PURIFICATION AND CHARACTERIZATION OF A CHOLINERGIC TROPHIC FACTOR FROM RAT SKELETAL MUSCLES

by

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A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science.

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To my parents

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ABSTRACT

Studies have shown that a soluble protein factor in muscle extracts causes a significant increase in choline acetyltransferase (CAT) activity in dissociated spinal cord neurons in culture. A reproducible procedure for the isolation of CSF from rat skeletal muscle extracts has been established. A bioassay using the neuroblastoma x glioma hybrid cell line NG108-15 was used to screen for the CSF at different steps of the isolation procedure.

The CSF in the muscle extract was first precipitated by 50% of ammonium sulfate, followed by adsorbing onto a CM-Cellex column at pH 6.2, and eluted stepwise by increasing concentrations of NaCl. The active fraction was subsequently fractionated from other contaminating proteins by DE-Cellex chromatographies at pH 7.4, and eluted by a linear and a continuous compound gradient of increasing concentrations of NaCl. The CSF was finally purified by gel filtration on Sephadex G-100.

The muscle CSF has been purified to 13,333-fold and appears to be a homogeneous preparation consisting of only one major protein band upon polyacrylamide gel electrophoresis. By analytical thin gel isoelectric focusing, the isoelectric point of CSF has been determined to be 4.75. The molecular weight of CSF has been estimated to be 30,000 daltons by the method of gel filtration on Sephadex G-100, and 28,000 daltons by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. CSF stimulated CAT activity in dose-dependent manner with a half maximal effect at 15 ng/ml.

These physical properties distinguish the CSF from the Nerve Growth Factor and other putative neuronotrophic factors. The availability of a highly purified CSF preparation is essential for further elucidation of the biochemical properties and physiological role(s) of this neuronotrophic factor. It is speculated that CSF may have an important role in the differentiation and maturation of spinal cord neurons.

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LIST OF ABBREVIATIONS

Units of Measurement

g = gram

mg = milligram

ug = microgram

ng = nanogram

ml = milliliter

ul = microliter

cm = centimeter

cm^2 = square-centimeter

mm = millimeter

min = minute

hr = hour

M = molar

mM = millimolar

pmole = picomole

uCi = microcurrie

i.u. = internationl unit

mA = milliampere

General Terms

PBS = phosphate buffer saline

NaCl = sodium chloride

dBcAMP = N^6, $O^{2'}$-dibutyryl-cyclic 3', 5'-adenosine monophosphate

EDTA = ethylenediamine tetracetate acid

TRIS = TRIS (hydroxymethyl) aminomethane

$(NH_4)_2SO_4$ = ammonium sulfate

NH_4HCO_3 = ammonium bicarbonate

KPO_4 = potassium phosphate

$NaPO_4$ = sodium phosphate

SDS = sodium dodecyl sulfate

PAGE = polyacrylamide gel electrophoresis

pI = isoelectric point

M.W. = molecular weight

PMSF = phenylmethyl-sulfonyl fluoride

CM- = carboxymethyl

DE- = diethylaminoethyl

DMEM = Dulbeco's modified Eagle's medium

FBS = fetal bovine serum

NGF = nerve growth factor

CAT = choline acetyltransferase

CSF = CAT-stimulating factor

CM = conditoned medium

GCM = glioma-conditioned medium

HCM = heart cell-conditioned medium

DRG = dorsal root ganglion

PORN = polyornithine

PNPF = polyornithine-attachable neurite-promoting factor

NE = noradrenaline

Ach = acetylcholine

TH = tyrosine hydroxylase

CSA = CAT-stimulating activity

CNS = central nervous system

GPA = growth promoting activity

SU = stimulation unit

g = unit of gravitational force

AchE = acetylcholinesterase

GAD = glutamic acid decarboxylase

INTRODUCTION

(I) CONTROL OF NERVE GROWTH AND DIFFERENTIATION

Neurons are highly evolved cells with specialized and specific functions. The evolution from an early somatic cell to a mature neuron must involve a number of complex interactions. Obviously, the acquisition of many specialized characteristics by neurons must be based ultimately on the gene expression during development. However, to what extent does neuronal development unfold according to rules built into each cell, and to what extent is it a result of influences impinging on developing neurons from without? The details of such modulation are just beginning to be understood at the molecular level in neurons (1).

Evidence for the importance of extrinsic influences in cellular development first came from some classical studies on induction in amphibian embryos. In 1938, Spemann (2) showed that if the optic vesicle was removed from an early frog embryo, the lens, normally arise from the overlying ectoderm, did not develop, and further that the introduction of a piece of amphibian embryo into a host embryo organized the development of an entire second embryo in the host at the site of transplantation.

Extrinsic influences for neuronal development were first demonstrated by studies on naturally occurring cell death in the nervous system (3, 4, 5, 6). These studies showed that early ablation of the target caused severe depletion of the final population of innervating neurons, and that some of the normal neuronal loss could be diminished if the amount of target tissue available to an innervating population of neurons was increased

compete among themselves for survival or maintenance through a substance(s) or factor(s), produced in limited quantities by their targets, and the losing neurons result in death.

The discovery of Nerve Growth Factor (NGF) in 1951 by Levi-Montalcini and Hamburger (9) raised the possibility that neuronal development, including target-dependent neuronal survival, might be mediated by diffusible molecules. In vivo experiments indicated that NGF could reverse naturally occurring as well as experimentally induced cell death in sympathetic and sensory neurons; moreover, NGF antibodies in vivo could block the development of the sympathetic nervous system (for reviews see 10, 11, 12). Furthermore, in vitro studies showed that NGF changed the direction of neuritic growth (13) and helped shape the neuritic tree maintained by the neuron (14), suggesting that NGF might also influence the choice of targets contacted by neurons.

NGF has been and remains the only neuronotrophic factor with an established physiological role. It has been purified and characterized and specific antibodies have been available for various studies (for reviews see 10, 11, 12). However, only sensory and sympathetic neurons have been convincingly shown to depend on NGF in normal development. Many other neurons are not affected by NGF for their development and maturation. It is obvious that there are other neuronotrophic factor(s) for the survival of other neuronal populations.

In the past few years, in vitro studies of neuronal growth and differentiation in the presence of extracts and cell

conditioned media (CM) have revealed other neuronotrophic factors that differ from NGF in promoting neuronal survival and development in cell culture. These factors do not appear to stimulate neuronal cell division, unlike most growth factors operative on non-neuronal cells; however, a possibility remains that the neurons used in these assays are postmitotic (for reviews see 15, 16, 17). Recently, a neuronotrophic factor, which stimulates survival of sympathetic neurons, has been purified from pig brain (18). This achievement adds further support to the existence of a family of neuronotrophic factors, helping to orchestrate neuronal development, and raises the possibility of identifying and isolating additional neuronotrophic factors from other sources.

(II) NEURONOTROPHIC FACTORS

Current concepts and information have shown us, among other perceptions, to distinguish two main general classes of neuronotrophic factors: First, trophic factors which control welfare and general growth capabilities of a neuron, and secondly, specifying factors which select particular behavior(s) to be expressed by the trophically-supported neuron. Trophic factors may regulate the balance between anabolic and catabolic activities and thus be equally reponsible for cell growth, degeneration and death, survival and maintenance. In turn, specifying factors will dictate to the cell whether it should channel its growth condition into increasing its own mass, extending neurite, or secretion. Under maintenance levels specifying factors will also determine what transmitter mode the neuron will use or what specialized products it will synthesize.

The distinctions among different categories of neuronotrophic factors do not exclude the possibility that the same molecule may be able to act in both trophic and specifying roles. Rather, such categories help us to maintain an operational distinction, reflecting the effect through which a given activity is monitored by the assay system used, and different mechanisms through which different responses are elicited under defineable sets of conditions.

(A) Trophic Factors

(1) Neuronal Survival Factors for Ciliary Ganglion Neurons

The chick ciliary gangion has been a useful preparation for identifying new neuronotrophic factors, partly because survival of these neurons appears not to be responsive to NGF. Extracts prepared from chick embryonic eye tissue, which contains all of the normal synaptic targets for the ciliary ganglion neurons, were found to have the highest levels of neuronal survival activity for the ciliary ganglion neurons (19). Subsequent studies have shown that high levels of survival activity for ciliary ganglion neurons was also present in extracts of chick heart (20), and in CNS wound fluids of developing and young adult rats (21). Bovine cardiac muscle has also been shown to contain components that stimulated survival of chick ciliary ganglion neurons (22).

Gel filtration of cardiac extracts under different experimental conditions revealed the activity was a complex of components. In low ionic strength buffers the activity migrated as a large molecular weight component (>40,000 daltons), while in

high ionic strength buffers or in the presence of EDTA the activity migrated primarly as a component of about 20,000 daltons (23). Gel filtration of the ciliary survival activity from embryonic chick eye tissue produced a spread of activities in large molecular weight fractions (>35,000 daltons) with low recoveries of activity (24), to a smaller molecular weight component of 20,000 daltons (25).

Isoelectric focusing has been used to compare the properties of survival factors in extracts prepared from chick embryo eye tissues, chick and bovine cardiac tissues. Values between pH 4.5 and 5.5 were obtained as the isoelectric points for the active components from the chick tissues (20, 26), and 6.2 for the component in the bovine tissue (23). From these observations of molecular weights and isoelectric points, it appears that these active components from different sources are similar but not identical.

Studies using a long term growth assay with ciliary ganglion neurons have revealed two kinds of stimulating activities: one stimulated development of choline acetyltransferase (CAT) activity in cultured neurons without influencing growth, and the other stimulated neuronal growth with no effect on levels of CAT activity. This latter activity, termed Growth-Promoting Activity (GPA), appeared to be associated with a molecular component of about 20,000 daltons upon gel filtration of crude embryonic chick eye extracts (27). These results suggest that the survival of ciliary ganglion neurons may be influenced by more than one active component in the tissue extracts.

(2) Neuronal Survival Factor for Dorsal Root Ganglion and Sympathetic Neurons

Barde and collaborators (28) first observed that a component in glioma-conditioned medium (GCM) could promote the survival of chick dorsal root ganglion (DRG) neurons in dissociated cell cultures. The component was not inactivated by antibodies against NGF and appeared to act on neurons different from those affected by NGF (28).

The finding that rat brain contains a component similar to that described for GCM raises the possibility that mammalian brain might be a convenient source for the purification of this active component (29, 30). Recently, Barde et al (18) have purified this active component to homogeneity from pig brain extracts. The component has a molecular weight of 12,300 daltons as determined by both SDS-polyacrylamide gel electrophoresis and gel filtration on Sephadex G-100, and an isoelectric point of approximately 10.1. In some respects, this active component is similar to NGF; however, this protein component fails to cross-react with NGF antibodies, and promotes the survival of certain populations of DRG neurons which are not supported by NGF. In addition, this component supports the survival of sympathetic neurons in culture (18). Given its molecular size and isoelectric point, this sensory neuron survival factor appears to be different also from other neuronotrophic factors that support ciliary ganglion neurons. This, however, is only tentative since the properties of the ciliary ganglion factors have been studied only with the crude materials and may not represent the exact properties of the purified component(s).

The survival of sympathetic neurons in response to NGF and GCM in cell culture has been demonstrated to be similar to that observed for DRG neurons (31). The effects of NGF and GCM appeared roughly additive when simultaneously supplied to the neurons (31). A factor present in heart cell-conditioned medium (HCM) produced a pattern of survival different from that obtained in the presence of either NGF or GCM (31). The effects of HCM and NGF on cultured neurons appeared additive. Neither the GCM nor the HCM activities were inactivated by incubating with NGF-antibodies. At present, it is not clear whether the different factors serve distinct populations of sympathetic neurons or whether individual neurons express changing requirements for the factors as a function of developmental age. The fact that the neurotransmitter enzymes associated with the surviving neurons differ according to the presence of NGF or HCM suggests the possibility that different populations of neurons are selected by each of the survival factors. Alternatively, the factors may induce different developmental destinies in the surviving neurons.

(3) Neuronal Survival Factors for CNS Neurons

Presence of diffusible factors in tissue extracts and conditioned media enhancing the survival of spinal cord neurons in culture has been demonstrated (32, 33, 34). Bennet et al (32) showed that survival of chick motoneurons was increased by culture medium conditioned by skeletal muscle cells but not by medium conditioned by kidney or smooth muscle cells. Similarly, Slack et al (34) demonstrated that the survival of motoneurons could be supported by extracts from non-endplate regions of

innervated muscle. Furthermore, Schnaar and Schaffner (33) fractionated spinal cord cells and observed that dissociated neuronal cells with the highest specific activity of choline acetyltransferase (CAT), putative motoneurons, required muscle-conditioned medium for survival in culture. Although the active components have yet to be characterized, these studies do suggest a pattern of specificity consistent with the hypothesis that target-derived components influence survival and development of motoneurons.

(B) Specifying Factors

(1) Neurite Extension Factors

Stimulation of neuritic outgrowth from ganglionic neurons in culture has been a traditional assay for NGF. Using similar assay systems, investigations have revealed new active factors in a variety of tissues stimulating neurite production by neurons. These components appear to be different from NGF, since they are not neutralized by NGF-antibodies, and in some cases they act on cell populations which are either unresponsive to or affected differently by NGF.

(a) Neurite Extension Factors for Ganglionic Neurons

Neurite promoting activity for ganglionic neurons other than NGF, has been described in tissue extracts and conditioned medium from a variety of sources (35, 36, 37, 38). Ebendal and co-workers (38) described a component in extracts of embryonic chick tissue stimulating neurite production by ciliary, sympathetic, and dorsal root ganglion explants in culture. Partial purification upon gel filtration of cardiac tissue extracts has

revealed a major component of molecular weight range of 40,000 daltons, and a small amount of activity of much larger molecular weight (39). This activity in chick tissue appeared to be associated with a component having an isoelectric point of 5 (39). These properties of neurite promoting activity appear similar to those reported for the ciliary ganglion survival factor from chick eye tissue (24,26)

It has been reported in several instances that fractionation of tissue extracts yielded a co-distribution of stimulating activities for neurite extension and neuronal survival (20, 38, 39). Although it is possible that the two activities are associated with the same protein component, given the precedent of NGF with its multiple effects on target neurons, isolation and further characterization of these neurite promoting and neuronal survival activities will be necessary to delineate their exact relationship.

(b) Neurite Extension Factors for CNS Neurons

Conditioned media from a number of cell types have been reported to stimulate neurite production by spinal cord neurons (33, 40, 41, 42, 43). Fractionation of rat fibroblast-conditioned medium upon gel filtration revealed two active components acting on rat spinal cord explants (44). One component of about 300,000 daltons stimulated neurite extension by adsorbing to the culture substratum; while the other of about 50,000 daltons acted through the culture medium and was not preadsorbed by the substratum. The two components were observed to produce a synergistic effect on spinal cord neurons in culture (45).

Studies with chick spinal cord neurons in dissociated cell culture have indicated a similar pattern (42). In this case, fractionation of chick muscle-conditioned medium upon gel filtration revealed a broad spread of neurite-promoting activity with peaks corresponding to components of approximately 40,000, 500,000, and $>10^6$ daltons. It is not certain whether any of these components acts by adsorbing to the culture substratum, similar to that of the large molecular weight component in rat fibroblast-conditioned medium.

Chick cerebral neurons in dissociated cell culture could be induced to extend neurites by components present in bovine brain extracts (46) and in chick heart-conditioned medium (47). The bovine brain activity has been prepared to 90% purity (46). Under nonreducing conditions, the major protein component in the partially purified material had a molecular weight of about 75,000 and 37,000 daltons upon native and reducing gel electrophoresis, respectively (46). Minor components of molecular weight ranging from 15,000 to 30,000 daltons were also revealed after gel electrophoresis under reducing conditions. However, the important question to be determined is whether the activity is associated with the major component or the minor protein bands. Despite its effect on neurite extension, the partially purified material had no effect on the survival of cerebral neurons in culture (46).

The mammalian brain has also been demonstrated to contain components that stimulate neurite production by retinal tissue in culture extracts prepared from pig brain which caused a

significant increase in neuritic outgrowth from rat retinal explants (48), and NGF-antibodies had no effect in blocking the neurite production by explants in response to pig brain extracts (48).

Chick retinal explants have also been shown to respond with significant increase in neuritic outgrowth to components in tissue extracts prepared from chick optic lobe and forebrain hemispheres (49). This activity in optic lobe extracts was shown to be associated with a fraction of macromolecular materials and could not be replaced by NGF in the assay (49). Little or no effect on retinal ganglion cells was observed with extracts prepared from skeletal muscle, heart, and yolk sac (49), which had been shown to induce neurite extension from peripheral ganglionic neurons (38). This, however, should be regarded as a tentative conclusion because crude tissue extracts may contain inhibitory components, which could have masked the action of the stimulatory factors (50).

(2) Polyornithine-Binding Neurite-Promoting Factors

Plastic substrata for neuronal cultures are usually precoated with collagen or other biological materials, to mimic some properties of cell surfaces or extracellular matrices encountered by cells in vivo. Polycationic coatings, such as polyornithine (PORN), have increasingly been employed because of their greater adhesiveness for dissociated cells. PORN-coated substrata have been found to bind proteins, which were unfavorable to neuritic growth (51). In contrast, however, pretreatment of PORN substrata with certain macromolecules markedly enhanced the regeneration of neurites from cultured

neurons (51). This component has been termed as polyornithine-attachable neurite-promoting factor (PNPF) by Varon and associates (52), who further demonstrated that a wide range of cell types could produce PNPF like material (52, 53, 54). PNPF has been shown to stimulate neurite production from a number of neuronal populations of peripheral origin, including parasympathetic, sympathetic, and sensory ganglia, but ineffective on most neurons from the CNS, except embryonic retinal neurons and a small fraction of spinal cord neurons (52).

The chemical properties of some partially purified preparations of this stimulatory component have been examined by several investigators. Lander et al (55) showed that bovine corneal endothelial cells produced a PNPF-like material; which appeared to be associated with a heparan sulfate proteoglycan, and had a molecular weight of just under 4×10^6 daltons. Conditioned medium from rat Schwannoma cell line has also been shown to contain a PNPF component (56), which appeared to contain sugar moieties because it bound to the lectins of Concanavalin A and wheat germ agglutinin (57). Upon gel filtration the molecular weight of the partially purified component from mouse heart cells was estimated to be 5×10^6 daltons (58).

The physiological role(s) of these large molecular weight neurite-extension factors *in vivo* remains to be determined. It is possible that these components normally promote cellular adhesion, and that the neurite production induced by these factors in culture is a result of an increased adhesion of the neuron to the substratum. A more interesting possibility is that

in vivo these neurite-promoting components may serve to guide neurite extension and thus contribute to the pattern and density of tissue innervation.

(3) Neuronal Developmental Factors

One of the primary choices a developing neuron makes is which neurotransmitter to produce, thereby determining the effect its synapses will have on its target cells. Neuronal developmental factors that specifically control the type and amount of transmitter made by the neurons have been identified in tissue extracts and conditioned media from a variety of sources (59, 60, 61, 62, 63,)

Patterson et al (60, 62) have identified a component that acted on dissociated sympathetic neurons. When grown in the virtual absence of other cell types, these neurons develop many of the properties expected of adrenergic neurons: They synthesize, store and release noradrenaline (NE) as do adrenergic neurons in vivo. However, they could be forced to produce considerable amounts of acetylcholine (Ach), in addition to or as a substitute for NE, when cultured with various types of nonneuronal cells or with medium conditioned over other nonneuronal cell cultures (60, 62).

Starting with serum-free conditioned medium of rat heart cells, Fukada (65) has recently purified this component 10,000-fold, obtaining a preparation active at about 50 ng/ml. The molecular weight of the protein appeared to be 45,000 daltons by gel filtration, and 50,000 daltons by SDS-polyacrylamide gel electrophoresis. Recently, this active component has also been purified 1500-fold from heart cells conditional medium (64),

using ammonium sulfate precipitation, followed by ion exchange chromatography and gel filtration, yielding a partially purified material active at 1 ug/ml with a molecular weight of 40,000-45,000 daltons by gel filtration (64).

Conditioned medium from chick heart cells has been reported to contain a neuronal developmental factor (31), since sympathetic neurons grown in the presence of chick heart-cell conditioned medium developed significantly higher levels of CAT activity and lower levels of tyrosine hydroxylase (TH) activity as compared to neurons grown in medium containing NGF. Furthermore, in the presence of a combination of NGF and heart-cell conditioned medium, the levels of CAT and TH activities developed in the cultured neurons were roughly additive to those grown in medium containing NGF or heart-cell conditioned medium alone (31).

More recently, another neuronal developmental factor has been identified in extracts of embryonic chick eye, inducing a two- to four-fold increase in the levels of CAT activity in ciliary ganglion neurons in culture (27). This CAT-stimulating activity (CSA) of the chick eye extracts migrated upon gel filtration as a component of 40,000-45,000 daltons and eluted upon DEAE ion-exchange chromatography at an ionic strength similar to that for the rat heart cholinergic factor (64, 65). It will be of interest to determine whether this chick eye factor shares other properties with the rat heart cholinergic factor. A rabbit antiserum raised against a crude fraction of the chick CSA has been shown to block specifically the increase in CAT activity

induced by CSA in cultured neurons, without influencing neuronal survival, neuronal growth, or the basal levels of CAT activity obtained in the absence of CSA (66).

Neuronal developmental factors affecting neurotransmitter synthesis in CNS have been identified in conditioned-medium from a number of cell types (59, 63). Studies on dissociated mouse spinal cord neurons have shown that conditioned medium of skeletal muscle cells as well as a number of other cell types increased dramatically the levels of CAT activity in developing neurons in culture (59, 63). This effect of conditioned medium on CAT activity has been demonstrated to be selective, since other neurotransmitter enzymes such as acetylcholinesterase and glutamic acid decarboxylase, present in the cultured neurons were not affected.

(III) CONCLUDING REMARKS

A wide array of new putative neuronotrophic factors have been described in tissue extracts and cell-conditioned media. It seems likely that in some cases further purification of these active components will demonstrate that a single component is responsible for diverse activities. It is also probable that in some instances in which partially purified material has been found to act on several types of neurons, further purification will reveal a mixture of active components, each with a unique specificity. In fact, one may question the value of carrying out extensive assays on impure components to test their specificity and distribution, since the results obtained are often critically dependent on obscure features of the assay and may even be compromised by toxic or inhibitory components in the test sample.

Therefore, it is absolutely imperative to obtain highly purified preparations of these new putative neuronotrophic factors for characterization studies on their biological and chemical properties, in order to gain further understanding on the physiological factors and biochemical events regulating the process of maturation and differentiation of neurons.

