCS activity was studied by preincubating synaptosomal membranes with FCS (1.2 μ g of protein per ml), NaF (10 mM), and MgSO₄ (5 mM) for 10 min. The membranes were washed once and assayed for adenylate cyclase activity. This resulted in a 2.6-fold increase in adenylate cyclase activity (560 pmol of cyclic AMP per mg of protein per min) as compared with controls in which

FCS was omitted from the preincubation step (214 pmol of cyclic AMP per mg of protein per min). When FCS was preincubated with synaptosomes in the absence of NaF, its effects were reversed by washing, as shown by subsequent incubation with NaF.

Preincubation with NaF and MgSO₄ without FCS also produced activation of adenylate cyclase, which was not reversed by washing; NaF produced adenylate cyclase activity of 214 pmol of cyclic AMP as compared with 96 pmol of cyclic AMP per mg of protein per min observed in the absence of a fluoride preincubation step. These findings are in agreement with experiments described previously by Perkins and Moore (5).

Effects of FCS on PDE Activity. The rat synaptic membrane preparation used for assay of FCS contained particulate PDE activity, which was inhibited about 60% by 0.5 mM isobutylmethylxanthine under the conditions of the cyclase assay. FCS neither stimulated nor inhibited PDE activity.

Comparison of FCS and Calmodulin. Calmodulin is a ubiquitous heat-stable protein that can activate adenylate cyclase and PDE in the presence of Ca²⁺ (15). Calcium (0.5 mM) and calmodulin (10 μ g/ml) in the presence of NaF increased adenylate cyclase activity by 80% over that of NaF alone (430 vs. 240 pmol of cyclic AMP per mg of protein per min). All cyclase assays were performed in the presence of EGTA (1 mM), conditions under which a sample of purified calmodulin (10 μ g/ml) (provided by Shiro Kakiuchi) had no effect on adenylate cyclase or PDE activity.

Tissue Responsiveness of FCS. Partially purified (Table 2, step 4) FCS and NaF were incubated with adenylate cyclases prepared from rat liver, rat renal medulla, silk moth brain, and rat myocardium. FCS enhanced NaF activation of adenylate

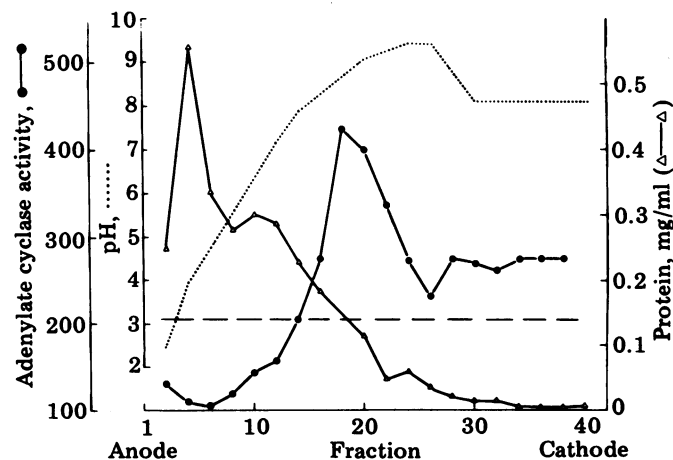


FIG. 1. Isoelectric focusing of FCS. Fractions (1 ml) of the glycerol gradient are assayed for FCS activity, which is expressed as adenylate cyclase activity in pmol of cAMP formed per mg of protein per min. FCS activity (●—●) is given as the increment in adenylate cyclase activity above that observed in the presence of 10 mM NaF (---).

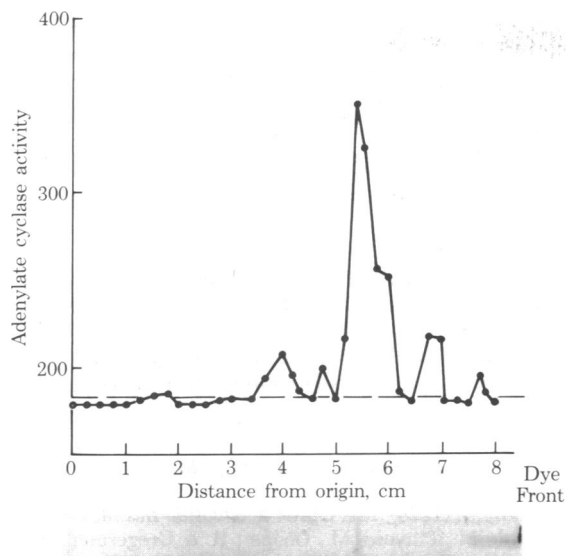


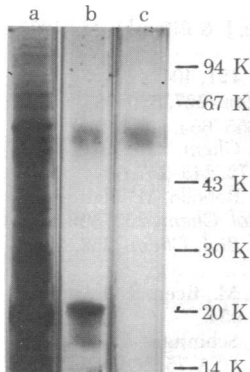
FIG. 2. Recovery of FCS activity from nondenaturing polyacrylamide gel electrophoresis. Gels are split longitudinally and assayed for FCS activity and Coomassie blue staining. FCS activity is expressed as in Fig. 1.

cyclase in these tissues. FCS was not found to influence Gpp[NH]p or hormone responsiveness in liver (glucagon) or kidney (vasopressin). FCS also enhanced NaF activation of adenylate cyclase activity in membranes prepared from rat hypothalamus, even though this preparation showed only a modest response to NaF alone (Table 3).

