Comparative Studies on Nerve Growth Factor
Isolated From the Mouse and Snake

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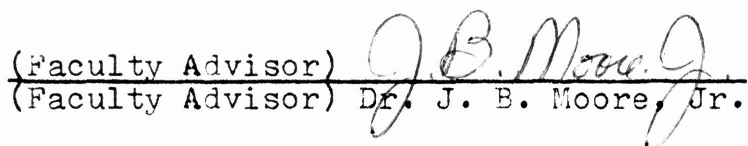

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ABSTRACT: Nerve Growth Factor (NGF) was isolated and purified from the male mouse submaxillary gland and from lyophilized snake venom. Biological activity, isoelectric point and molecular weight were compared. Mouse NGF showed a maximum activity between 4 ng/ml and 35 ng/ml whereas snake NGF showed maximum activity in the 60 -550 ng/ml range. The beta-subunit of mouse NGF has a pI of 9.2 and the snake NGF showed a pI of 8.3 - 8.5. When snake NGF was subjected to SDS gel electrophoresis, the minimum molecular weight was determined to be approximately 10,000 daltons.

Nerve Growth Factor (NGF) is a protein,first isolated from snake venom(Cohen, et al., 1956) and from the adult male mouse submaxillary gland (Cohen, 1960), which enhances the growth and differentiation of sympathetic and sensory ganglia. (Levi-Montalcini, 1966). The NGF activity isolated from the mouse is associated with a large protein complex, 7SNGF, which is composed of three subunits (Varon, et al., 1967). Only one of these subunits, the beta-subunit, has the NGF biological activity (Varon, et al., 1968). One of the other subunits, the gamma-subunit, displays arginine esteropeptidase activity(Greene, et al., 1969). The function of the third subunit, the alpha-subunit, is unknown at this time.

Another aspect of NGF is its similarity with the insulin molecule. The greatest similarities appear in structure, function and mechanism of action. (Bradshaw, et al., 1972).

Although NGF was first isolated from snake venom, little work has been done on the characterization of NGF from this source. This is partially due to the difficulties involved in its isolation from snake venom. The studies that have been with NGF isolated from snake venoms, (Banks, et al., 1968; Angeletti, 1970; Pearce, et al., 1972; Glass, et al., 1975) have suggested a possible evolutionary link with NGF from higher animals, and perhaps even with insulin(Bradshaw, et al. 1972).

This report describes a comparative study of NGF isolated from another snake, the western cottenmouth, in an effort to add knowledge in support of the theory mentioned above.

METHODS and MATERIALS

ISOLATION AND PURIFICATION. The procedure used for the isolation of NGF from the mouse was that described by Varon, et al.,(1967). Submaxillary glands were dissected from 45 adult male Swiss Webster mice. Columns with Sephadex G-100 and G-150 (Pharmacia) were equilibrated with 0.05 M Tris_Cl buffer (pH 7.4). The G-100 column (4x100 cm) was operated at a flow rate of 100 ml/hr and the G-150 column (3x95 cm) at 15 ml/hr. The DEAE-Cellulose (Whatman) column (5x17 cm) was also equilibrated with the same Tris-Cl buffer. Effluents from these columns were collected fractionally and the absorbance at 280 mu was determined by a Beckman DB Spectrophotometer. The pooled fractions

were concentrated by reverse pressure dialysis.

The isolation procedure used for the snake venom was that described by Cohen (1959). 1 gram of lyophilized snake venom from Agkistrodon piscivorus leucostoma (The Miami Serpentarium Laboratories) was dissolved with urea and subjected to ammonium sulfate precipitation before being loaded on a combination of DEAE- and CM-Cellulose columns (Whatman). Again, the effluents were collected fractionally and absorption at 280 mu was determined to locate the protein peaks. The flow sheets for the isolation procedures are shownn in figures 1 and 2.

TISSUE CULTURE ASSAY FOR NERVE GROWTH FACTOR ACTIVITY. Dorsal root ganglia from 8-day old chick embryos were explanted onto collagen treated culture plates and incubated with 5% fetal calf serum in Hank's Balanced Salt Solution according to the method described by Levi-Montalcini, et.al. (1954). The cultures were examined after an 18 hr. incubation at 37 degrees and the responsive ganglia were graded according to the size and morphology of their neurite outgrowth.