(IV) RATIONALE AND AIMS OF INVESTIGATION

Possible role(s) of target tissues on the development of specific neurotransmitters in CNS neurons has been studied by various investigators (59, 61, 67, 68). Developmental increases in choline acetyltransferase (CAT) activity have been correlated with the appearance and maturation of synaptic connections between spinal cord motoneurons and skeletal muscle in the rat and the chick (67) and between ciliary ganglion cells and iris muscle in the chick (68). Using cell cultures from fetal mouse spinal cord, Giller et al (59, 61) demonstrated the role of peripheral tissues causing significant increases in CAT activity when co-cultured with skeletal myotubes. Similarly, large increases in CAT activity were seen when conditioned-medium from muscle cultures was added to spinal cord cultures (61). This effect of conditioned-medium on CAT activity was selective, as acetylcholinesterase (AchE) and glutamic acid decarboxylase (GAD) activities were not increased. This CAT-Stimulating Factor (CSF) has been shown to be a macromolecule of molecular weight >50,000 daltons and was relatively heat stable (61). Further, this CAT-Stimulating Factor has been identified in conditioned medium from cell cultures of muscle, heart, and kidney; and its action

appeared to be specific on neurons from medulla and spinal cord on the CNS (63). It has been suggested that the CAT-Stimulating Factor in conditioned medium from cultures of mouse muscle affecting spinal cord cells (61, 63) is not identical to the factor (s) in the conditioned medium from cultures of rat heart muscle increasing CAT activity in cultured sympathetic neurons (60, 62). Therefore, it is imperative to isolate the molecules involved in these phenomena, compare their biological and chemical properties, and to determine their functional mechanism and physiological significance in the developing neurons.

The aims of this study were:

1. To establish a rapid and reproducible bioassay for the CAT-Stimulating Factor.
2. To characterize the CAT-stimulating activity in rat skeletal muscle.
3. To purify the CAT-Stimulating Factor from rat skeletal muscle extract.
4. To elucidate the physical and chemical properties of the highly purified muscle CAT-Stimulating Factor.

MATERIALS AND METHODS

(I) MATERIALS

Tissue culture supplies, including Dulbeco's modified Eagles's medium-high glucose (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and L-glutamate were purchased from GIBCO Canada (Calgary, Alberta, Canada). Sephadex G-100, Spectrapor dialysis tubing and standards for isoelectric focusing were products of Pharmacia Fine Chemicals (Dorval, Quebec, Canada). DE-Cellex, CM-Cellex, molecular weight standards for SDS-PAGE, coomassie brilliant blue R-250, bis-acrylamide, ammonium persulfate, and sodium dodecyl sulfate were products of Bio-Rad Canada Laboratories (Mississauga, Ontario, Canada). ^{3}H acetyl-coenzyme A was obtained from New England Nuclear (Boston, Mass., USA). Isoelectric focusing kits (pH 3.5-9.5) were purchased from LKB (Bromma, Sweden). Sprague-Dawley rats (250-300 g) were purchased from Charles River Breeding Lab. (Wilmington, Mass., USA).

All other standard laboratory reagents were obtained either from Fisher Scientific Co. (Winnipeg, Canada) or Sigma Chemical Co. (St. Louis, MO, USA).

(II) METHODS

(1) TISSUE CULTURE METHODS

(a) Hybrid NG108-15 Cell Line. The neuroblastoma x glioma hybrid cell line NG108-15 was a gift from Dr. B. Schrier, Laboratory of Developmental Neurobiology, National Institute for Health, USA., the hybrid NG108-15 cell was maintained in medium D (90% DMEM, 10% FBS, 1×10^{-4} M hypoxanthine, 1×10^{-6} M aminopterin, 1.6×10^{-5} M thymidine, 100 i.u./ml penicillin, 100 ug/ml

streptomycin, and 2 mM L-glutamate) at 37°C in a humidified atmosphere of 10% CO_2 and 90% air. The growth medium was changed 2-3 times weekly and the cells were passaged at confluency.

(b) Bioassay for CAT-Stimulating Factor. The bioassay for CAT-Stimulating Factor (CSF) was based on the ability of various fractions to stimulate or increase the choline acetyltransferase (CAT) activity in NG108-15 cells when cultured in the presence of 1 mM of cyclic 3', 5'-AMP. For assays, hybrid NG108-15 cells were inoculated into Falcon Multiwells (2.1 cm^2 surface area) at 3.3×10^4 cells/cm^2 in 1.5 ml of medium E (95% DMEM, 5% FBS, 1 mM $N^6,O^{2'}$-dibutyryl-cyclic 3',5'-AMP, and hypoxanthine, aminopterin, thymidine, penicillin-streptomycin, and L-glutamate as medium D). Tissue extracts or eluants after chromatographies to be assayed were filtered (0.22 um) and introduced into the hybrid cells after 1 day of culture. After an additional 5 days incubation, the CAT activity of the NG108-15 hybrid cells was assayed in dupolicates or triplicates.

(2) ANALYTICAL PROCEDURES

(a) Protein Determination. The protein distribution in various fractions after ion exchange chromatographies or gel filtration steps of purification was monitored with a Beckman Model 25 Spectrophotometer by absorbance at 278 nm. For more accurate determination of protein concentrations the method of Bradford (69) was used.

(b) Measurement of Choline Acetyltransferase Activity. Choline acetyltransferase was assayed by the radiochemical assay of Schrier et al (70) with slight modifications, Briefly, for

assay the culture medium was replaced by 0.2 ml of assay buffer (50 mM KPO_4 buffer pH 6.8 with 0.75 mM EDTA contained 240 mM NaCl, and 0.56% Triton X-100). The cells were dissolved in the assay buffer by incubating at 37°C for 10 min, and 40 ul of the reaction cocktail (50 mM KPO_4 buffer, pH 6.8 with 1 mM EDTA contained 0.29 uM ^3H acetyl Co-enzyme A [0.08 uCi/reaction mixture], 0.046 mM acetyl co-enzyme A, 13.4 mM choline iodide, 200 mM NaCl, 0.6 mM neostygmine bromide, and 0.25% Triton X-100) was added followed by an additional incubation of 10 min at 37°C. The reaction was stopped by adding 1 ml of ice cold H_2O, and the mixture was then passed through a small AG 1-X8 ion exchange column, followed by washing two times with 1 ml of H_2O. The eluant was collected directly into a scintillation vial, and, after mixing with 10 ml of Scinti Verse, was counted in a LKB scintillation beta-counter.

(c) Determination of Cell Number. After centrifugation, the cell pellet was resuspended in the appropriate medium, and cell number was counted with a hemacytometer under a microscope.

(3) ISOLATION OF THE CSF FROM RAT SKELETAL MUSCLE

(a) Starting Materials. Rat skeletal muscles were removed from hindlimbs of male or female Sprague-Dawley rats of body weight (250-300 g). The homogenization and extraction and all subsequent steps of isolation were carried out at 4°C.

(b) Extraction Procedure. Rat skeletal muscle was minced and homogenized in 8 volumes of PBS with 1 mM PMSF at pH 7.4. The homogenate was stirred for 60 mins at 4°C, filtered through layers of cheese cloth, and centrifuged at 15,000g for 60 min. The supernatant was saved, and the pellet was re-extracted and

centrifuged. The supernatants were pooled for further fractionation.

(c) Ammonium Sulfate Precipitation. Ammonium sulfate was added to the supernatant to obtain 50% saturation. The mixture was stirred at 4 °C for 60 min and the precipitate was pelleted by centrifugation at 15,000 g for 60 min. The 0-50% ammonium sulfate precipitate was dissolved in 1 mM $NaPO_4$ buffer at pH 6.2, and dialysed against 20 volumes of the same buffer with several changes. The dialysed solution was centrifuged at 100,000 g for 60 min, the supernatant was applied onto a CM-cellex ion exchange column for fractionation of proteins.

(d) Stepwise Elution on CM-Cellex Chromatography. A CM-Cellex column was equilibrated in 1 mM $NaPO_4$ buffer at pH 6.2. The supernatant fraction of the dialysed 0-50% ammonium sulfate precipitate of muscle extracts was loaded onto the CM-Cellex column. After washing extensively with the 1 mM $NaPO_4$ buffer, the adsorbed proteins on the column was eluted in stepwise with 0.2 M, and 0.5 M NaCl in 1 mM $NaPO_4$ buffer. The fractions were monitored for proteins by absorbance at 278 nm and for CAT-Stimulating activity by the NG108-15 bioassay. The appropriate fractions were pooled and dialysed in 0.01 M $NaPO_4$ at pH 7.4 for the next fractionation step.

(e) Linear Gradient Elution on DE-Cellex Chromatography. A DE-cellex column was equilibrated in 0.01 M $NaPO_4$ buffer at pH 7.4. The pooled fractions after CM-Cellex chromatography were loaded onto the DE-Cellex column. After washing with 0.01 M $NaPO_4$ buffer, the adsorbed proteins on the column were first

eluted by 0.05 M NaCl in the $NaPO_4$ buffer, and the remaining proteins were then eluted by a linear gradient of 0.05 M, to 0.15 M NaCl in the $NaPO_4$ buffer. Finally the column was eluted stepwise by 0.15 M, and 0.25 M NaCl in the $NaPO_4$ buffer. The fractions were monitored for protein by absorbance at 278nm and for CAT-Stimulating activity by the NG108-15 bioassay. The appropriate fractions were pooled and dialysed in 0.01 M $NaPO_4$ at pH 7.4 for the next fractionation step.

(f) Compound Gradient elution on DE-cellex Chromatography.

The dialysed fraction from the first DE-Cellex chromatography was loaded onto another DE-Cellex column in the same experimental conditions. After washing the column with 0.01 M $NaPO_4$, the adsorbed proteins on the column was first eluted by 0.05 M NaCl in the $NaPO_4$ buffer, and then followed by a continuous compound gradient of 9 chambers, consisting of 0.05 / 0.15 / 0 / 0.10 / 0.10 / 0.15 / 0.15 / 0.15 / 0.10 M NaCl in the $NaPO_4$ buffer, generated by a Varigrad apparatus. Finally, the column was washed by the stepwise elution with 0.10 M, and 0.15 M NaCl in the $NaPO_4$ buffer. The fractions were monitored for protein by absorbance at 278 nm and for CAT-stimulating activity by the NG108-15 bioassay. The appropriate fractions were pooled, dialysed in 0.25% NH_4HCO_3, and lyophilized.

(g) Gel Filtration on Sephadex G-100. Gel filtration was carried out on a Sephadex G-100 column equilibrated in 0.25% NH_4HCO_3. The lyophilized sample from the second DE-Cellex column was dissolved in 2 ml of 0.25% NH_4HCO_3, centrifuged at 20,000 g for 30 min, and loaded on the Sephadex G-100 column. The column was eluted with the same buffer, and the distribution of proteins

and CAT-Stimulating activity after gel filtration were monitored, and the appropriate fractions were pooled and lyophilized.

(4) CHARACTERIZATION OF THE CSF

(a) Polyacrylamide Gel Electrophoresis. Analytical PAGE was performed on a 7.5% separating gel and a 4% stacking gel at pH 8.9 (71). The electrophoresis was carried out at a current of 10 mA/cm^2 of gel surface. At the end of electrophoresis, the gel was stained with 0.25% coomassie brilliant blue, in 45% methanol and 9% glacial acetic acid. And destained by diffusion in 10% methanol and 7% glacial acetic acid.

To demonstrate the CAT-stimulating activity of the purified CSF, samples of 20 ug each of CSF were electrophoresed in duplicates in 7.5% polyacrylamide gel at pH 8.9. One gel column was stained for protein; and the other gel column was cut into 2 mm segments, and the protein in each segment was eluted by 1 ml of PBS at pH 7.4, with 0.2% fatty acid free BSA, overnight at 4^{o}C. The eluant of each segment was diluted 1:20 with the same buffer, filter-sterilized and assayed for CAT-stimulating activity by adding an equal volume of 200 ul each to the NG108-15 cells.

(b) Estimation of Molecular Weight

(i) Gel Filtration on Sephadex G-100. A Sephadex G-100 column was calibrated with protein markers of known molecular weights: Bovine serum albumin (67K), ovalbumin (45K), trypsinogen (25K), and cytochrome C (12K). The purified CAT-Stimulating Factor was then applied onto the same column of Sephadex G-100 column, and the molecular size of CAT-Stimulating

Factor was estimated from its elution volume against those of the marker proteins of known molecular weights.

(ii) SDS-Polyacrylamide Gel Electrophoresis. The molecular weight of the CAT-Stimulating Factor was also estimated by SDS-Polyacrylamide gel electrophoresis (72). The molecular weight markers (phosphorylase B [93K], bovine serum albumin [66K], ovalbumin [45K], carbonic anhydrase [31K], soybean trypsin inhibitor [22K], and lysozyme [14K]) and the samples to be tested were pre-treated by heating at 100 ^{o}C for 5 min. in the presence of 1% sodium dodecyl sulfate and 5% B-mercaptoethanol and electrophoresis was carried out in 10% polyacrylamide containing 1% SDS. The gels were electrophoresed at a current of 10 mA/cm^{2} of gel surface. At the end of electrophoresis the gels were stained with 0.1% coomassie brilliant blue in 50% trichloroacetic acid, and destained by diffusion in 10% glacial acetic acid. The relative mobilities (Rf values) of the protein markers were plotted against their molecular weights. The molecular weight of the CAT-Stimulating Factor was estimated by plotting its electrophoretic mobility (Rf value) to compare with those of the protein markers.

(c) Gel Isoelectric Focusing. Analytical thin layer polyacrylamide gel (LKB) isoelectric focusing was carried out using a LKB 2117 multiphor apparatus and a pre-cast 5% polyacrylamide gel with a pH range of 3.5-9.5. After focusing for 4.5 hrs at 4 watts, the gel containing the isoelectric point (pI) markers (amyloglucosidase [3.50], soybean trypsin inhibitor [4.55], B-lactoglobulin A [5.20], bovine carbonic anhydrase B [5.85], human carbonic anhydrase B [6.55], horse myogloblin-

acidic band [6.85], horse myoglobin-basic band [7.35], lentil lectin-acidic band [8.15], lentil lectin-basic band [8.65], and trypsinogen [9.30]) and sample was placed in a fixing solution containing 57.5 g TCA and 17.25 g sulphosalicylic acid in 500 ml distilled water for 30-60 mins., and was then stained overnight with coomassie brilliant blue R-250 (0.230g in 200 ml destaining solution: 500 ml ethanol plus 160 ml acetic acid diluted to 2 liters with distilled water) After staining, the gel was placed in the destaining solution until the stained protein bands were clearly visible. To determine the pH gradient formed after focussing, an additional gel lane was cut into 5 mm segments, each of which was individually soaked and eluted in 1 ml double distilled water for 60 min under continuous shaking. The pH in each gel eluant was determined by the pH meter.

RESULTS

(I) CONDITIONS FOR ASSAYING THE CHOLINE ACETYLTRANSFERASE STIMULATING ACTIVITY USING THE HYBRID NG108-15 CELL LINE

For successful isolation of biological active principles, a relatively simple and specific assay is essential. The original assay for the CAT-stimulating activity by Giller et al (59, 61) using primary cultures of mouse spinal cord neurons is specific but time consuming. However, the more recently developed assay using the hybrid cell line NG108-15 (63), though less sensitive, takes only a few days of cultures. The NG108-15 cell line was derived from a Sendai-virus-induced fusion of a mouse neuroblastoma cell line (N18 TG-2) and a rat glioma cell line (C6 BU-1). This hybrid cell line is one of the most highly characterized and widely studied cell lines of neural origin. It has been shown to synthesize, store, and secrete acetylcholine (73), and form functional synapses with striated muscle cells *in vitro* (74). In addition, NG108-15 cells possess a number of receptors for neuroactive compounds: opiate receptors (75,76), muscarinic acetylcholine receptors (77), as well as receptors for prostaglandins PGE_1 (78). Since the NG108-15 cells possess all of these neuronal characteristics, it is evident that this cell line behaves as neuronal cells in culture.

In adapting this NG108-15 cells into our routine system for the CAT-stimulating activity, a series of preliminary experiments was carried out to establish a simple and reproducible bioassay.

(1) Effect of the rat muscle extract on NG108-15 cells at various time intervals

Cells from confluent culture of NG108-15 cells in medium D were inoculated into Falcon Multiwells at 5.0×10^4 cells/well in

1.5 ml of medium E. After 1 day of culture rat skeletal muscle extract was introduced into the cells and CAT activity of the NG108-15 cells was assayed after 1, 2, 3, 4, and 5 days of additional incubation in triplicates.

Figure 1 shows that the CAT enzyme activity of the NG108-15 cells continued to increase for at least up to day 5 of culture in both the extract treated and untreated cells. The CAT-Stimulating Factor (CSF) appeared to have the maximal effect on the NG108-15 cells after 5 days in culture.

Figure 2 shows that cell number in both muscle extract treated and untreated cells continued to increase slightly but gradually up to 4 days in culture. On day 5 of culture there was a slight decrease in cell number probably due to overcrowding. Most importantly, there appeared to be no significant difference in cell number between the treated and untreated cells.

Figure 3 shows the phase contrast photomicrography of the NG108-15 cells in culture. Treatment of the NG108-15 cells with dBcAMP (medium E), whether alone or in combination with muscle extracts resulted in dramatic morphological changes with elongation and thickening of nerve processes (Fig. 3B,C). In contrast, the NG108-15 cells with no treatment (medium D) have relatively short nerve processes and frequently resembled fibroblast processes rather than neurite (Fig. 3A).

(2) Determination of the optimal plating cell number for the NG108-15 cell bioassay

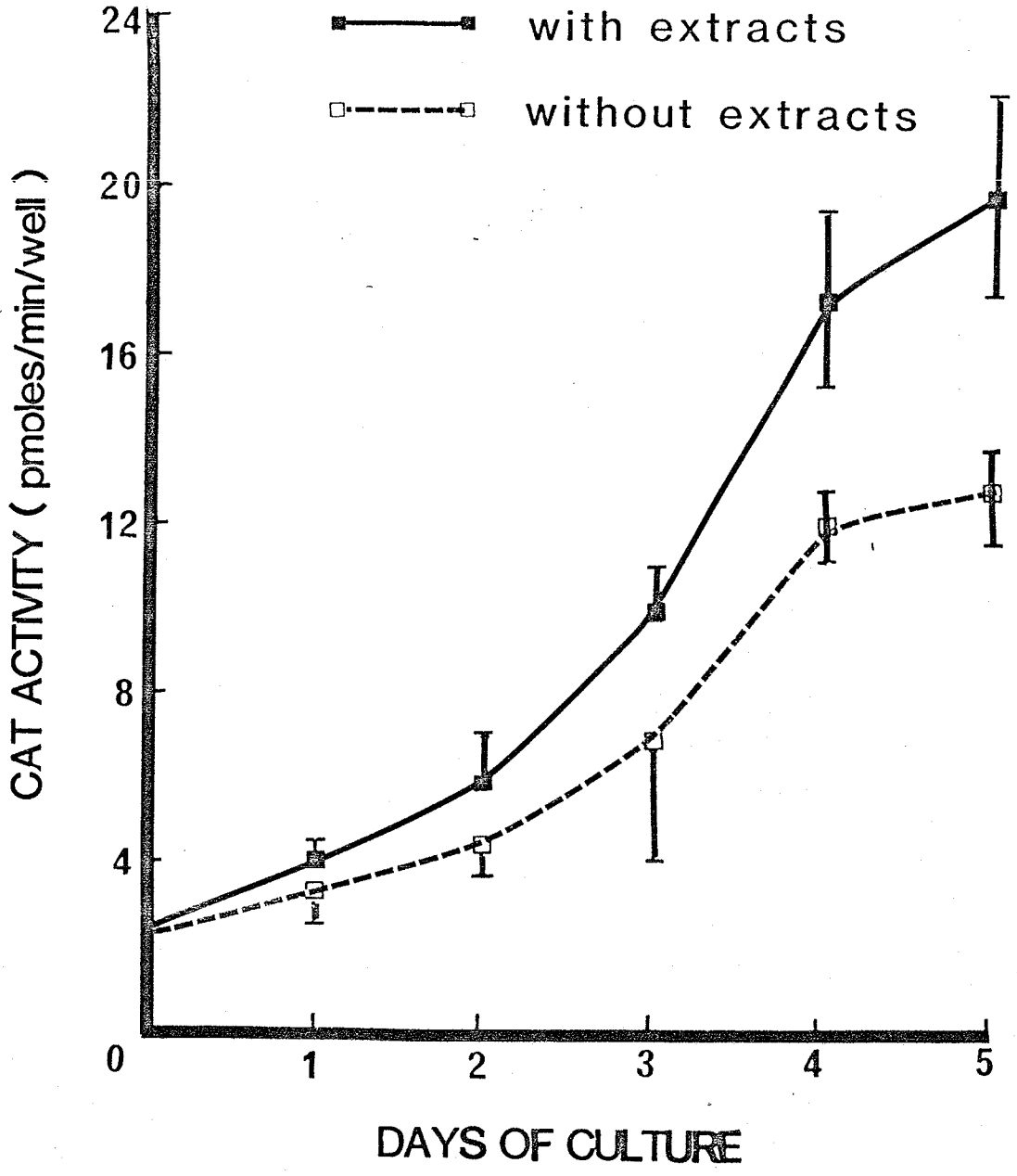
Cells from confluent culture of NG108-15 cells were inoculated into Falcon Multiwells at 2.5, 5.0, 7.5, 10.0, and 12.0×10^4 cells/well. Rat skeletal muscle extract was

Figure 1

Effects of rat skeletal muscle extract on the CAT activity in NG108-15 cells at various time intervals. Hybrid NG108-15 cells from confluent culture were inoculated into Falcon Multiwells at 5.0×10^4 cells/well in 1.5 ml of medium E. Sterilized rat skeletal muscle extract (500 ug) was introduced into the cells after 1 day of culture. CAT activity of the extract-treated NG108-15 cells was assayed in triplicates after 1, 2, 3, 4, and 5 days of additional incubation.