Purification of FCS: Ammonium Sulfate Fractionation and Column Chromatography. FCS was purified about 3000-fold from the $105,000 \times g$ synaptosomal supernatant, DS₃ (Table 2). The first purification step was a 10-min immersion in boiling water. This achieved a 9-fold purification without significant loss of activity (Tables 1 and 2). FCS activity remained in the $40,000 \times g$ supernatant fraction of a 60% saturated ammonium sulfate solution, and was entirely precipitated by 80% (NH₄)₂SO₄.

A variety of chromatographic materials (including DEAE-Sephadex, Sephadex G-100, G-150, G-200, Sephacryl S-200, and Bio-Gel P-100) were tried for purification of FCS. FCS displayed anomalous behavior with all of these materials, showing a smeared elution pattern and significant retention within the column. This problem was not ameliorated by varying the pH or ionic strength. A similar problem has been observed during the purification of other basic proteins such as interferon (32) and the synaptic phosphoprotein Protein I (33). Exposure of FCS to detergents (cholate, digitonin, and lubrol: 0.05% to 1%),

FIG. 3. Tentative identification of FCS in NaDodSO₄/polyacrylamide gel electrophoresis. Coomassie blue-staining of FCS preparations after various steps in the purification process. The molecular weight scale is derived from marker proteins run simultaneously. Marker proteins are: phosphorylase, M_r 94,000; albumin, M_r 67,000; ovalbumin, M_r 43,000; carbonic anhydrase, M_r 30,000; trypsin inhibitor, M_r 20,100; and α -lactalbumin, M_r 14,400. Lanes: a, ammonium sulfate fractionation (Table 2, step 3); b, isoelectric focusing (Table 2, step 4); c, polyacrylamide gel active peak (Table 2, step 5; Fig. 2).



acid (1 M acetic), or urea (2–8 M) at 4°C or 23°C produced >90% loss of activity.

Isoelectric Focusing. After ammonium sulfate fractionation, $100,000 \times g$ centrifugation, and Amicon CF 50 filtration (which decreased the amount of <50,000-dalton components), a complex protein mixture remained (see Fig. 3, lane a). Isoelectric focusing considerably reduced the number of protein species (see Fig. 3, lane b) and revealed an FCS activity peak with a pI of 8.9 (Fig. 1). This step provided a 1300-fold purification and a recovery of 7% (Table 2).

Polyacrylamide Gel Electrophoresis. The active fractions from isoelectric focusing were concentrated (15-fold) in an Amicon CF 50 filter cone and electrophoresed in a 4–30% polyacrylamide gradient gel. Following electrophoresis, the FCS activity peak fraction was eluted, concentrated with an Amicon CF 50 filter cone, and electrophoresed in an 8.5% polyacrylamide gel. FCS activity comigrated with the major Coomassie blue staining species in this gel (Fig. 2).

Molecular Weight in NaDodSO₄/Polyacrylamide Gel Electrophoresis. The gel region of FCS peak activity (54–56 mm from the origin; see Fig. 4) was excised from replicate gels. These regions were eluted into NaDodSO₄ sample buffer, reduced, alkylated (29), and electrophoresed (Fig. 3, lane c). The visible Coomassie blue-stained component in this preparation is a monomer with an apparent M_r of 59,000. Molecular weight determination in sucrose density gradient.

Molecular Weight Determination in Sucrose Density Gradients. Isoelectrically focused FCS was centrifuged in a (5–20%) sucrose density gradient to estimate the M_r under nondenaturing conditions. When compared to standards of known M_r and sedimentation velocity, after the method of Martin and Ames (30), FCS had an $S_{20,w}$ of 4.20 and an apparent M_r of 59,000.

FCS Activity from Rat Liver. FCS isolation was also carried out from rat liver homogenate through the isoelectric-focusing step (Table 2, step 4). This produced an activity profile similar to that observed with material isolated from rat brain (see Fig. 1). In the peak fraction, FCS activity was 4×10^4 units/mg of protein as compared with 5.8×10^4 units/mg for FCS from rat brain.