Figure 2

Effects of rat skeletal muscle extract on the cell number of the NG108-15 cells at various time intervals. Hybrid NG108-15 cells were inoculated into Falcon Multiwells at 5.0×10^4 cells/well in 1.5 ml of medium E. Sterilized rat skeletal muscle extract (500 ug) was introduced into the cells after 1 day of culture. Cell number of the NG108-15 cells was counted in triplicates after 1, 2, 3, 4, and 5 days of additional incubation.


with extracts
without extracts
CAT ACTIVITY (pmoles/min/well)
24
20
16
12
8
4
0
1
2
3
4
5
DAYS OF CULTURE

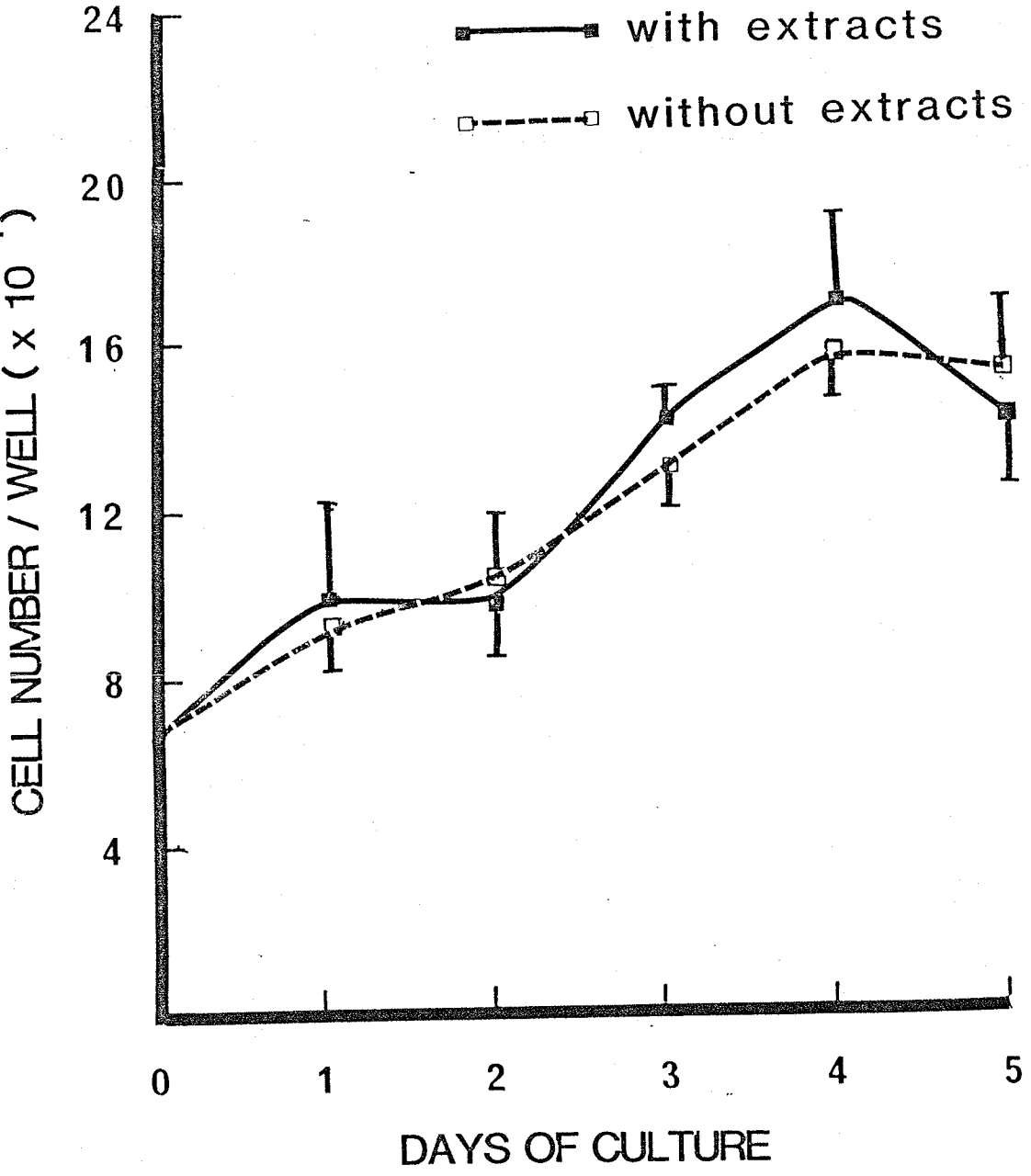
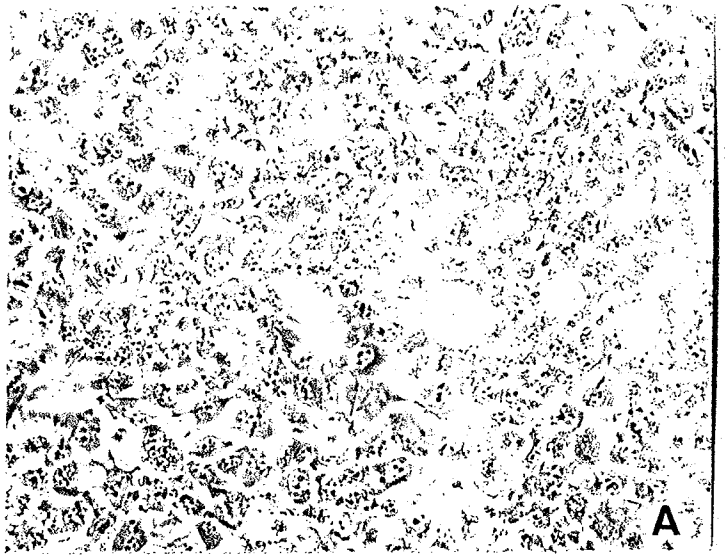
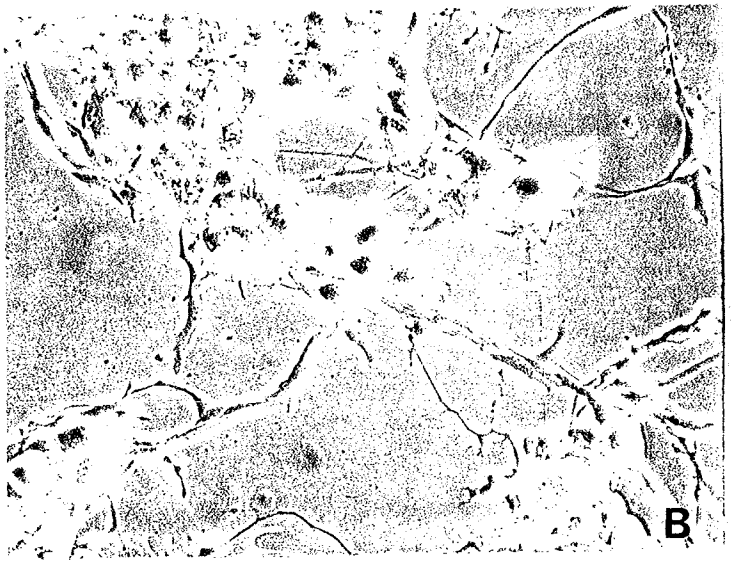
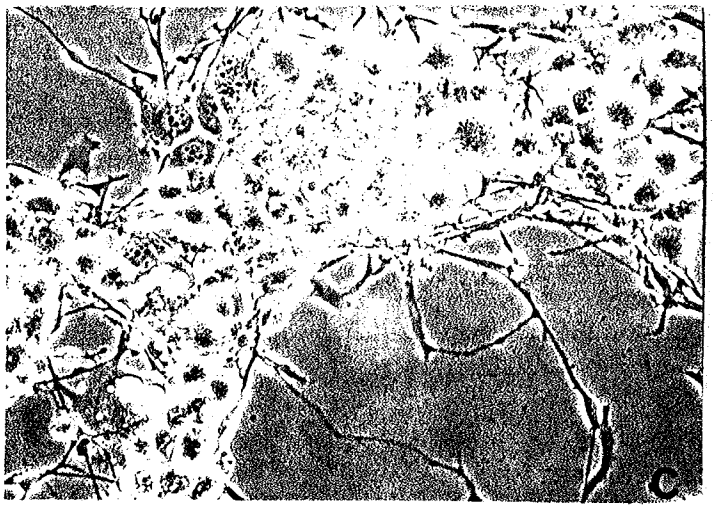

with extracts
without extracts
24
20
16
12
8
4
CELL NUMBER / WELL (x 10^{-4})
0
1
2
3
4
5
DAYS OF CULTURE

Figure 3

Phase contrast photomicrography of the hybrid NG108-15 cells in culture. A = cells cultured in medium D, B = cells cultured in medium E, C = cells cultured in medium E in the presence of 500 ug proteins of rat skeletal muscle extract.


A


B


C

introduced after 1 day of culture. CAT activity of the NG108-15 cells was assayed after an additional incubation of 5 days.

Figure 4 shows that the increase of CAT activity by muscle extracts was the highest in NG108-15 cells of a plating number of 7.5×10^4 cells/well; and the increase of CAT activity declined in wells plating at greater cell numbers, probably due to overcrowding. These results indicate that $7.0\text{-}8.0 \times 10^4$ cells/well was the optimal plating number of the NG108-15 bioassay cell for the bioassay of the CAT-stimulating activity.

(3) Dose response of the CAT-stimulating activity on the NG108-15 cells

The effect of the rat skeletal muscle stimulating the CAT activity in NG108-15 cells at various dilutions was examined. Rat skeletal muscle extract was diluted to the appropriate concentrations, and 200ul of each dilution were added to the cells to make up a final concentration of : 83, 167, 333, 500, 667, and 833 ug protein/ml of culture medium.

Figure 5 shows that 500 ug protein/ml of culture medium appeared to be the optimal concentration of muscle extracts to stimulate the CAT activity in the NG108-15 cells.

(II) PRELIMARY STUDIES ON THE PROPERTIES OF THE CAT-STIMULATING ACTIVITY IN RAT SKELETAL MUSCLE EXTRACT

In order to isolate the CAT-Stimulating Factor (CSF), some knowledge of the properties of the CSF is imperative. Preliminary studies were carried out to establish some of the properties of the CAT-stimulating activity in the rat skeletal muscle extract.

Figure 4

Determination of the optimal plating cell number of the NG108-15 cells for bioassay of CAT-stimulating activity. The NG108-15 cells were inoculated into falcon Multiwells at 2.5, 5.0, 7.5, 10.0, and 12.0×10^4 cells/well. Sterilized rat skeletal muscle extract (500 ug) was introduced into the cells after 1 day of culture. CAT activity of the cells was assayed in triplicates after 5 days of additional incubation.

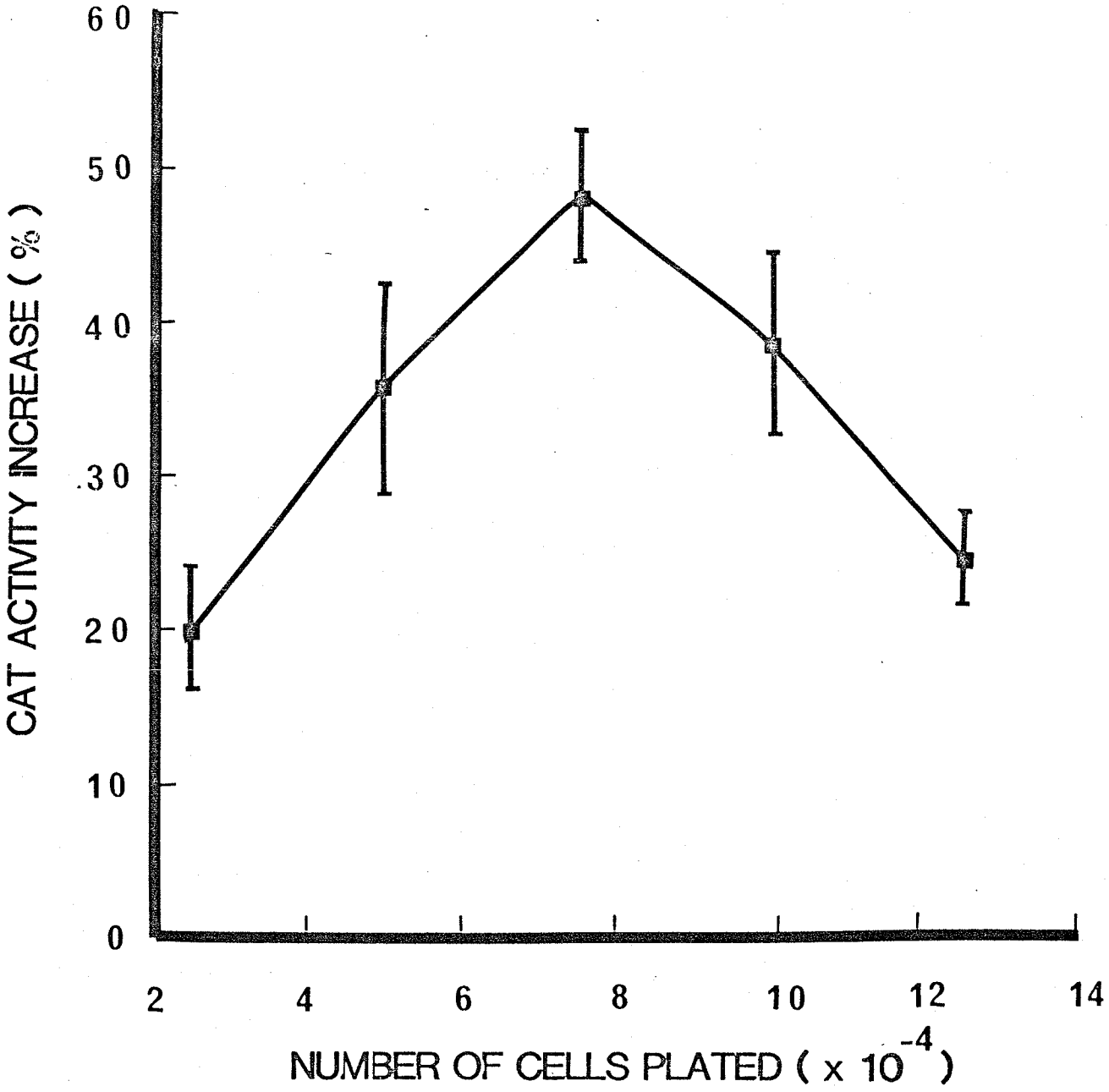
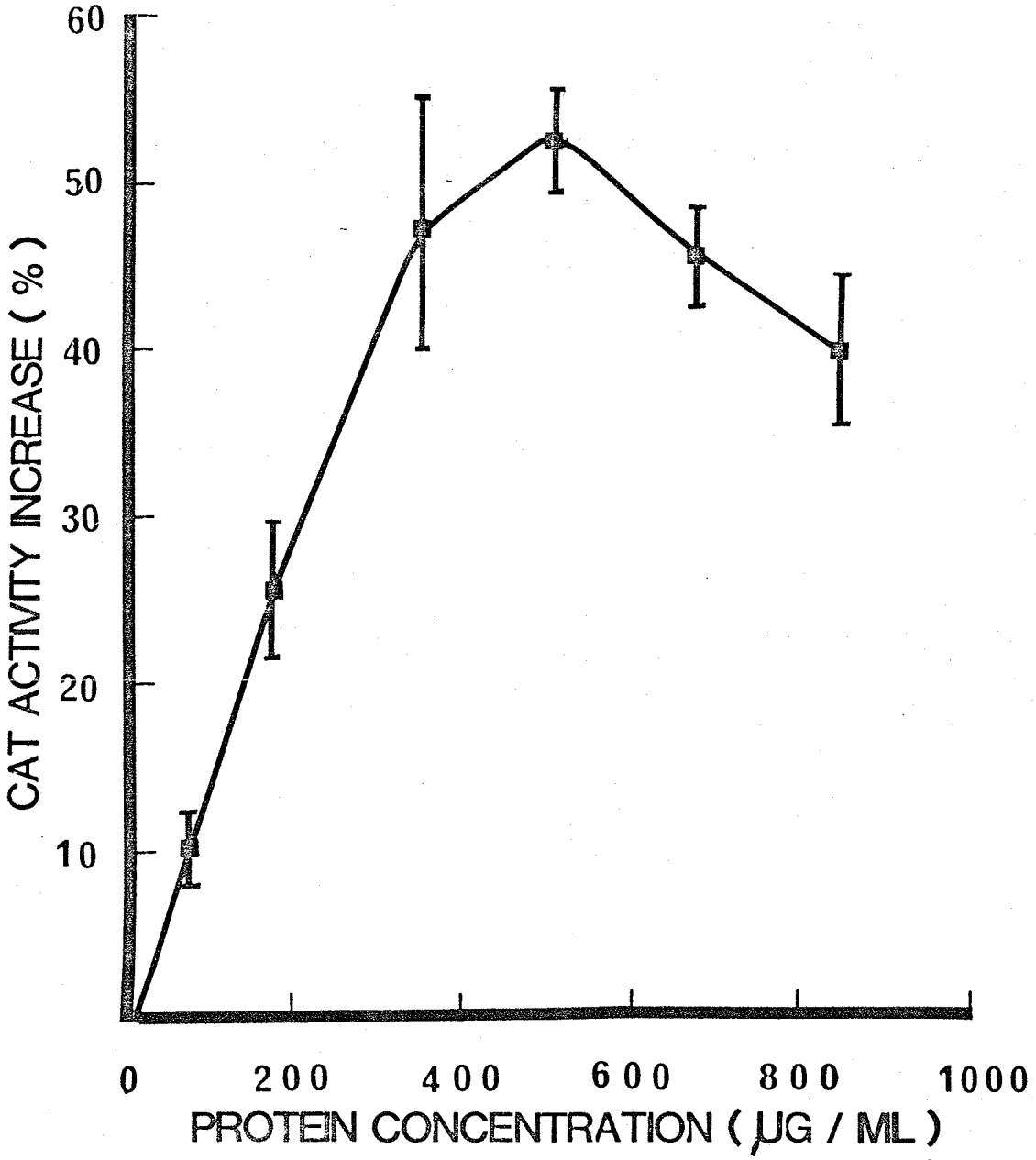

60
50
40
30
20
10
0
CAT ACTIVITY INCREASE (%)
2
4
6
8
10
12
14
NUMBER OF CELLS PLATED (x 10^-4)

Figure 5

Dose-response curves of the CAT-stimulatiang activity in rat skeletal muscle extract on the NG108-15 cells. Sterilized rat skeletal muscle extract was diluted to the appropriate concentrations,and 200 ul of each dilution was added to the cells to give a final concentration of: 83, 167, 333, 500, 667, and 833 ug/ml of culture medium. CAT activity of the NG108-15 cells was assayed in triplicates after 5 days of additional incubation.


60
50
40
30
20
10
CAT ACTIVITY INCREASE (%)
0
200
400
600
800
1000
PROTEIN CONCENTRATION (μG / ML)

(1) Precipitations of the CAT-Stimulating Factor by ammonium sulfate

Crude rat skeletal muscle extracts was precipitated by ammonium sulfate from 30-70% saturation. The various fractions were redissolved and dialysed over night against PBS, and assayed for CSF in the NG108-15 cell bioassay.

Figure 6 shows that ammonium sulfate at saturation between 30-50% has relatively small amount of proteins (§ 10% of total protein), and contained most of the CAT-stimulating activity. However, since the 0-30% fraction also contained significant amount of activity with little proteins (§3% of total protein), it was decided that the 0-50% fraction would be used for later purification steps.

(2) Stability of the muscle CAT-stimulating activity

The stability of CSF was tested at various experimental conditions to establish the optimal conditions for extraction and storage of the CAT-Stimulating Factor during and after purification. CAT-stimulating activity was compared among fresh rat skeletal muscle extract, extracts after 5 days storage, extracts in the presence of 1 mM PMSF after 5 days of storage at $4^{o}C$, 0-50% ammonium sulfate fraction after 5 days of storage at $4^{o}C$, and 0-50% ammonium sulfate fraction after being dialysed and lyophilized.

Figure 7 depicts that after 5 days of storage at $4^{o}C$, the CAT-stimulating activity in the crude extract decreased markedly, and significantly, and that the addition of the proteolytic enzyme inhibitor, phenylmethyl-sulfonyl fluoride (PMSF), could stabilize the activity. The 0-50% ammonium sulfate fraction

Figure 6

Fractionation of the CAT-stimulating activity in rat skeletal muscle extract by ammonium sulfate precipitation. The crude rat skeletal muscle extract was precipitated by ammonium sulfate at 30, 50, and 70% final saturations. The final supernatant (S/N), and the precipitated fractions were re-dissolved in PBS and dialysed extensively against PBS at pH 7.4. An aliquot of 200 ug protein of the dialysates was added to the NG108-15 cells. CAT activity of the NG108-15 cells was assayed in triplicates after 5 days of additional incubation.

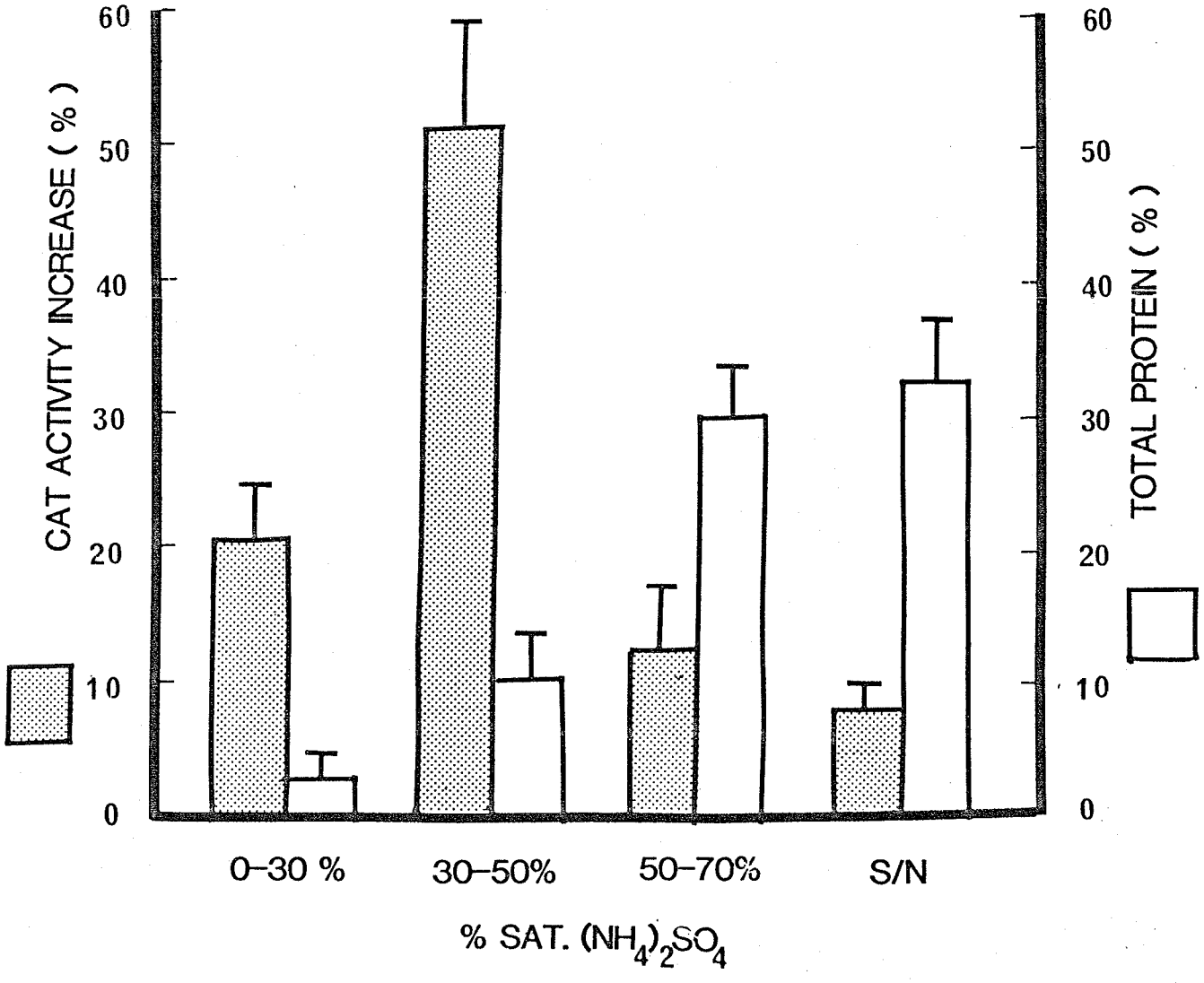
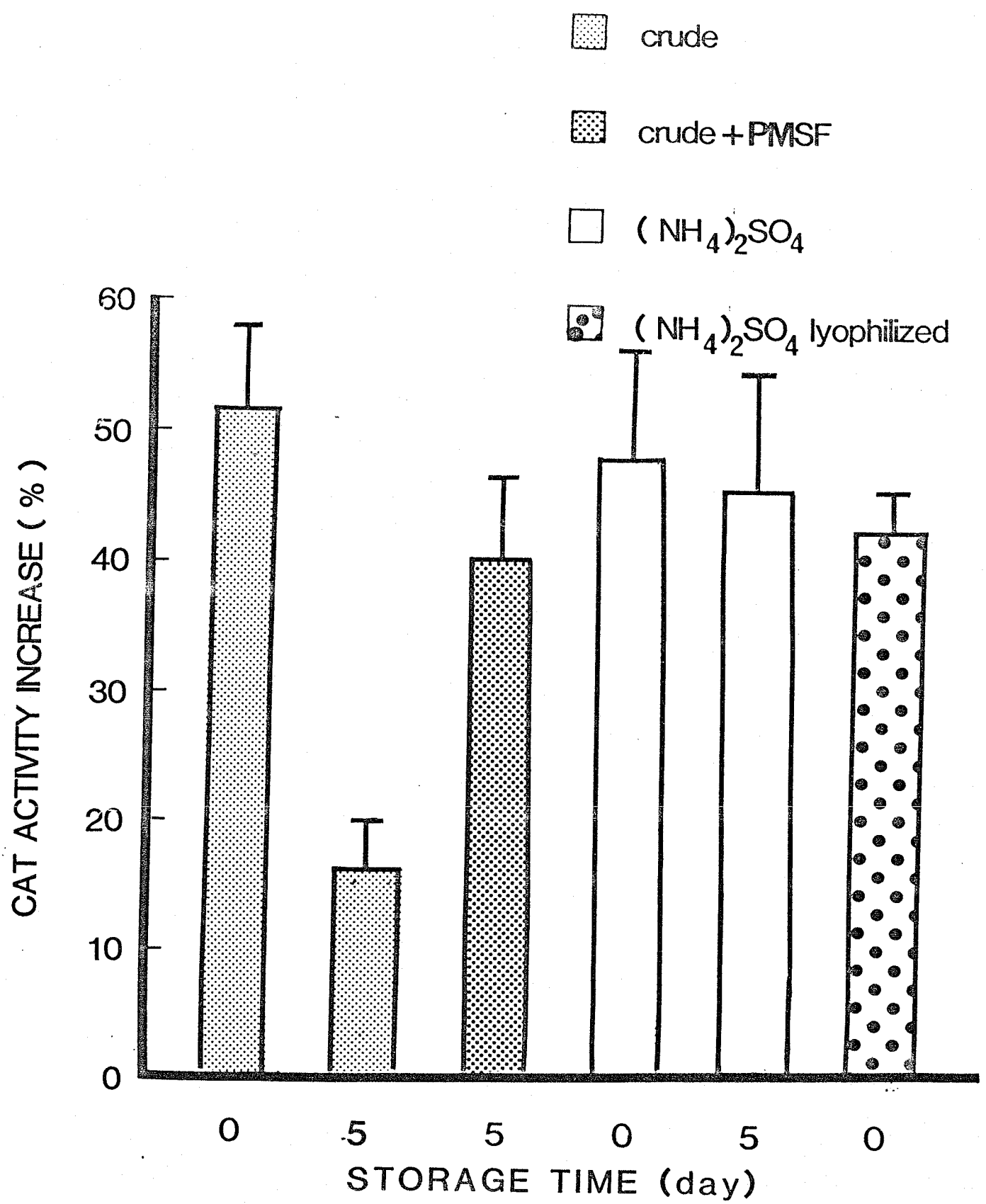

CAT ACTIVITY INCREASE (%)
TOTAL PROTEIN (%)
60
50
40
30
20
10
0
0–30 %
30–50%
50–70%
S/N
% SAT. $(NH_4)_2SO_4$

Figure 7

Stability of the CAT-Stimulating activity in rat skeletal muscle extract. Control = fresh crude muscle extract without storage; crude + PMSF = crude muscle extract with 1 mM PMSF in PBS for 5 days at 4 °C; $(NH_4)_2SO_4$ = sample of re-dissolved 50% saturation of ammonium sulfate precipitate of crude muscle extract in PBS for 5 days at 4 °C; $(NH_4)_2SO_4$ lyophilized = sample of re-dissolved lyophilized 50% saturation of ammonium sulfate precipitate of crude muscle extract in PBS. An aliquot of 200 ug protein of each sample was tested in triplicates for CAT-stimulating activity in NG108-15 cells.


crude
crude + PMSF
$(NH_4)_2SO_4$
$(NH_4)_2SO_4$ lyophilized
CAT ACTIVITY INCREASE (%)
60
50
40
30
20
10
0
0
5
5
0
5
0
STORAGE TIME (day)

after storage at 4°C for 5 days or stored lyophilized showed no difference in the CAT-stimulating activity compared to fresh extracts.

(3) Effects of temperature on the muscle CAT-stimulating activity

Aliquots of the CAT-stimulating activity in the 0-50% saturation of ammonium sulfate fraction was monitored by the NG108-15 bioassay after incubating at temperatures of: 4, 22, 37, and 55°C for 30 or 60 minutes.

Figure 8 depicts that there was no difference in CAT-stimulating activity in extracts after incubating at 4, 22, or 37°C for 60 min, but the activity decreased slightly after 30 min of incubation at 55°C. These results indicate that the CSF is relatively heat stable.

(4) Effects of pH on the muscle CAT-stimulating activity

Aliquots of the 0-50% ammonium sulfate fraction of the muscle extracts were stored in: Sodium acetate buffer at pH 4.4 or 5.4, sodium phosphate buffer at pH 6.4 or 7.4, TRIS buffer at pH 8.4, or glycine buffer at pH 9.4 for 5 days at 4°C, and the CAT-stimulating activity was assayed by the NG108-15 cell bioassay.

The results in figure 9 shows that the CAT-stimulating activity was decreased significantly less at pH 4.4, and dramatically less at pH 5.4, but relatively stable at a pH range of 6.4-9.4.

(5) Effects of enzymic digestion and chemical treatment on the CAT-stimulting activity

For enzymic digestion, 1 mg each of the 0-50% ammonium sulfate fraction was incubated in 1 ml of PBS, pH 7.4, with 1 mg

Figure 8

Effects of temperature on the CAT-stimulating activity in rat skeletal muscle extract. Samples of 2 ml each of the 0-50% ammonium sulfate fraction were kept at 4, 22, 37, and 55 °C for 30 or 60 min, and an aliquot of 200 ug protein of each sample was added in triplicates to the NG108-15 cells to assay the CAT-stimulating activity.

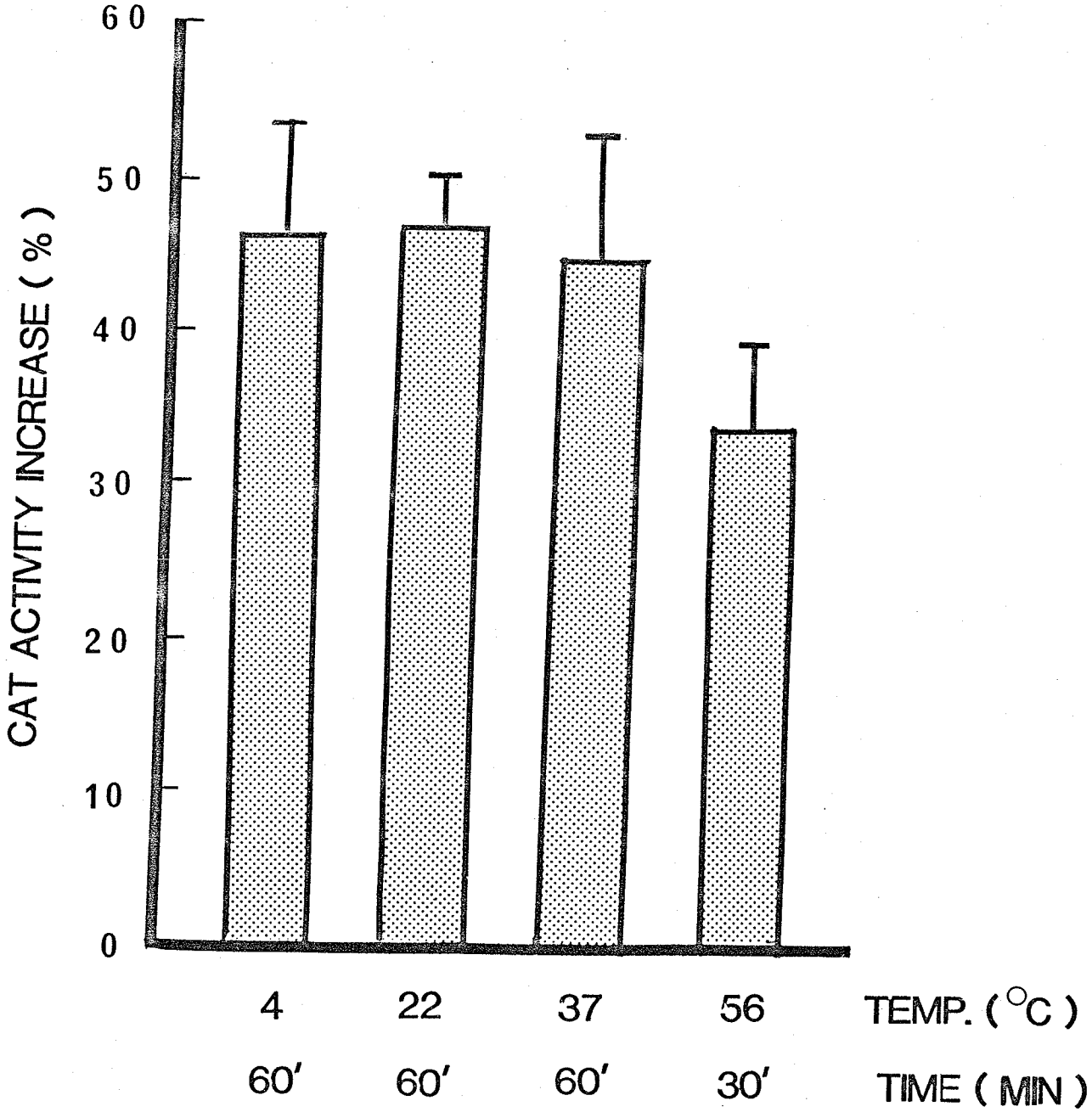
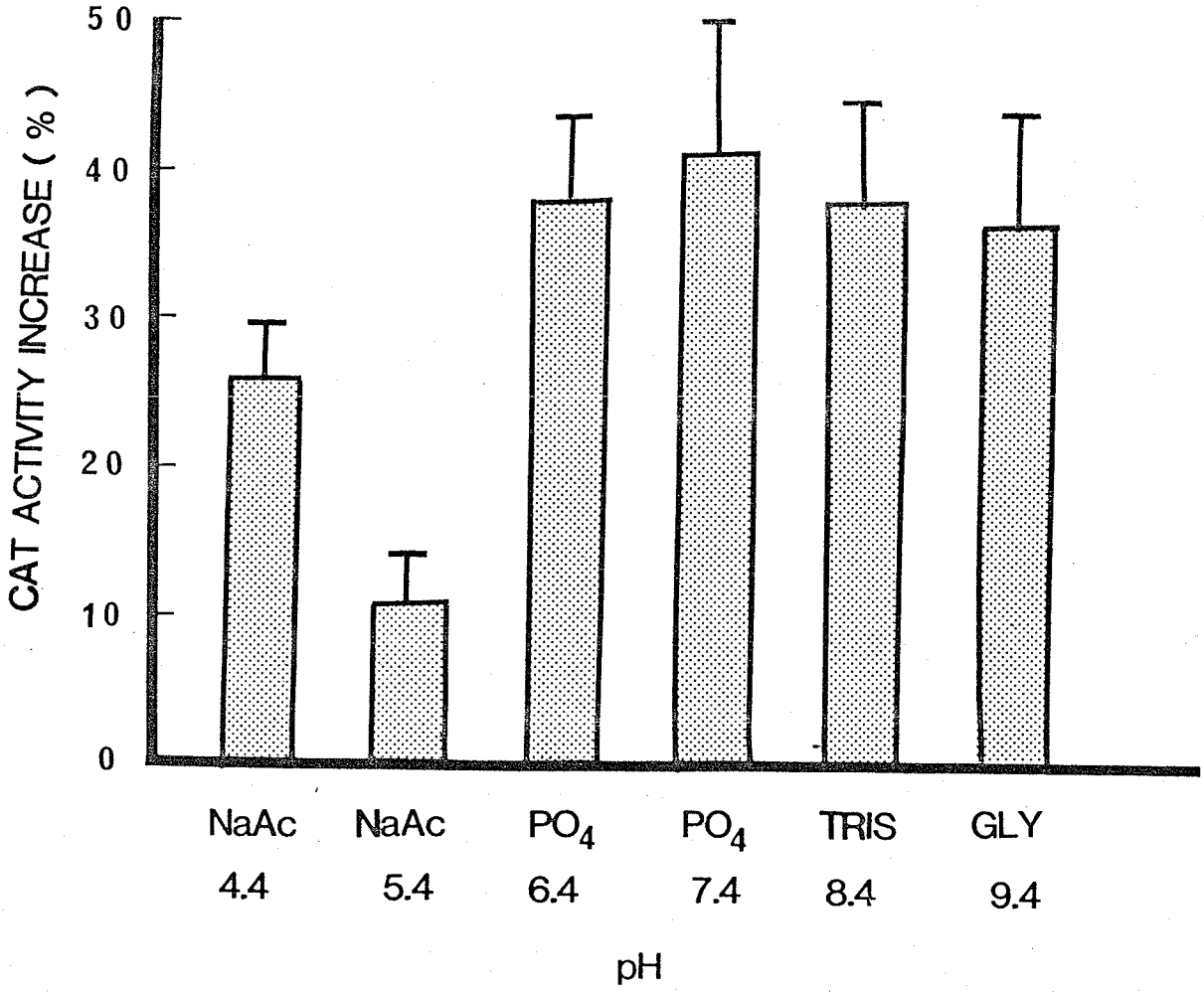

60
50
40
30
20
10
0
CAT ACTIVITY INCREASE (%)
4
22
37
56
TEMP. (°C)
60′
60′
60′
30′
TIME (MIN)

Figure 9

Effects of various pH on the CAT-stimulating activity in rat skeletal muscle extract. Samples of 2 ml each of the 0-50% ammonium sulfate fraction were dialysed against sodium acetate, pH 4.4 and 5.4; sodium phosphate, pH 6.4 and 7.4; TRIS, pH 8.4; and glycine, pH 9.4, and then stored for 5 days at 4 °C. An aliquot of 200 ug of protein from each samples was added in triplicates to the NG108-15 cells to assay the CAT-stimulating activity.


CAT ACTIVITY INCREASE (%)
50
40
30
20
10
0
NaAc
4.4
NaAc
5.4
PO4
6.4
PO4
7.4
TRIS
8.4
GLY
9.4
pH

of either trypsin, neuramindase, or phospholipase D, overnight at 4 °C. The action of trypsin was terminated at the end of incubation by the addition of soybean trypsin inhibitor (1.5 mg). For chemical treatments; samples were incubated in the presence of 8 M Urea, 1% B-mercaptoethanol, or 1 M NaCl overnight at 4 °C, followed by dialysis . Controls containing only the enzyme or chemical without the crude samples were treated exactly the same. All samples were assayed at 100 ug/ml for the ability to stimulate CAT activity using the NG108-15 bioassay.

Figure 10 shows that incubation of the CAT stimulating activity with trypsin and 8M Urea abolished most of the CAT-stimulating activity; however, treatments with 1 M NaCl, 1% B-mercaptoethanol, neuraminadase, and phospholipase D did not affect the activity. These results suggest that the biological component is protein in nature and can be denatured by 8 M Urea. Furthermore, disulphide bonds are not essential for biological activity and the molecule probably does not require carbohydrate or lipid residues for its biological activity.

(III) Purification of the CAT-Stimulating Factor in the rat skeletal muscle

(1) Extraction procedure

Rat skeletal muscles (890 g) were removed from hindlimbs of 50 male or female Sprague-Dawley rats of body weight (250-300 g), processed to obtain 40 g of crude protein in the rat skeletal muscle extract.

(2) Fractionation by ammonium sulfate precipitation

The CAT-stimulating activity in the rat skeletal muscle extract was first concentrated by ammonium sulfate

Figure 10

Effects of enzymic digestion and chemical treatment on the CAT-stimulating activity in rat skeletal muscle extract. Approximately 1 mg protein of the 0-50% ammonium sulfate fraction was incubated with 1 mg of trypsin (Trypsin), neuraminadase (Neura), or phospholipase D (Phos) in PBS at pH 7.4, overnight at 4 °C. The sample containing trypsin was terminated by the addition of soybean trypsin inhibitor (1.5 mg). Additional samples were incubated in the presence of 8 M Urea (Urea), 1% B-mercaptoethanol (Mercap), or 1 M NaCl (NaCl) in PBS at pH 7.4 overnight at 4 °C, followed by dialyzing against PBS. Control consisted of buffer samples treated identically with enzymes or chemicals except without the muscle extract proteins. An aliquot of 200 ug protein of each sample was added in triplicates to the NG108-15 cells to assay CAT-stimulating activity.

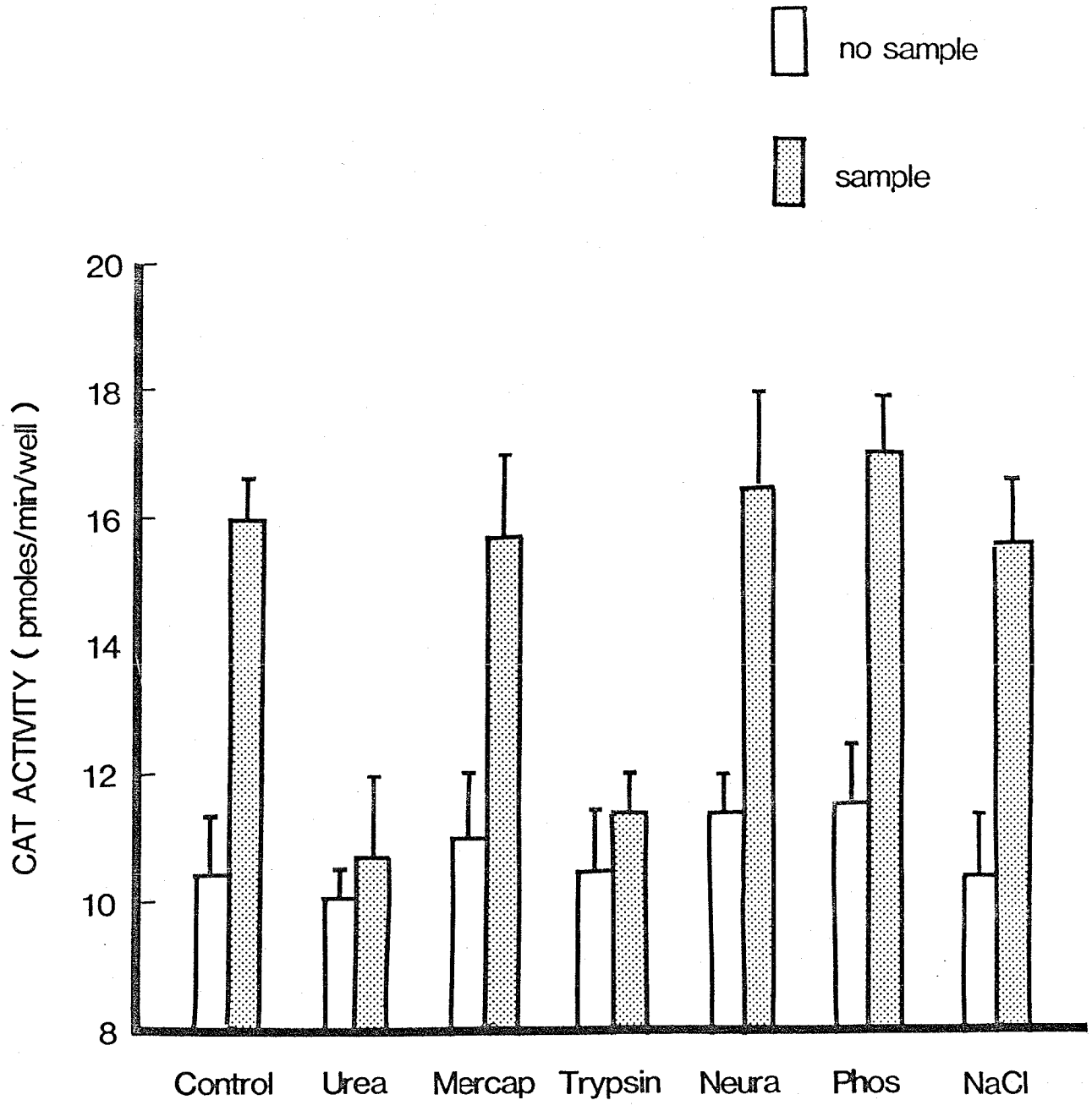
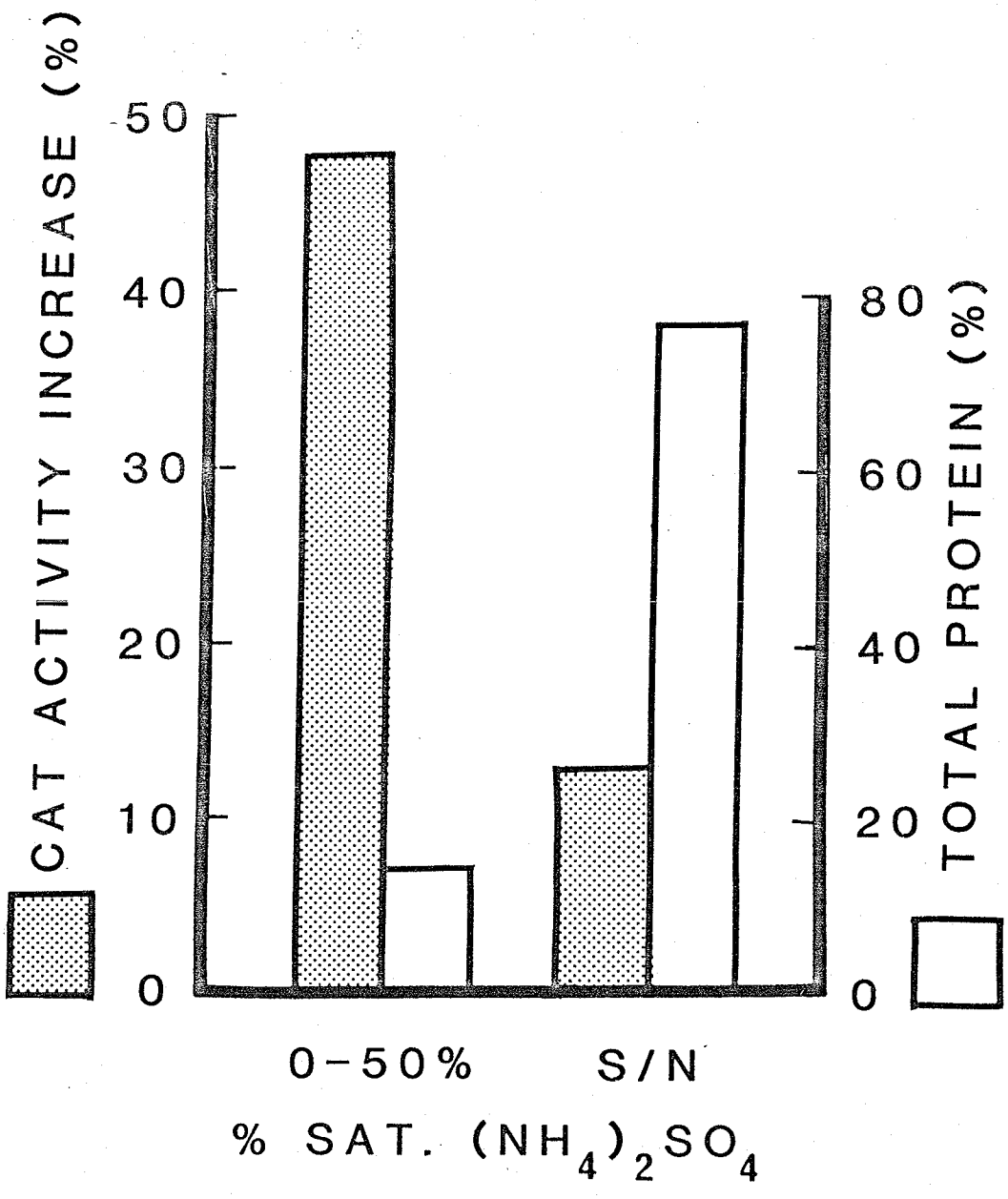

no sample
sample
CAT ACTIVITY (pmoles/min/well)
20
18
16
14
12
10
8
Control
Urea
Mercap
Trypsin
Neura
Phos
NaCl

Figure 11

Fractionation of the muscle CAT-stimulating activity by ammonium sulfate precipitation. Rat skeletal muscle extract (40 g of proteins) was precipitated by adding solid ammonium sulfate slowly to a 50% saturation to precipitate proteins with CAT-stimulating activity. After stirring for 60 min at 4 °C, precipitated proteins were harvested by centrifugation at 15,000 g for 60 min. The redissolved precipitate and supernatant were dialysed and assayed for CAT-stimulating activity in NG108-15 cell assay.