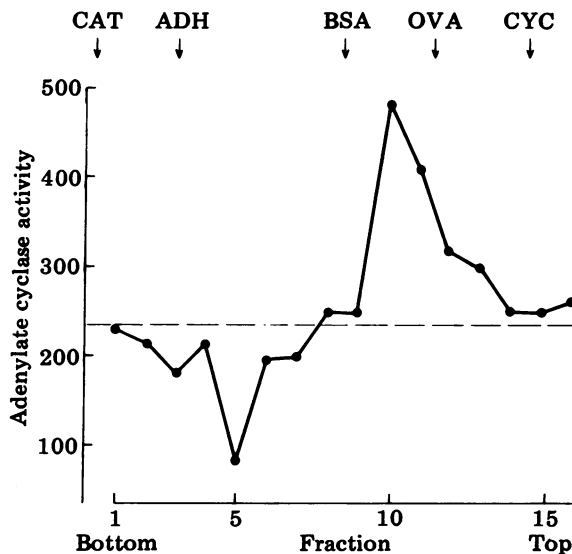


FIG. 4. Sedimentation of FCS on a continuous sucrose density gradient. Isoelectrically focused FCS is loaded onto the top of a 5–20% (wt/wt) continuous sucrose gradient centrifuged and assayed as described. FCS activity is expressed as in Fig. 1. Markers are catalase (CAT), 240,000; alcohol dehydrogenase from yeast (ADH) 140,000; bovine serum albumin (BSA), 67,000; chicken ovalbumin (OVA), 43,000; and cytochrome c (CYC), 12,500.

DISCUSSION

FCS is a heat-stable protein from rat brain (and liver) that enhances fluoride stimulation of adenylate cyclase in a variety of tissues and that appears unrelated to previously reported endogenous adenylate cyclase-activating factors (15–18). Data obtained with NaDodSO₄/polyacrylamide gel electrophoresis and sucrose density gradient centrifugation suggest that FCS is a monomer with an apparent *M_r* of 59,000. Because we did not recover FCS activity from NaDodSO₄ gels, we cannot entirely discount the possibility that a weakly staining protein comigrates with the 59,000 molecular weight material(s) through all previous purification steps and the sucrose density gradient analysis. Such material in the presence of NaDodSO₄ could dissociate into subunits and escape detection in stained gels. Further studies are needed on the molecular weight, subunit composition, and chemistry of FCS.

Adenylate cyclase inhibitory activity was consistently found in fractions 4–8 of the isoelectric focusing procedure (Fig. 1) and fractions 4–6 of the sucrose density gradient (Fig. 4). The material recovered in these fractions had no effect upon PDE activity in synaptic membranes. The inhibition of adenylate cyclase is associated with heat-stable factor(s), because the starting material for FCS purification was subjected to 90°C for 10 min (Table 2). This inhibitory activity did not require NaF; a similar depression of basal- and Gpp[NH]p-stimulated adenylate cyclase activities was noted when the above fractions (4–8) were added to membranes from cerebral cortex, hypothalamus, or renal medulla (data not shown).

Fluoride stimulation of adenylate cyclase is not reversed by washing (5), and the stimulatory effect of FCS in the presence of NaF is also irreversible. Neither fluoride nor fluoride in combination with FCS changed the adenylate cyclase *K_m* for ATP (2). The concentration of NaF that gives half-maximal activation of adenylate cyclase is also unaffected by FCS.

Several investigators have found an absolute requirement for the GTP-binding unit in the fluoride activation of adenylate cyclase (10, 11, 34). In the absence of FCS, fluoride and guanyl nucleotides (GTP or Gpp[NH]p) (34) appear to compete for a common locus in the activation of adenylate cyclase. Guanyl nucleotides, however, cannot substitute for fluoride in the FCS-mediated enhancement of adenylate cyclase activation.

The GTP-binding unit of rat brain adenylate cyclase behaves as an integral membrane protein (35). On the other hand, FCS behaves as a soluble protein. It is not clear that the brain synaptosomal preparation used for adenylate cyclase assay is entirely free of endogenous FCS. Contamination with endogenous FCS would reduce the apparent efficacy of the purified material added *in vitro*.

Some simple alternatives appear possible as models for FCS activity: (i) FCS could increase the catalytic efficiency of those GTP-binding unit–cyclase catalytic-moiety complexes that are stimulated by fluoride; (ii) FCS could function to recruit additional GTP-binding unit–cyclase catalytic-moiety complexes that, in the absence of FCS, are unavailable for interaction with fluoride. This seems unlikely since cyclase activation by Gpp[NH]p is not enhanced by FCS (Table 1); (iii) FCS might act as a coupling agent to join those guanyl nucleotide-binding units that have interacted with fluoride to previously unavailable catalytic moieties of adenylate cyclase. Identification of the actual mechanism of FCS action awaits further study.

The activation of adenylate cyclase by fluoride has been regarded as an *in vitro* phenomenon; it has been difficult to attribute physiological meaning to an activation that depends upon concentrations of fluoride not ordinarily encountered in the cell. However, the possibility exists that the activation produced by fluoride and enhanced by FCS could mirror en-

dogenous activation produced by a putative physiological analogue of fluoride. Although both fluoride and Gpp[NH]p are thought to activate adenylate cyclase via interaction with a GTP-binding unit, FCS enhanced only that activation that was mediated by fluoride (Tables 1 and 3). FCS thus may serve as a useful tool for the study of those specific aspects of fluoride-dependent cyclase activation that are not shared by GTP and its analogues.

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