CAT ACTIVITY INCREASE (%)
50
40
30
20
10
0
80
60
40
20
0
TOTAL PROTEIN (%)
0-50%
S/N
% SAT. $(NH_4)_2SO_4$

precipitation. Figure 11 shows that the 0-50% ammonium sulfate fraction contained most of the CAT-stimulating activity, but only approximately 14% of total protein in the extracts (Table 2). This precipitate was redissolved in 1 mM $NaPO_4$ at pH 6.2 and dialysed extensively against the same buffer for the next step of purification by ion-exchange chromatography.

(3) Stepwise elution on CM-Cellex chromatography

The dialysed concentrate of the 0-50% ammonium sulfate fraction (5.5 g) was applied onto a CM-Cellex column, equilibrated in 1 mM sodium phosphate buffer at pH 6.2. After washing extensively, the bulk of the adsorbed proteins with low CAT-stimulating activity were eluted with 0.2 M NaCl in the buffer, and most of the stimulating activity was then eluted with 0.5 M NaCl in the same buffer as shown in figure 12. The 0.5 M NaCl fractions were pooled, and dialysed against 0.01 M phosphate buffer at pH 7.4, for the next step of isolation.

(4) Linear gradient elution on DE-Cellex chromatography

A DE-Cellex column was equilibrated in 0.01 M sodium phosphate buffer at pH 7.4. The dialysed sample (490 mg) after CM-Cellex chromatography (Fig. 12) was loaded onto the DE-Cellex column. After the unadsorbed fractions, the adsorbed proteins on the column were first eluted by 0.05 M NaCl in the phosphate buffer, and then by a linear gradient of 0.05 M to 0.15 M NaCl in the phosphate buffer. Finally, the column was eluted stepwise by 0.15 M and 0.25 M NaCl in the same buffer for the remaining proteins. Various elutions were monitored for protein distribution and CAT-stimulating activity as depicted in figure 13. It was observed that the broad protein peak of 0.09-0.15 M

Figure 12

CM-Cellex chromatography of the 50% ammonium sulfate precipitated fraction of the rat skeletal muscle extract. The redissolved precipitate of 50% saturation of ammonium sulfate (Fig.11) was dialysed extensively against 1 mM $NaPO_4$ buffer at pH 6.2, and centrifuged at 100,000 g for 60 min. The supernatant (5.5 g) was applied onto a CM-Cellex ion exchange column (6.6x16 cm) in 1 mM $NaPo_4$ at pH 6.2. After extensive washing of the unadsorbed fractions, the adsorbed proteins were eluted stepwise by 0.2 M, and 0.5 M NaCl in 1 mM $NaPO_4$ buffer. Fractions were collected in 20 ml/tube at a flow rate of 150 ml/hr. Aliquots of appropriate fractions were dialysed against PBS, filter-sterilized and assayed for CAT-stimulating activity by adding an equal volume of 200 ul each to the NG108-15 cells.

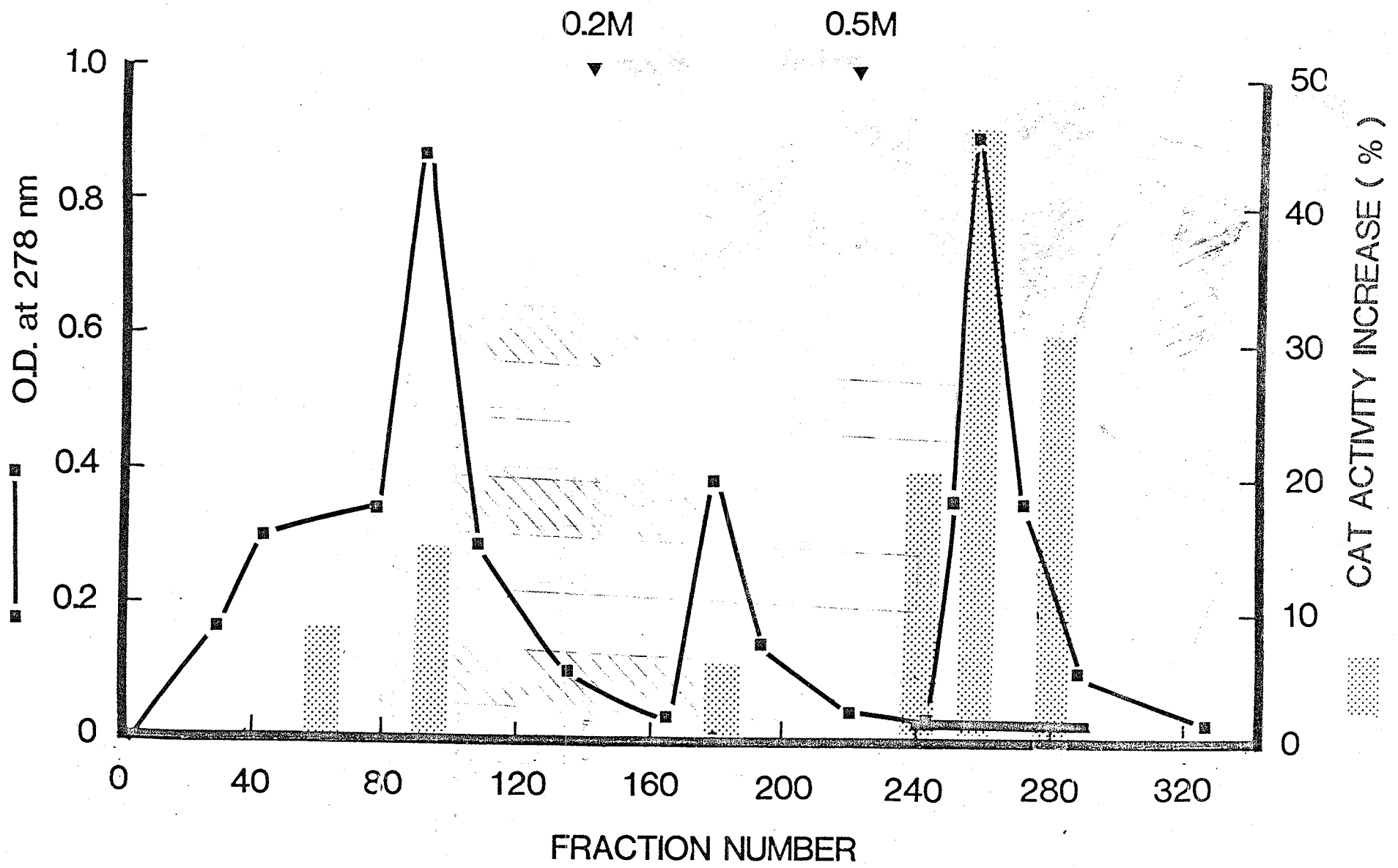
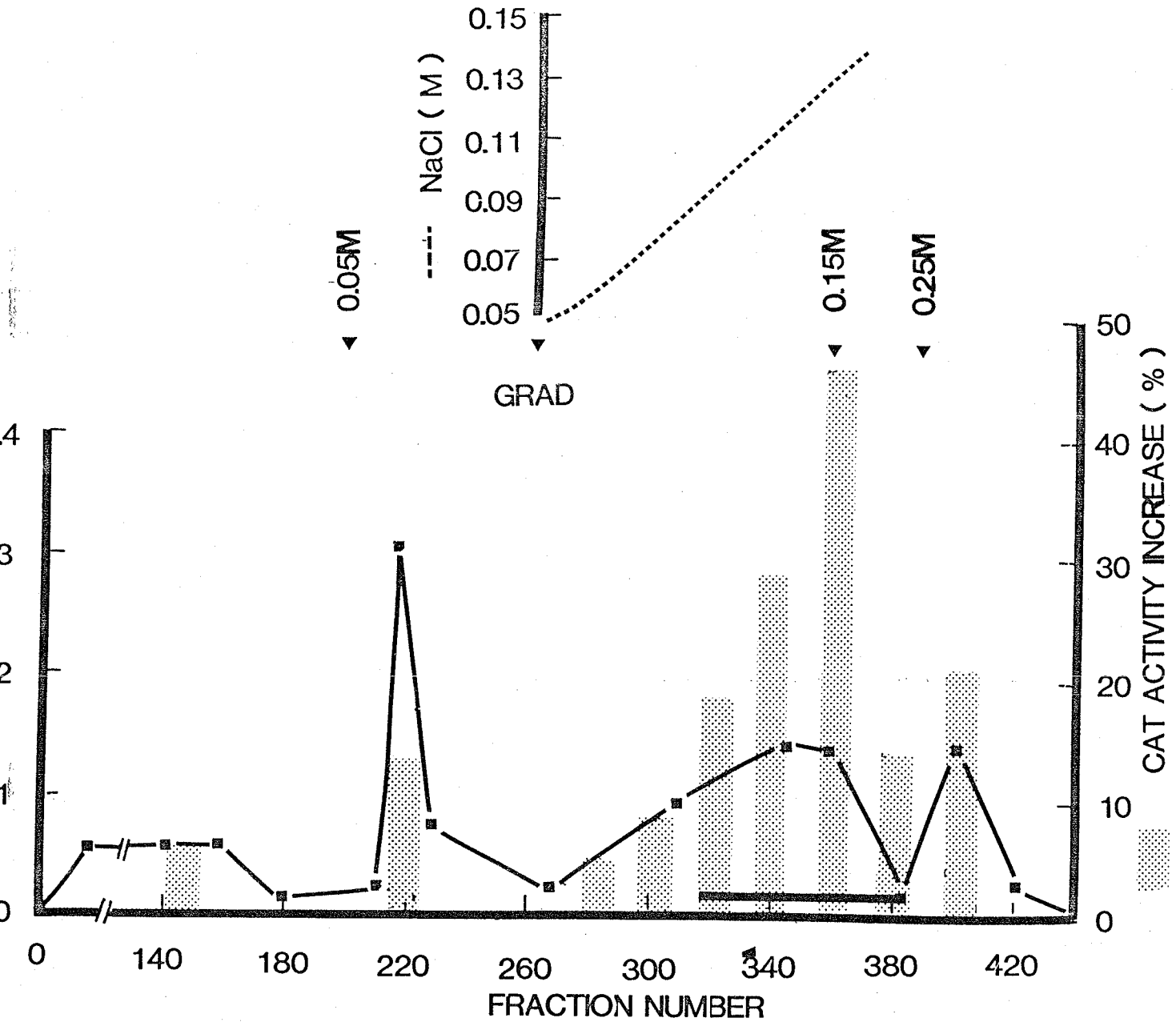

0.2M
0.5M
1.0
0.8
0.6
0.4
0.2
0
O.D. at 278 nm
50
40
30
20
10
0
CAT ACTIVITY INCREASE (%)
0
40
80
120
160
200
240
280
320
FRACTION NUMBER

Figure 13

DE-Cellex chromatography of the muscle CSF fractions after CM-Cellex chromatography. Pooled active fractions of CSF (490 mg) eluted by 0.5 M NaCl upon CM-Cellex chromatography (Fig. 12), after dialysis, were loaded onto a DE-Cellex column (3x16 cm) equilibrated in 0.01 M $NaPO_4$ at pH 7.4. After washing the unadsorbed fractions, the adsorbed proteins were first eluted by 0.05 M NaCl in the $NaPO_4$ buffer, and then by a linear gradient of 0.05 M to 0.15 M NaCl in the $NaPO_4$ buffer. Finally the remaining proteins were eluted stepwise by 0.15 M and 0.25 M NaCl in the $NaPO_4$ buffer. Fractions were collected in 10 ml/tube at a flow rate of 120 ml/hr. Aliquot of appropriate fractions were dialysed against PBS, filter-sterilized, and assayed for CAT-stimulating activity by adding an equal volume of 200 ul each to the NG108-15 cells.


0.15
0.13
0.11
0.09
0.07
0.05
NaCl (M)
0.05M
0.15M
0.25M
GRAD
50
40
30
20
10
0
CAT ACTIVITY INCREASE (%)
0
140
180
220
260
300
340
380
420
FRACTION NUMBER

NaCl fraction contained most of the CAT-stimulating activity. These fractions were pooled and dialysed against 0.01 M sodium phosphate buffer at pH 7.4 for the next step of purification.

(5) Compound gradient elution on DE-Cellex chromatography

The fractions (92 mg) eluted by 0.09-0.15 M NaCl (Fig. 13) containing proteins with CAT-stimulating activity were loaded onto another DE-Cellex column equilibrated with 0.01 M sodium phosphate buffer at pH 7.4. A more refined gradient of NaCl was inititated in order to get rid of more contaminated protein. A small amount of adsorbed proteins were first eluted by 0.05 M NaCl in phosphate buffer, and remaining proteins were then eluted by a continuous compound gradient of 0.05 / 0.15 / 0 / 0.10 / 0.10 / 0.15 / 0.15 / 0.15 / 0.10 M NaCl in the same buffer, generated by a nine chambers Varigrad apparatus. Finally, the column was washed by a stepwise elution with 0.10 M and 0.15 M NaCl in the phosphate buffer.

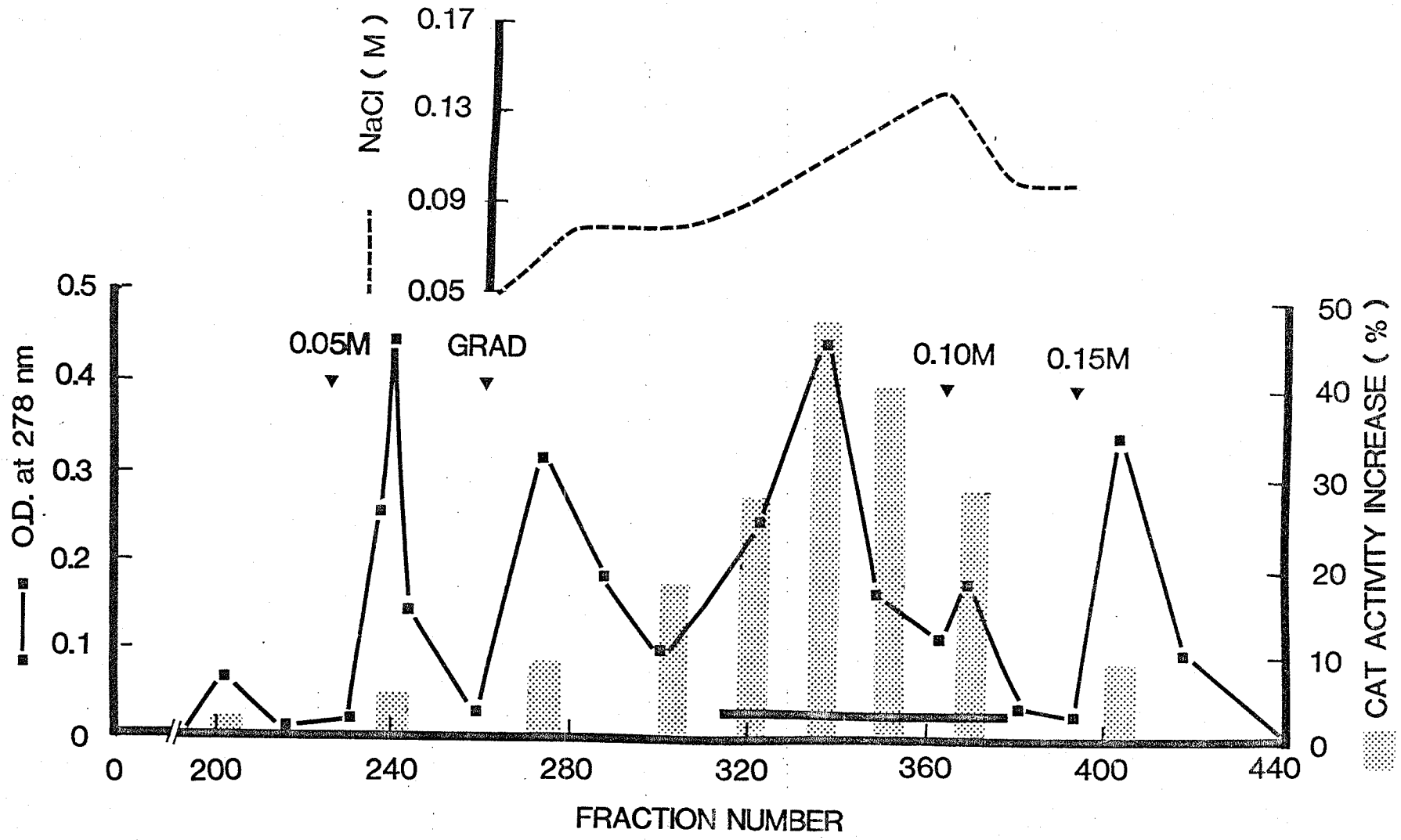
Figure 14 shows that the second peak of the eluted proteins between 0.09-0.14 M NaCl contained most of the CAT-stimulating activity. This step of ion-exchange chromatography proved to be very useful in separating a major portion of contaminating proteins of low activity from the proteins of high CAT-stimulating activity. The appropriate fractions were pooled, dialysed in 0.25% NH_4HCO_3, and lyophilized for the next step of purification by gel filtration on Sephadex G-100.

(6) Gel filtration on Sephadex G-100

The lyophilized sample of partially purified CSF (23 mg) after DE-cellex chromatography was redissolved in 2 ml of NH_4HCO_3

Figure 14

Recycling upon DE-Cellex chromatography of the muscle CSF fractions after DE-Cellex chromatography. The pooled active fractions of CSF (92 mg) eluted by 0.09 M to 0.15 M NaCl upon DE-Cellex chromatography (Fig. 12), after dialysis, were loaded onto another DE-Cellex column (1.8x9 cm) in 0.01 M $NaPO_4$ at pH 7.4. After washing the unadsorbed froactions, the adsorbed proteins were first eluted by 0.05 M NaCl in $NaPO_4$ buffer, the remaining proteins were then eluted by a continuous compound gradient of 0.05 / 0.15 / 0 / 0.10 / 0.10 / 0.15 / 0.15 / 0.15 / 0.10 M NaCl in $NaPO_4$, generated by a nine chambers Varigrad apparatus. Finally, the remaining proteins were washed by the stepwise elution with 0.10 M and 0.15 M NaCl in the $NaPO_4$ buffer. Fractions were collected in 3 ml/tube at a flow rate of 30 ml/hr. Aliquots of appropriate fractions were dialysed against PBS, filter-sterilized, and assayed for CAT-stimulating activity by adding an equal volume of 200 ul each to the NG108-15 cells.


NaCl (M)
0.17
0.13
0.09
0.05
0.05M
GRAD
0.10M
0.15M
O.D. at 278 nm
0.5
0.4
0.3
0.2
0.1
0
CAT ACTIVITY INCREASE (%)
50
40
30
20
10
0
0
200
240
280
320
360
400
440
FRACTION NUMBER

then subjected to gel filtration on Sephadex G-100. Figure 15 shows that 70-80% of the material in the sample was eluted with the high molecular weight fractions, but the majority of the CAT-stimulating activity was eluted with a small amount of proteins with the lower molecular weight fractions. The activity-containing fractions were pooled, lyophilized and recycled in the same Sephadex G-100 column.

Figure 16 shows that the pooled fractions from the first Sephadex G-100 column (Fig. 15) were separated into 3 distinct protein peaks. The first protein peak contained relatively low activity; the second and the third peak were only partially separated, and with most of the CAT-stimulating activity concentrated in the third peak. The third peak of proteins was pooled and recycled on another Sephadex G-100 column.

Figure 17 shows that the materials in the third peak (Fig. 16) could be separated into 2 distinct peaks of protein upon recycling, and the CAT-stimulating activity was eluted , coincident with one symmetrical peak of protein. Accordingly, the appropriate fractions were pooled and lyophilized as the final product of purified CSF.

(IV) SUMMARY OF THE PURIFICATION PROCEDURES FOR CSF

The established procedure for the purification of CSF is is summarized in table 1, and the yields and recoveries of materials and activities of the CAT-stimulating activity at different steps of purification are summarized in table 2. From 890 g of rat skeletal muscle, after 4 steps of isolation procedure, a preparation of 0.40 mg of highly purified CSF was achieved.

Figure 15

Gel filtration on Sephadex G-100 of the muscle CSF fractions after recycling on DE-Cellex chromatography. The pooled fractions of CSF (23 mg) eluted by 0.09 M to 0.14 M NaCl upon DE-Cellex chromatography (Fig. 13), were dialysed against 0.25% NH_4HCO_3 at pH 8.2 and lyophilized. The lyophilized sample was dissolved in 2 ml of 0.25% NH_4HCO_3, and centrifuged at 20,000 g for 30 min, and loaded onto a Sephadex G-100 column (2.2x 110 cm), equilibrated in 0.25% NH_4HCO_3. Fractions were collected in 2 ml/tube at a flow rate of 15 ml/hr. Aliquots of appropriate fractions were diluted 1:10 with PBS at pH 7.4, filter-sterilized, and assayed for CAT-stimulating activity by adding an equal volume of 200 ul each to the NG108-15 cells.

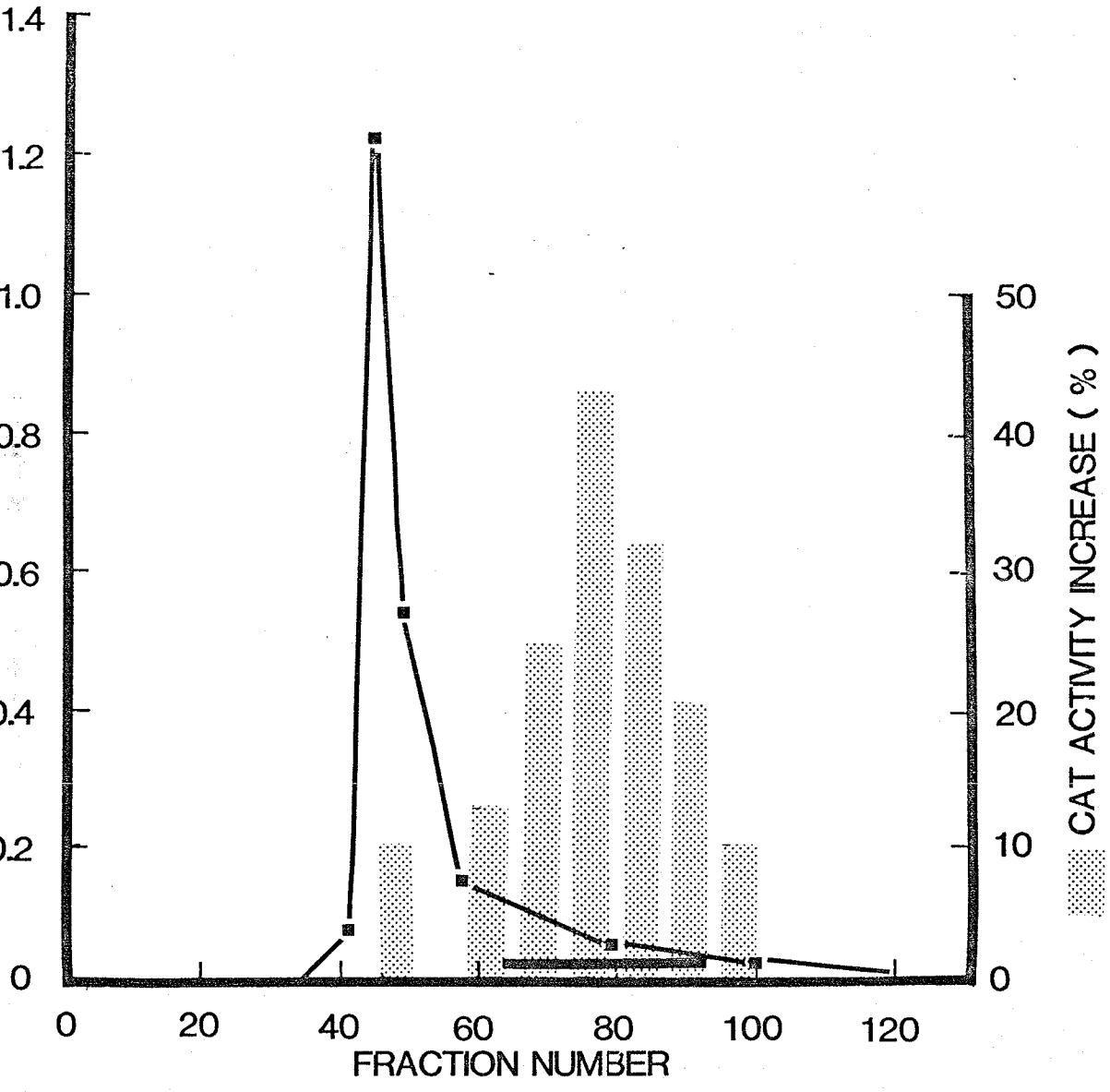

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
50
40
30
20
10
0
CAT ACTIVITY INCREASE (%)
0
20
40
60
80
100
120
FRACTION NUMBER

Figure 16

Recycling upon Sephadex G-100 of the muscle CSF fractions after gel filtration on Sephadex G-100. The pooled active fractions (3.1 mg) after gel filtration on Sephadex G-100 (Fig. 14) were lyophilized. the lyophilized sample was dissolved in 2 ml 0.25% NH_4HCO_3, centrifuged at 20,000 g for 30 min, and loaded onto the same Sephadex G-100 column (2.2x110 cm) and eluted with the same buffer. Fractions were collected in 2 ml/tube at a flow rate of 15 ml/hr. Aliquots from appropriate fractions were diluted 1:20 with PBS, filter-sterilized, and assayed for CAT-stimulating activity by adding an equal volume of 200 ul each to the NG108-15 cells.

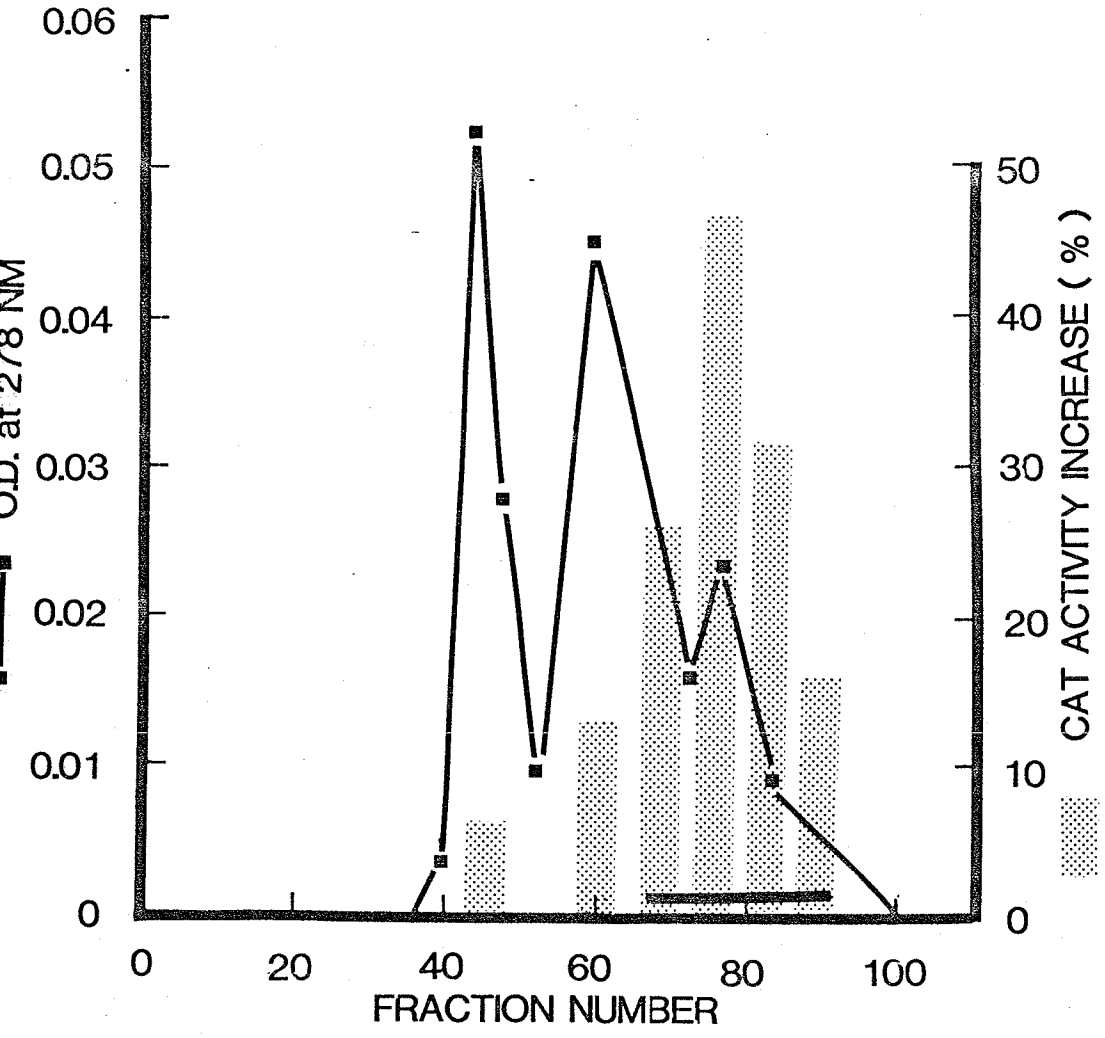

0.06
0.05
0.04
0.03
0.02
0.01
0
O.D. at 278 NM
50
40
30
20
10
0
CAT ACTIVITY INCREASE (%)
0
20
40
60
80
100
FRACTION NUMBER

Figure 17

Recycling upon Sephadex G-100 of the muscle CSF fractions after the second gel filtration on Sephadex G-100. The pooled active fractions (0.95 mg) after recycling on the second Sephadex G-100 column (Fig. 15), were lyophilized. The lyophilized sample was dissolved in 1 ml of 0.25% NH_4HCO_3 at pH 8.2, centrifuged at 20,000 g for 30 min, and loaded onto another Sephadex G-100 column (1.0x110 cm) and eluted with the same buffer. Fractions were collected in 1 ml/tube at a flow rate of 15 ml/hr. Aliquots from appropriate fractions were diluted 1:20 with PBS, filter-sterilized, and assayed for CAT-stimulating activity by adding an equal volume of 200 ul each to the NG108-15 cells.

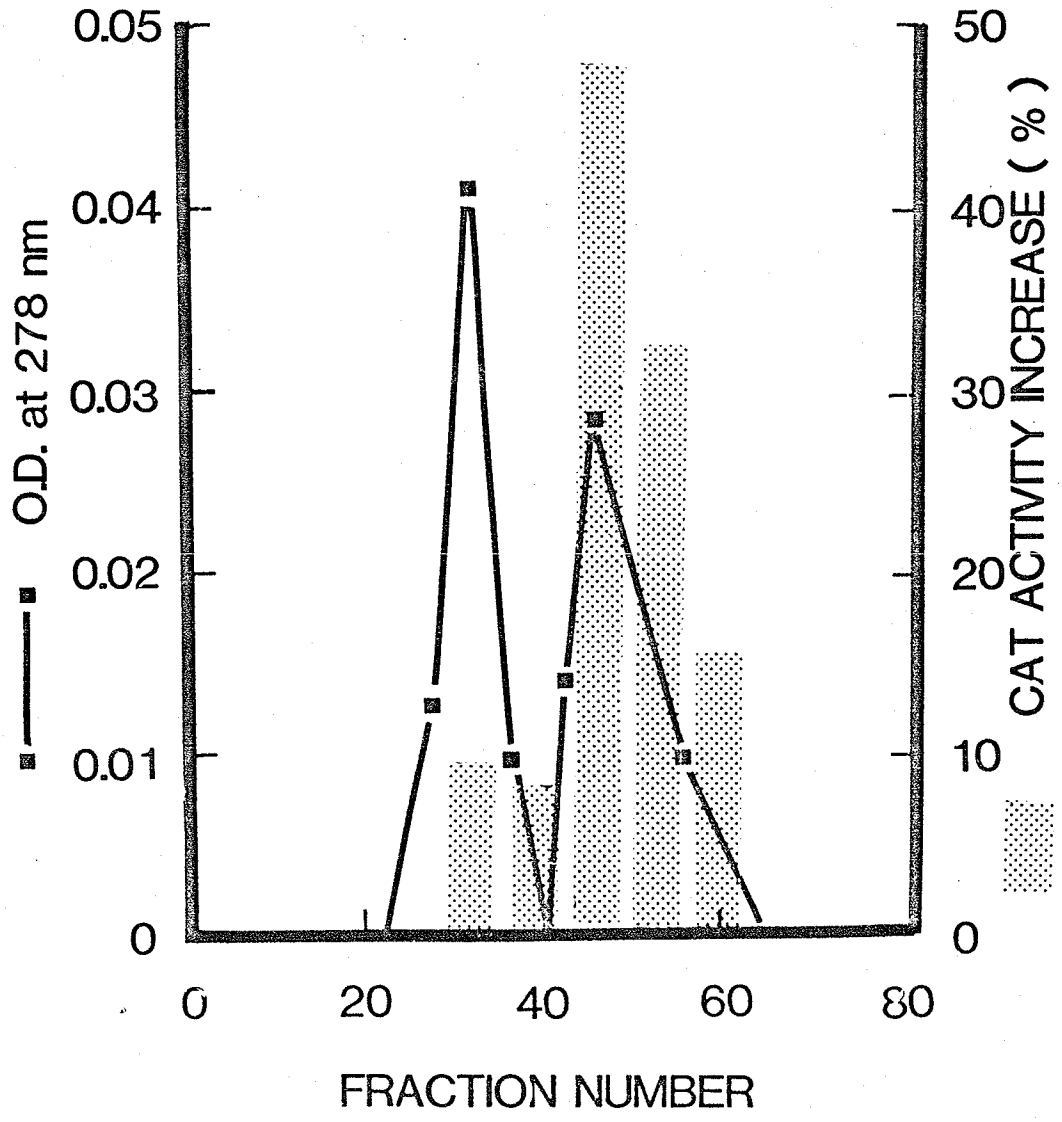

0.05
0.04
0.03
0.02
0.01
0
O.D. at 278 nm
50
40
30
20
10
0
CAT ACTIVITY INCREASE (%)
0
20
40
60
80
FRACTION NUMBER

TABLE 1 SUMMARY OF CSF PURIFICATION

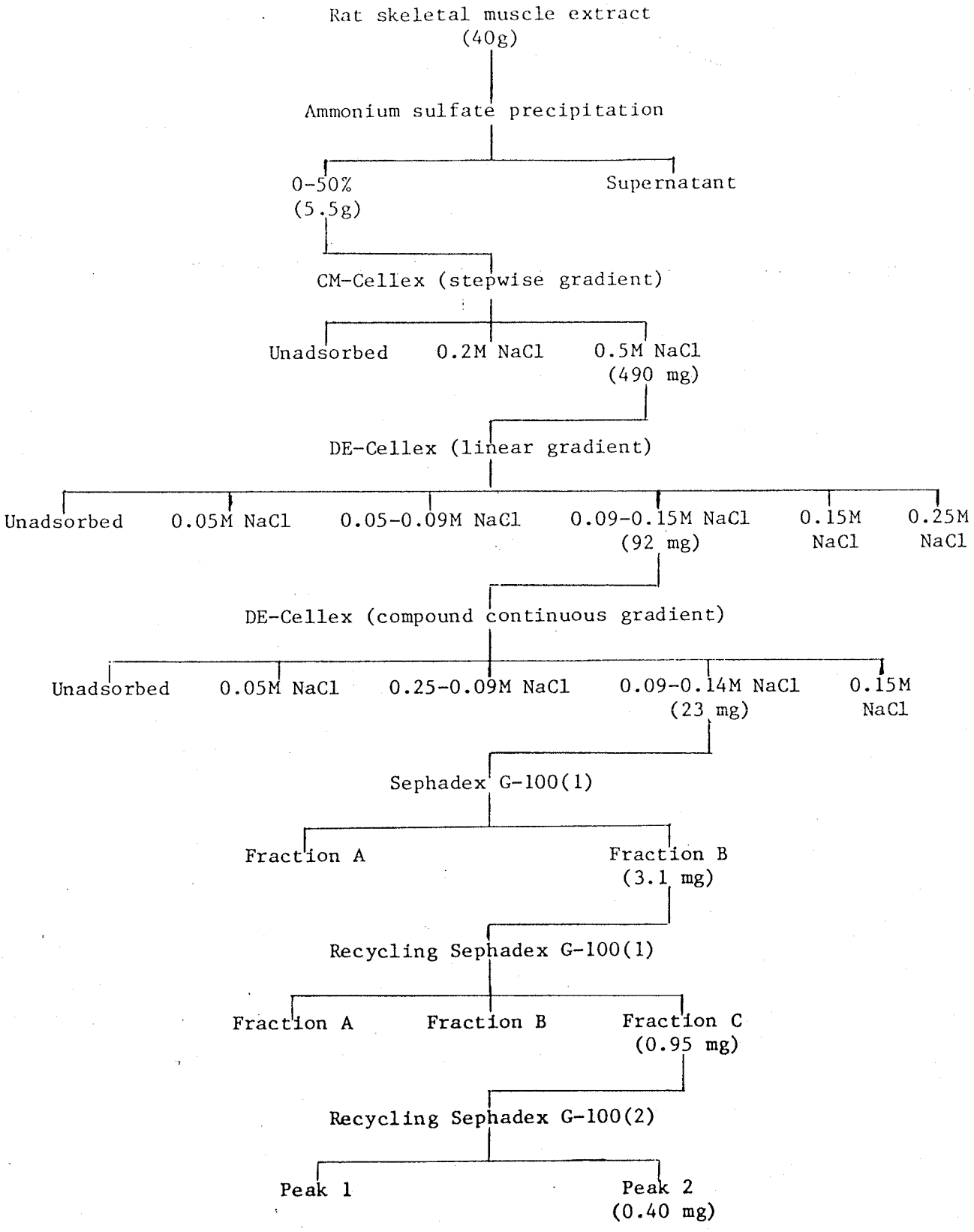


TABLE 2 SUMMARY OF CAT-STIMULATING FACTOR PURIFICATION

PURIFICATION STEPS	PROTEIN MG	PROTEIN YIELD %	SU (μg/ml) OF CULTURE MEDIUM	ACTIVITY RECOVERY %	SPECIFIC ACTIVITY (SU X mg^{-1})	TOTAL UNITS (X 10^{-3})	PURIFICATION FOLD
Crude extract	40,000	100	200	100	5.0	200	1
$(NH_4)_2 SO_4$ 0-50% fraction	5,500	14	60	46	17	92	3.4
CM-cellex stepwise gradient	490	1.2	7.8	32	128	63	26
DE-cellex(I) linear gradient	92	0.23	1.7	27	588	54	118
DE-cellex(II) compound gradient	23	0.058	0.50	23	2,000	46	400
G-100(1)	3.1	0.0078	0.080	19	12,500	38	2,500
G-100(1) Recycling	0.95	0.0024	0.030	16	33,333	32	6,667
G-100(2) Recycling	0.40	0.0010	0.015	13	66,667	27	13,333

One stimulation unit (SU) is defined as the amount of protein per ml of culture medium which provided a stimulation equal to half of the maximal stimulation obtained from the dose-response curve (Fig. 17).

(IV) CHARACTERIZATION OF THE CAT-STIMULATING FACTOR

(1) Dose response curves of muscle CSF fractions obtained at various steps in the purification procedure

Figure 18 shows the dose responses of major active fractions obtained during the purification procedure. The purified CSF was active at 5 ng/ml, and has a half maximal dosage of 15 ng/ml.

(2) Polyacrylamide gel electrophoresis of the purified CSF

Upon polyacrylamide gel electrophoresis the highly purified CSF exhibits one major band (Fig. 19), with the CAT-stimulating activity coincident with the protein band (Fig. 20).

(3) Analytical gel isoelectric focusing

Analytical thin layer polyacrylamide gel (LKB) isoelectric focusing was carried out using LKB 2117 multiphor apparatus and a pre-cast 5% polyacrylamide gel with a pH range of 3.5-9.5. Sample and markers of various known pI (amyloglucosidase, 3.50; soybean trypsin inhibitor, 4.55; B-lactoglobulin A, 5.20; bovine carbonic anhydrase B, 5.85; human carbonic anhydrase B, 6.55; horse myogloblin-acidic band, 6.85; horse myoglobin-basic band, 7.35; lentil lectin-acidic band, 8.15; lentil lectin-basic band, 8.65; and trypsinogen, 9.30) were focused for 4.5 hrs at 4 watts and stained for protein. After focusing, the gel was fixed and stained with coomassie brilliant blue to visualize proteins. To determine the pH gradient formed after focusing, an additional gel lane was cut into 5 mm segments, which was individually soaked and eluted in 1 ml double distilled H_2O for 60 min. The pH in each gel eluants was determined by the pH meter.

Figure 21a shows that purified CSF consisted of only one

Figure 18

Dose-response curves of the muscle CSF fractions obtained at various steps in the purification procedure. The pooled samples representing the CSF activity at each step of purification were measured at various concentrations in the NG108-15 cell bioassay. One hundred percent stimulation is defined as the maximal stimulation obtained with the fresh crude extract.

crude extract = ▼——▼

0-50% $(NH_4)_2SO_4$ fraction = ○——○

CM-Cellex pool = ▽——▽

DE-Cellex [1] pool = □·····□

DE-Cellex [2] pool = ■——■

Sephadex G-100 [1] pool = ●——●

recycling Sephadex G-100 [1] = ■·····■

recycling Sephadex G-100 [2] pool = □——□

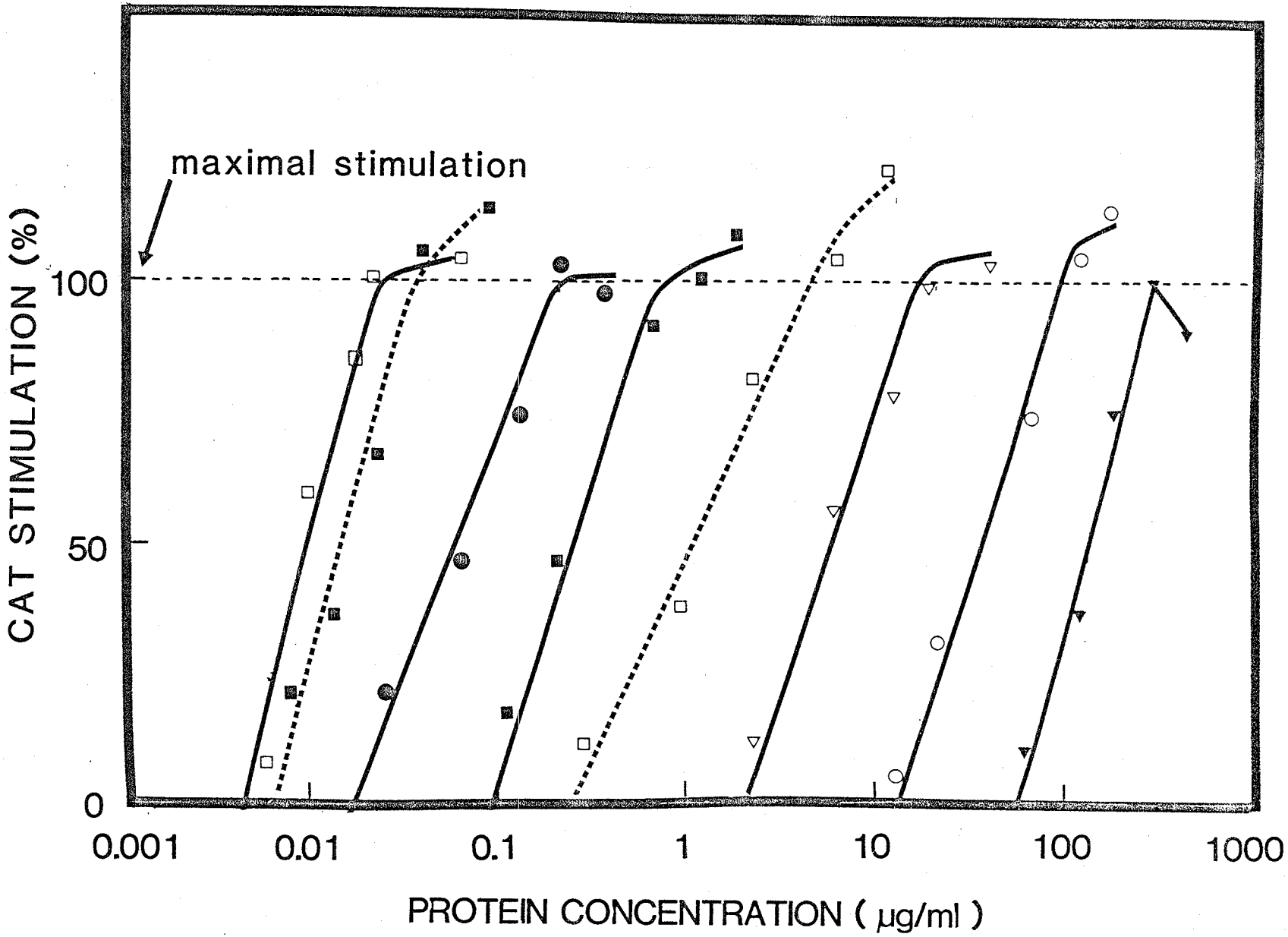

maximal stimulation
CAT STIMULATION (%)
100
50
0
0.001
0.01
0.1
1
10
100
1000
PROTEIN CONCENTRATION (µg/ml)

Figure 19

Polyacrylamide gel electrophoresis of the highly purified rat skeletal muscle CSF. A sample of purified CSF (20 ug) was electrophoresised in 7.5% polyacrylamide gel electrophoresis at pH 8.9.

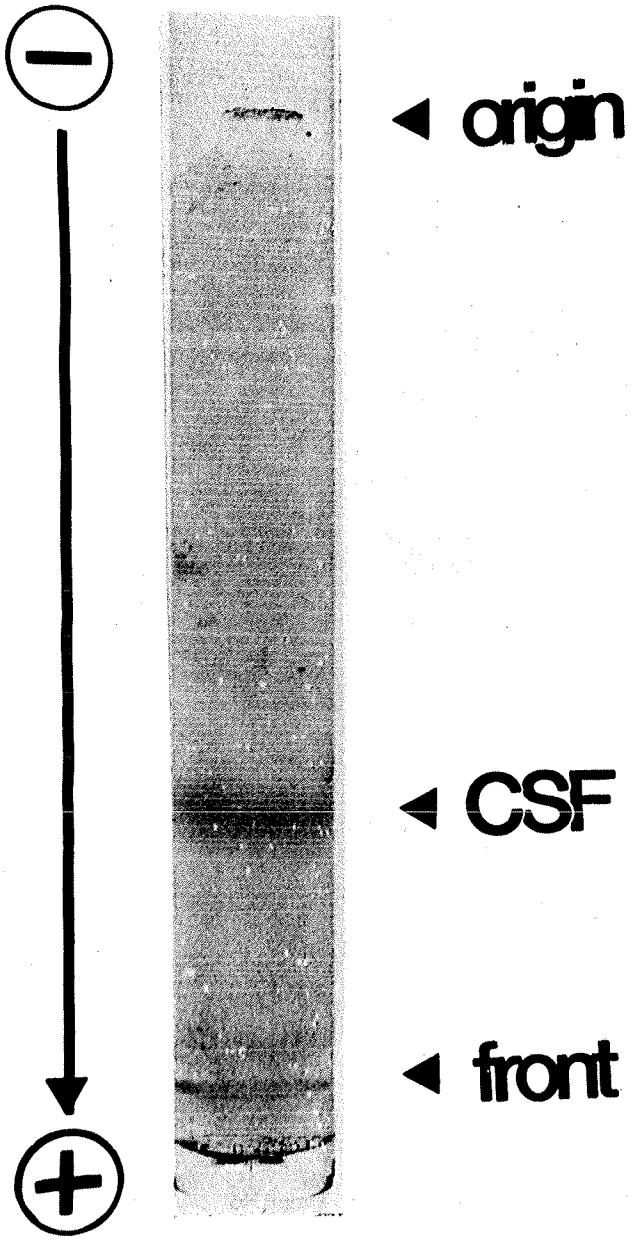
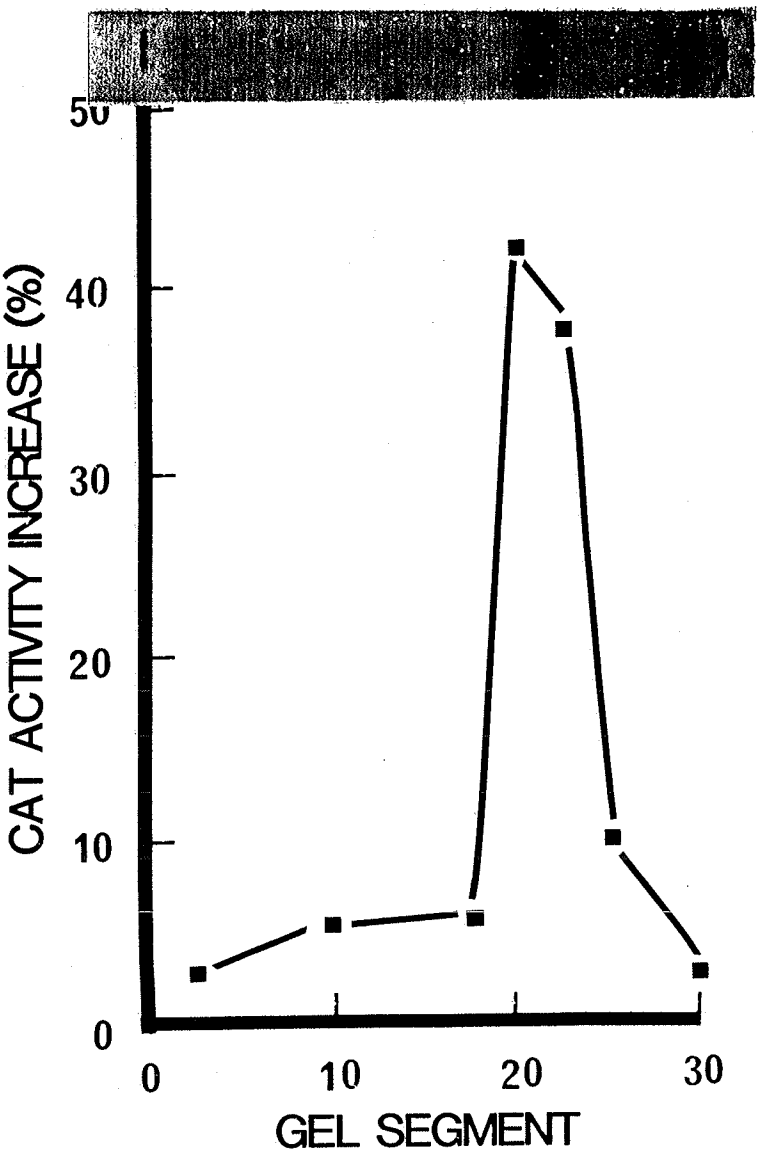

origin
CSF
front

Figure 20

Analysis of the purified muscle CSF by polyacrylamide gel electrophoresis. Samples of purified CSF (20ug) was analysed in duplicates by 7.5% polyacrylamide gel electrophoresiss at pH 8.9. One gel was stained for protein, another gel was cut into 2 mm segments and eluted by 1 ml of PBS with 0.2% fatty acid free BSA at pH 7.4, overnight at 4 ^{o}C; and diluted 1:20 with the same buffer, filter-sterilized and assayed for CAT-stimulating activity by adding an equal volume of 200 ul each to the NG108-15 cells.


50
40
30
20
10
0
CAT ACTIVITY INCREASE (%)
0
10
20
30
GEL SEGMENT

protein band, and was focused at pH 4.7-4.8. Upon isoelectric focusing, highly purified CSF was confirmed to be homogeneous and isoelectric point (pI) of CSF was estimated to be 4.75 (Fig. 21b).

(2) Determination of Molecular Weight

(a) Gel filtration on Sephadex G-100. The molecular weight of highly purified CSF was estimated by the method of gel filtration on Sephadex G-100. The elution volume of CSF was compared with those of protein markers of known molecular weight (Bovine serum albumin, 67K; ovalbumin, 45K; trypsinogen, 25K; and cytochrome C, 12K) calibrated in the same column under identical experimental conditions (Fig. 22a).

The molecular weight of CSF was estimated by plotting the elution volumes of these proteins against their molecular weights as shown in figure 22a. By this method of gel filtration on Sephadex G-100, the molecular weight of CSF was determined to be 30K (Fig. 22b).

(b) Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

The molecular weight of the purified CSF was also determined by the method of PAGE in the presence of 1% SDS. Figure 23a shows the electrophoretic protein pattern of purified CSF and the protein markers of phosphorylase B (93K), bovine serum albumin (66K), ovalabumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (22K), and lysozyme (14K). Figure 23b shows the determination of molecular weight by plotting the electrophoretic mobilities (Rf) of the protein markers and their molecular weights. The highly purified CSF appeared to be a

Figure 21a

Analysis of the purified muscle CSF by thin layer polyacrylamide gel isoelectric focusing. Protein markers of known isoelectric points (amyloglucosidase, 3.50; soybean trypsin inhibitor, 4.55; B-lactogloblin A, 5.20; bovine carbonic anhydrase B, 5.85; human carbonic anhydrase B, 6.55; horse myogloblin-acidic band, 6.85; horse myogloblin-basic band, 7.35; lentil lectin-acidic band, 8.15; lentil lectin-basic band, 8.65; and trypsinogen, 9.30) and a sample of purified CSF (20 ug) were focused for 4.5 hr at 4 watts and stained with coomassie brilliant blue.

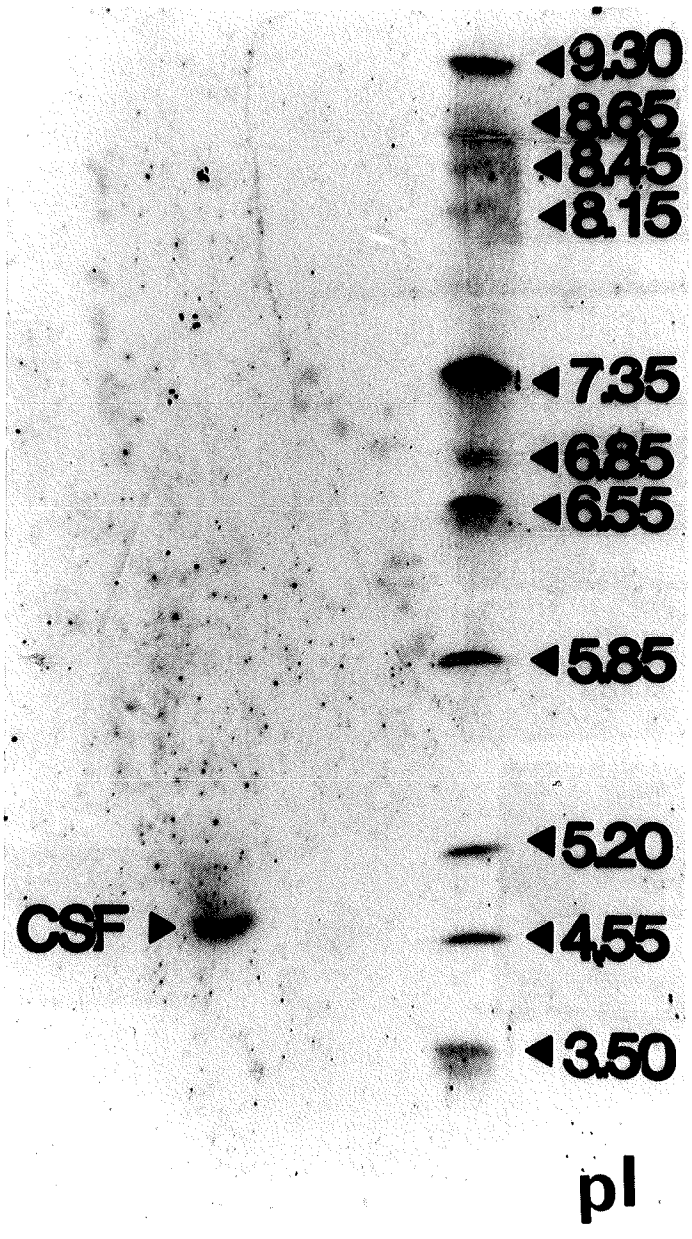

9.30
8.65
8.45
8.15
7.35
6.85
6.55
5.85
5.20
CSF
4.55
3.50
pI

Figure 21b

Determination of the isoelectric point for purified muscle CSF by thin layer polyacrylamide gel isoelectric focusing. An additional gel lane from the same thin layer polyacrylamide gel (Fig. 20) was sliced into 5 mm segments and each soaked in 1 ml double distilled water for measurement of pH.

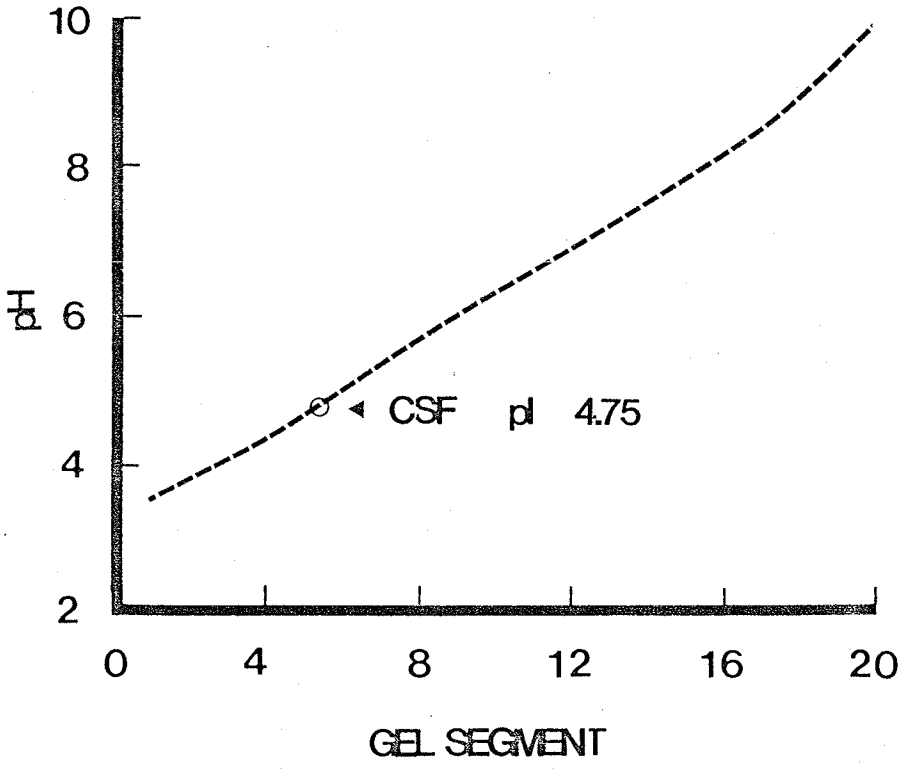

10
8
6
4
2
pH
CSF pI 4.75
0
4
8
12
16
20
GEL SEGMENT

Figure 22a

Determination of the molecular weight of purified CSF by gel filtration on Sephadex G-100. (Elution profiles of proteins). A column of Sephadex G-100 (1x110 cm) was equilibrated in 0.25% NH_4HCO_3 at pH 8.2. Molecular weight protein markers (BSA=bovine serum albumin, 67K; OVAL=ovalbumin, 45K; TRYP=trypsinogen, 25K; and CYT=cytochrome C, 12K) and the purified CSF were separately applied to the column. Fractions were collected in 1 ml/tube at a flow rate of 15 ml/hr.

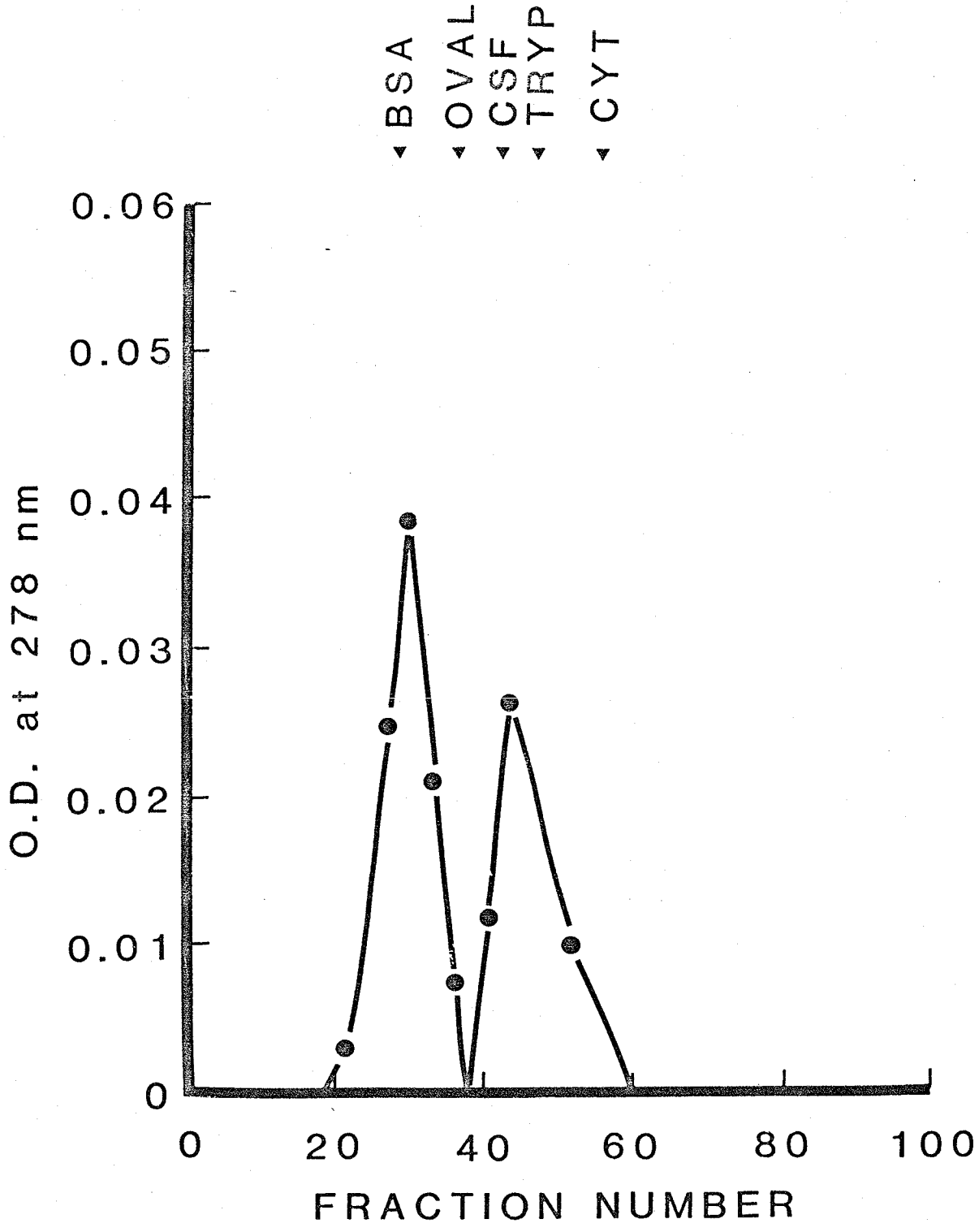
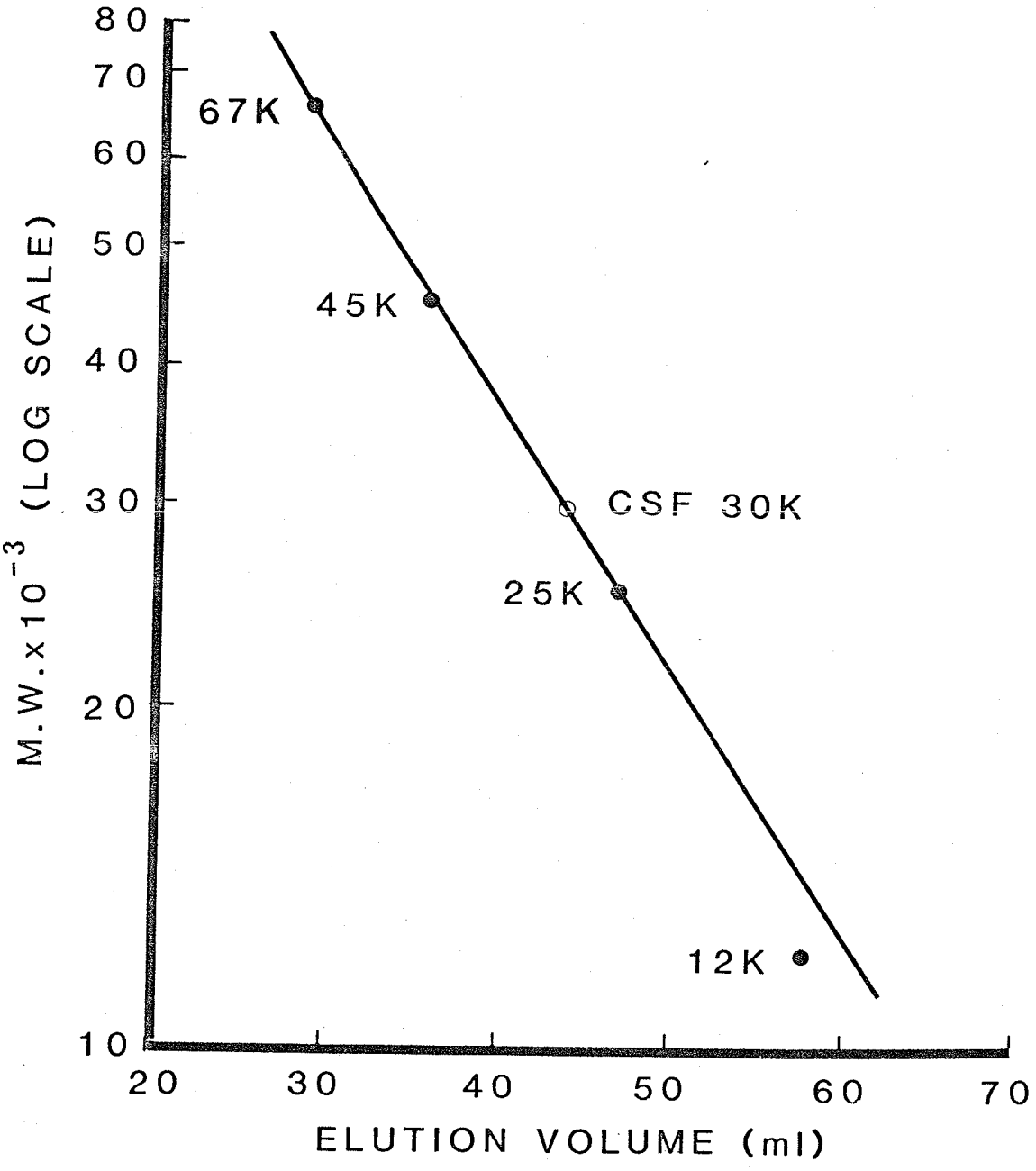

BSA
OVAL
CSF
TRYP
CYT
0.06
0.05
0.04
0.03
0.02
0.01
0
O.D. at 278 nm
0
20
40
60
80
100
FRACTION NUMBER

Figure 22b

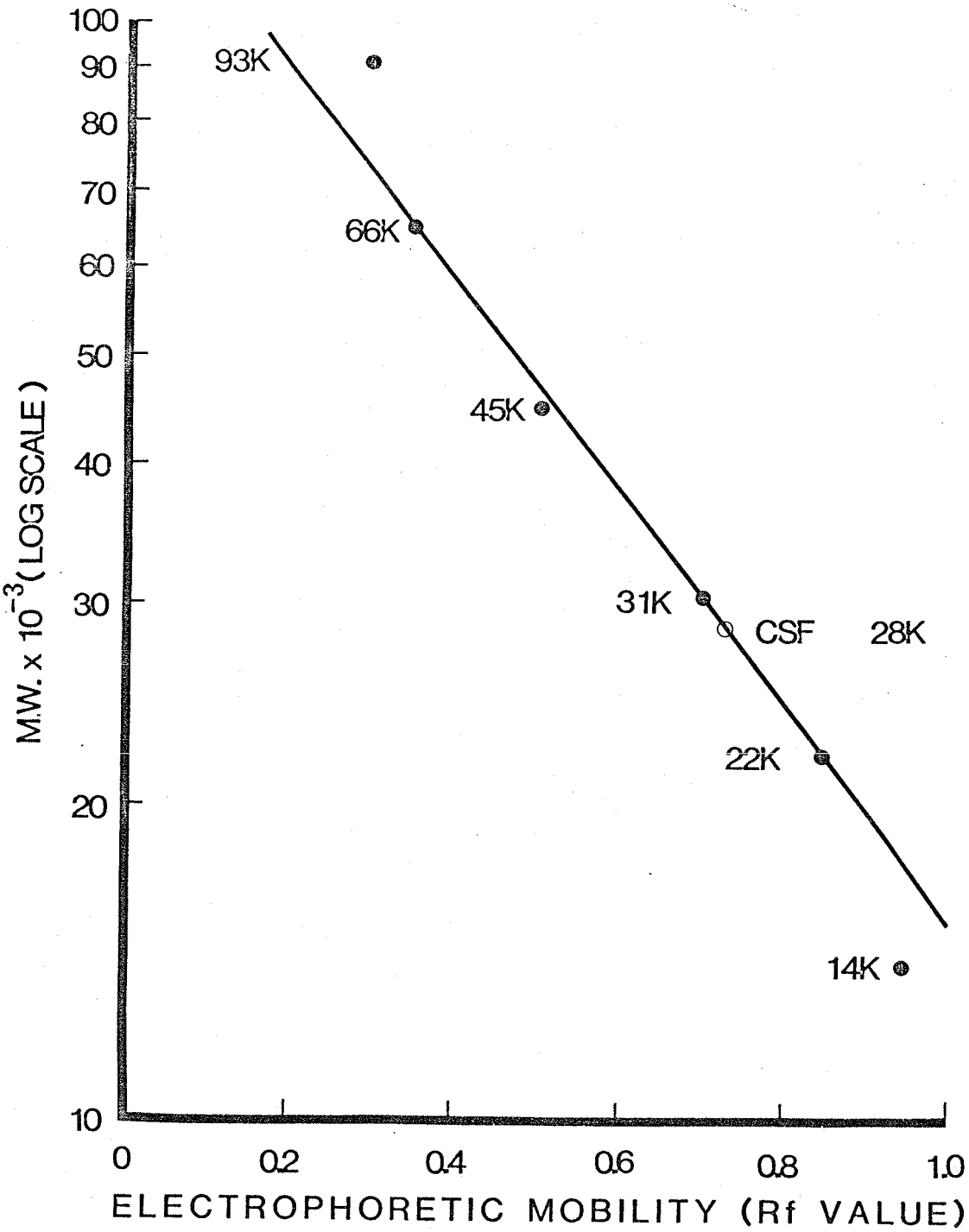
Determination of the molecular weight of purified CSF by gel filtration on Sephadex G-100. (Estimation of molecular weight). The elution volumes of the molecular weight protein markers of known molecular weights were plotted against their molecular weight (log scale). The molecular weight of muscle CSF was estimated from its elution under identical experimental conditions. 67K=bovine serum albumin, 45K=ovalbumin, 25K=trypsinogen, 12K=cytochrome C.


80
70
60
50
40
30
20
10
M.W.x10^{-3} (LOG SCALE)
67K
45K
CSF 30K
25K
12K
20
30
40
50
60
70
ELUTION VOLUME (ml)

monomeric protein with no subunit structures since there was only one major band of protein upon SDS-PAGE (Fig. 23a). The molecular weight of CSF was estimated to be 28K by the method of SDS-PAGE (Fig. 23b). The molecular weights obtained by both methods agree very well, being 28K and 30K by SDS-PAGE and gel filtration on Sephadex G-100, respectively.

Figure 23a

Determination of the molecular weight of purified muscle CSF by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. (Electrophoretic pattern of proteins). Protein markers of known molecular weights (phosphorylase B, 93K; bovine serum albumin, 66K; ovalalbumin, 45K; carbonic anhydrase, 31K; soybean trypsin inhibitor, 22K; and lysozyme, 14K) and the purified CSF (20 ug) were analysed by 10% polyacrylamide gel in the presence of 1% SDS.


100
90
80
70
60
50
40
30
20
10
M.W. x 10^{-3} (LOG SCALE)
93K
66K
45K
31K
CSF
28K
22K
14K
0
0.2
0.4
0.6
0.8
1.0
ELECTROPHORETIC MOBILITY (Rf VALUE)

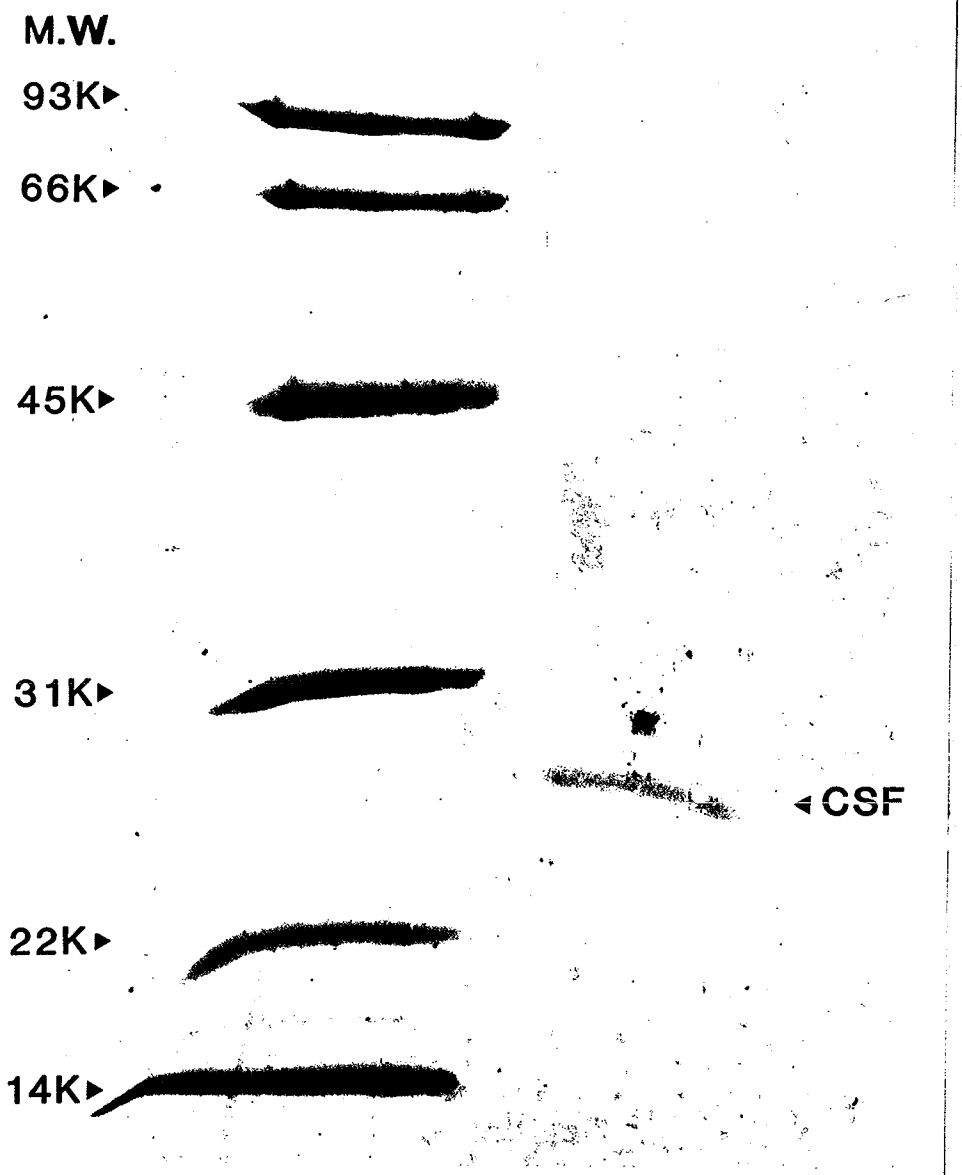

M.W.
93K
66K
45K
31K
CSF
22K
14K

Figure 23b

Determination of the molecular weight of purified muscle CSF by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. (Estimation of molecular weight). The electrophoretic mobilities (Rf values) of the protein markers were plotted against their molecular weight in log scale. The molecular weight of CSF was estimated from its Rf values.

93K=phosphorylase B, 66K=bovine serum albumin, 45K= ovalbumin, 31K=carbonic anhydrase, CSF 28K=CAT-Stimulating Factor, 22K=soybean trypsin inhibitor, and 14K=lysozyme.

DISCUSSION

(A) CHARACTERIZATIION OF THE NG108-15 BIOASSAY

The bioassay for CAT-stimulating activity, using the hybrid cell line NG108-15, originally developed by Godfrey et al (63) has been adapted with slight modifications into our routine assay system for the activity in various fractions at different steps of our purification procedure. A simple and reproducible assay system to follow the distribution of the activity is absolutely essential for the successful purification of the protein factor. Treatment of the NG108-15 cells with dBcAMP (medium E), whether alone or in combination with muscle extracts, resulted in dramatic morphological changes (Fig. 3), and the stimulating effect on CAT activity in the NG108-15 cells in the presence of muscle extracts (Fig. 1), was not due to changes in cell number of the NG108-15 cells in culture (Fig. 2). These results are in complete agreement with the findings of Godfrey et al (63) on the CAT-stimulating activity in muscle CM, as well as observations on the cholinergic factors identified in other culture systems (27, 64, 65). When comparing the NG108-15 bioassay with the original assay of primary culture of mouse spinal cord neurons by Giller et al (59, 61) for the CAT-stimulating activity the NG108-15 cell bioassayis clearly a much more convient assay system (Table 3). However, the NG108-15 bioassay has one liability, the increases in CAT activity seen were generally smaller than those seen using the primary culture of spinal cord neurons.

TABLE 3 COMPARISON OF THE RESPONSES OF THE HYBRID NG108-15 CELL LINE AND THE PRIMARY CULTURE OF SPINAL CORD NEURONS TO MUSCLE CSF AS A BIOASSAY SYSTEM

Property	NG108-15 Cells	Spinal Cord Neurons
A. Biological responses to CSF		
(1) Cat activity increase	50%	200%
(2) Cell number increase	no difference	no difference
(3) Treatment period for maximum effect of CSF	4-5 days	>12 days
B. Technical features of assay systems		
(1) Steps to prepare for cat assay	2 steps	5 steps
(2) Medium changes with CSF	1	6
(3) Days of culture required	5-6 days	13 days
(4) Days of CSF treatment required	4-5 days	12 days
(5) Culture inoculated in 1 hr. work	100 (wells)	10 (dishes)

(B) PRELIMINARY CHARACTERIZATION OF THE PROPERTIES OF THE CAT-STIMULATING ACTIVITY IN SKELETAL MUSCLE EXTRACTS

We observed that rat skeletal muscle extracts increased the CAT activity in cultured NG108-15 cells in the presence of dBcAMP, and this CAT-stimulating activity was precipitated at 50% saturation of ammonium sulfate (Fig. 6). This finding is different from previous reports (64, 65) on cholinergic factor(s), which was 60-100% saturation , but in good agreement with results from Godfrey et al (63), the CAT-stimulating activity in CM over mouse muscle cells, being relatively unstable upon storage at 4 °C (Fig. 7) and relatively heat resistant (Fig. 8). The instabilities of the CAT-stimulating activity was resolved by addition of proteolytic inhibitor (PMSF) to the extracts (Fig. 7), and fractionation by 50% saturation of ammonium sulfate (Fig. 6). The CAT-stimulating activity was also found to be relatively stable in storage at a pH range of 6-9, and unstable at a pH of 4-5 (Fig. 9). The latter finding of the pI of CSF being 4.75 might help to explain this result: CSF is isoelectrically precipitated in pH 4-5. Furthermore, the CAT-stimulating activity was sensitive to trypsin and Urea treatments, but not affected by B-mercaptoethanol, neuraminadase, phospholipase D, and NaCl treatments (Fig. 10). This results suggest that CSF is protein in nature, and the activity does not require disulphide bonds, carbohydrate and lipid residues. This conclusion, however, should be regarded as tentative since the activity and purity of the enzymes and Urea used were not known. In comparison, the partially purified rat heart cholinergic factor was trypsin sensitive (64, 65) but resistant

to Urea treatment (65).

In general, the CAT-stimulating activity in the rat skeletal muscle extract is similar to the factor in mouse muscle cell CM identified by Godfrey et al (63), but different from cholinergic factors reported by others (64, 65). However, this is only a tentative conclusion, because the properties of the crude impure materials being compared might not reflect the exact nature of the purified component(s).

(C) ISOLATION OF THE CAT-STIMULATING FACTOR

The purification of CSF to homogeneity from rat skeletal muscle extracts was made possible by four steps of conventional isolation procedure (Table 1), including: Ammonium sulfate precipitation, CM-Cellex chromatography, DE-Cellex chromatographies, and gel filtration on Sephadex G-100. Several steps of isolation were found to be necessary since rat skeletal muscle extracts contain only small amounts of CSF, but relatively large amount of contaminating proteins.

Similar isolation techniques have been used to isolate from CM over rat heart cells the cholinergic factor, which stimulated the dissociated rat superior cervical ganglion neurons. Using ammonium sulfate precipitation, followed by ion-exchange chromatographies upon CM- and DEAE-Cellulose, and gel filtration on Sephadex G-100, the component has been purified to 1,500- and 10,000-fold by Weber (64), and Fukada (65), respectively. This rat heart cholinergic factor was precipitated at 60-100% saturation of ammonium sulfate, eluted in the low salt fractions upon CM- and DEAE-Cellulose chromatographies, and was estimated by gel filtration to have a molecular weights between 40,000-

45,000 daltons (64) and 45,000 daltons (65).

A component that stimulates cholinergic development of ciliary ganglion neurons in cell culture has also been partially purified from extracts of embryonic chick eye (27). This component was found to have a molecular weight of 40,000-45,000 daltons and eluted from a DEAE-Cellulose column at an ionic strength similar to that found for the cholinergic factor from rat heart cell CM (27).

In contrast to the rat and chick cholinergic factors, CSF was precipitated at 0-50% saturation of ammonium sulfate (Fig. 6), eluted in the relatively high salt fractions of 0.5 M NaCl upon CM-Cellex chromatography (Fig. 12), and was estimated by gel filtration on Sephadex G-100 to have a molecular weight of 30,000 daltons (Fig. 22a).

Taking all together our findings and the results from other investigators, it becomes evident that CSF is structurally different from, but biologically similar to other cholinergic factor(s) reported. However, this is a tentative conclusion, because, the experimentally conditions and cell culture systems used in various laboratories are often quite different.

The success in purifying the CSF to apparent homogeneity lay in the recycling steps of the ion-exchange chromatographies and gel filtration on Sephadex G-100. Recycling upon DE-Cellex chromatography using continuous compound gradient has separated two groups of similarly charged proteins (Fig. 14), apparently inseparable by a simple linear gradient elution upon DE-Cellex chromatography (Fig. 13). Furthermore, a bulk of contaminating

proteins of slightly larger molecular weight was fractionated from CSF by recycling on the Sephadex G-100 columns (Fig. 16,17). An alternative to the recycling steps might be the employment of the high performance liquid chromatography (HPLC) in a preparative scale. Preparative ion-exchange and gel filtration columns for HPLC are available and supposedly could better resolved proteins of similar charges and molecular weights. Up to the present time, other investigators (27, 64, 65) have relied mostly on one or two steps of purification procedure without any refined adjustments in experimental conditions. This is probably the major reason for failure of other investigators to obtain a highly purified preparation of the cholinergic factor.

Finally, it is worth noting that the yield of the homogeneous CSF is 0.4 mg from 890 g of rat skeletal muscle and a 13% recovery of the CAT-stimulating activity (Table 2). An estimated 13,333-fold of purification has been achieved on the basis of the specific activity of the purified CSF compared to the crude muscle extract (Table 2).

(D) CHARACTERIZATION OF THE CAT-STIMULATING FACTOR

Upon electrophoresis in polyacrylamide gel, purified muscle CSF has been shown to be homogeneous (Fig. 19), with the CAT-stimulating activity coincident with the protein band (Fig. 20). The molecular weight of highly purified CSF has been estimated to be 30,000 daltons by gel filtration on Sephadex G-100 (Fig. 22b), and 28,000 daltons by polyacrylamide gel electrophoresis in the presence of SDS (Fig. 23b). Thus, the molecular weight of muscle CSF appears to be slightly smaller than the other cholinergic factors reported. The molecular weight of the rat heart

cholinergic factor has been reported to be 40,000-45,000 daltons (64) and 45,000 daltons (65) by gel filtration, and 50,000 daltons by SDS-polyacrylamide gel electrophoresis (65). The chick eye cholinergic factor has been shown to migrate as a component of 40,000-45,000 daltons upon gel filtration (27). Furthermore, both muscle CSF (Fig. 23a) and the rat heart cholinergic factor (65) have no subunit structures upon SDS-polyacrylamide gel electrophoresis, indicating some possible similarities between CSF and the other cholinergic factor.

By analytical thin layer polyacrylamide gel isoelectric focusing, CSF has again been shown to be homogeneous (Fig. 21a) and has a pI of 4.75 (Fig. 21b). Since the pI of the other cholinergic factors are not available, it is not possible to compare them.

Given its molecular weight (28,000-30,000 daltons) and isoelectric point (4.75), rat muscle CSF appears to be different from other purified neuronotrophic factors (Table 4). NGF is a 140,000 daltons protein complex (7S NGF) (79, 80), consisting of subunits designated α, B, and γ. The NGF activity is associated with the B-subunit composed of 2 non-convalently linked polypeptide chains of 13,000 daltons (81), with an isoelectric point of 9.3 (82). The neuronal survival factor from pig brain has a molecular weight of 12,300 daltons and an isoelectric point of 10.1 (18).

Our rat skeletal muscle CSF appears to be structurally different but biologically similar to other partially purified neuronotrophic factors on the basis of their molecular weights

TABLE 4 COMPARISON OF PHYSICAL PROPERTIES OF CSF AND OTHER PURIFIED NEURONOTROPHIC FACTORS

NEURONOTROPHIC FACTOR	TARGET	SOURCE	M.W.	pI	REFERENCE
Nerve Growth Factor	sensory and sympathetic neurons	mouse submaxillary gland	140K		79, 80
			3 subunits α, β & γ		81
			β subunit (active subunit) 13K	9.3	82
Neuronal Survival Factor	dorsal root ganglion neurons	pig brain	12K	10.1	18
CAT-Stimulating Factor	spinal cord neurons	rat skeletal muscle extracts	28-30K	4.75	(present studies)

and isoelectric points as summarized in table 5 and 6. However, since these neuronotrophic factors have not been prepared in a highly purified state, the possibility of these factors being identical or similar to muscle CSF cannot be conclusively evaluated.

Therefore, the development of a specific radioimmunoassay for the purified CSF is absolutely essential for further immunological characterization and subsequent in vivo and in vitro biological studies in order to delineate more exclusively the biological identity of this protein factor. Recently, a rabbit serum raised against a crude fraction of the chick CAT stimulating activity (CSA) has been shown to block specifically the stimulatory effect of CSA, the increase of CAT activity, without influencing neuronal survival, neuronal growth, or the basal levels of CAT activity in cultured ciliary ganglion neurons (66). Likewise, the availability of a specific antibody against CSF would help to identify and assess the biological specificity of the apparently similar neuronotrophic factor(s) from the muscle for spinal cord neurons and superior cervical ganglion neurons: whether the active components are neutralized or not by incubating with the specific CSF antibody. Furthermore, with the development of a specific and sensitive radioimmunoassay, the structural and immunological identities of the muscle CSF within and between species can easily be established. The availability of a specific antibody and a sensitive radioimmunoassay will open up a new area of physiological research on the interrelationship between neurons and their target organs.

TABLE 5 COMPARISON OF PHYSICAL PROPERTIES OF CSF AND OTHER PARTIALLY PURIFIED NEURONOTROPHIC FACTORS

NEURONOTROPHIC FACTOR	TARGET	SOURCE	M.W.	pI	REFERENCE
Neuronal Survival Factor	chick ciliary ganglion neurons	bovine heart extracts	20K and >40K	6.2	23, 25
		embryonic chick eye extracts	20K and >35K	4.5-5.5	20, 24, 26, 27
Neurite Extension Factor	ciliary, sympathetic and dorsal root ganglion explants	embryonic chick heart extracts	40K	5	39
	spinal cord explants	rat fibroblast CM	50K and 300K	--	45
	spinal cord neurons	chick muscle CM	40K, 500K and $>10^6$	--	42
	cerebral neurons	bovine brain extracts	37K	--	46
CAT-Stimulating Factor	spinal cord neurons	rat skeletal muscle extracts	28-30K	4.75	(present studies)

TABLE 6 COMPARISON OF PHYSICAL PROPERTIES OF CSF AND OTHER PARTIALLY PURIFIED NEURONOTROPHIC FACTORS

NEURONOTROPHIC FACTOR	TARGET	SOURCE	M.W.	pI	REFERENCE
Polyornithine-Binding Neurite-Promoting Factor	parasympathetic, sympathetic and sensory neurons	bovine corneal endothelial cells CM	4×10^3K	--	55
	retinal and spinal cord neurons	rat Schwannoma cell CM	5×10^3K	--	58
Neuronal Developmental Factor	sympathetic neurons	rat heart cell CM	45-50K	--	64, 65
	ciliary ganglion neurons	chick embryonic eye extracts	40-45K	--	27
CAT-Stimulating Factor	spinal cord neurons	rat skeletal muscle extracts	28-30K	4.75	(present studies)

(E) POSSIBLE FUNCTIONAL ROLES OF THE CAT-STIMULATING-FACTOR

In normal conditions, muscle CSF may play important roles in the development of cholinergic neurons which innervate peripheral cells. The role of CSF could be analogous to that of NGF in sensory and sympathetic neuronal development. NGF has been shown to include effects on the survival, maintenance, and differentiation of these neurons (for reviews see 10, 11, 12). Similarly, CSF appears to be involved in the developmental increase of neurotransmitter-synthesizing enzymes. A related function of CSF might be the prevention of neuronal death for neurons to form peripheral synapses. NGF has been shown to be retrogradely transported from its target tissues to cell bodies in sensory and sympathetic ganglia (83, 84, 85). Likewise, CSF could also be transported retrogradely from its muscular or other tissue sources, to the innervating neurons and thereby control the development of these neurons.

CSF may also play an important role in adult neurons. Since adult neurons are postmitotic cells which have exhausted their encoded development very early, their subsequent function and survival become largely dependent on the nature and extent of extrinsic influences. It can be envisaged that under normal circumstances, CSF is adequately available at all times to the neurons and can be supplied at greater levels under conditions of increased need. On the other hand, under diseased or aging circumstances, there might be an inadequate supply of CSF from the peripheral sources to the neurons or a reduced utilization of CSF by these neurons. In fact, the role of CSF may be most relevant in neurological diseases of the motor neurons, such as

amyotrophic lateral sclerosis (ALS), and Alzheimer's disease, and aging of the normal brain. Recently, it has been speculated that ALS and Alzheimer's disease could be caused by an insufficient trophic supply from the corresponding innervating territories to motor neurons (ALS) (86), and cholinergic CNS neurons (Alzheimer's disease) (86). Furthermore, the neurons affected the most in brain aging have been reported to be the cholinergic neurons of septal nuclei and the nucleus basalis of Meynert (87). However, one must continue to stress the conjectural nature of such speculations; but it is reasonable to expect that evidence to support them may be gathered in the not too distant future given the fast-growing research on neuronotrophic factors.

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