ELECTROPHORESIS. Isoelectric Focusing electrophoresis was done according to the method of Greene, et. al. (1971). The gels were made by mixing pH 3-10 Ampholines (LKB Aminkemi), acrylamide and bis-acrylamide (Sigma) and TEMED with water. The upper buffer was made by diluting 4 ml conc. Sulfuric acid to 2 liters with water. The lower buffer was made by diluting 16 ml monoethanolamine to 2 liters with

Homogenate(5%w/v) of 45 submaxillary glands in water was centrifuged at 50,000 g for 30 min. to give app. 360 ml of supernatant which was then lyophilized. The powder was taken up in 20 ml of 0.05 M Tris-Cl buffer (pH 7.4)

Lyophilized supernatant was loaded on a G-100 Sephadex column (4x100 cm), equilibrated and eluted with the Tris-Cl buffer. App. 40 ml of effluent at 100 ml/hr was collected according to figure 3a to give the G-100 fraction.

G-100 fraction was loaded onto a DEAE-Cellulose column (5x17 cm) equilibrated with the Tris-Cl buffer. The flow rate was 360 ml/hr. The column was washed with 2 volumes of 0.01 M NaCl in the same buffer and eluted with 350 ml of 0.08 M NaCl in the same buffer to give the DEAE fraction. This was concentrated by pressure dialysis to about 4 ml.

Sucrose was added to the concentrated DEAE fraction to a final concentration o 5gm/100ml and this was loaded onto a G-150 Sephadex column (3x95 cm) equilibrated and eluted with the Tris-Cl buffer. App. 180 ml in two pools were collected at a flow rate of 15 ml/hr to give the G-150 fraction (figure 3b) which was concentrated by pressure dialysis to about 2ml/pool. Pool 1 contained the biological activity.

FIGURE 1. Flow sheet for the isolation of Nerve Growth Factor from the mouse.

1 gm of lyophilized venom + 18 gm urea was mixed with 37 ml of water containing 3.5 ml of 0.1 N NaOH for 90 min. at 0 degrees. Saturated ammonium sulfate was added to a final concentration of 46.5% saturation for 30 min. This was centrifuged at 10,000g for 5 min. to give supernatant.

Saturated ammonium sulfate was added to the supernatant to a final concentration of 685 saturation for 15 min. and centrifuged at 10,000 g for 5 min. to give a precipitant containing NGF. This was dissolved in 25 ml water and dialysed overnite against distilled water.

This solution was fractionated again with saturated ammonium sulfate and the active material was precipitated between 45.5% and 59% saturation. This was centrifuged and the precipitant was dissolved in ml water and dialyzed overnite against distilled water.

This solution was loaded on a DEAE column (2cm diameter, 3 gm resin) equilibrated with 0.2 M potassium phosphate buffer, pH 6.0 and washed with distilled water at a flow rate of 0.2 ml/hr. This was followed by a wash of 1.5 column volumes of water to give DEAE eluant.

DEAE eluant was applied at a flow rate of 0.2 ml/hr to a CM-Cellulose column (2cm diameter, 3 gm resin) washed with a mixture of NaCl and NaOH, 0.5 M, followed by distilled water. The inactive fraction was eluted off with 20 ml of water and the active fraction appeared in tubes 3-7 (5 ml fractions)(Figure 4a). This pool was dialyzed against distilled water.

The CMC fraction was applied to a second DEAE column as above except equilibrated with water after washing with the NaCl-NaOH mixture. The column was first washed with 20 ml 0.001 M NaCl and the eluant discarded. Then it was washed with 1.0 M NaCl and 5 ml fractions were collected according to figure 4b.

Figure 2. Flow sheet for the isolation of Nerve Growth Factor from snake venom.

water. The gels were run for 3 hrs. at 100 volts.

SDS gel electrophoresis was done according to the method described by Webster and Osborn (1969) for molecular weight determination. The proteins used as standards for both the molecular weight and isoelectric point determinations were from Sigma except for beta-Lactoglobulin which was a gift from Dr. Nick Pace.

RESULTS

ISOLATION OF NERVE GROWTH FACTOR FROM THE MOUSE SUBMAXILLARY GLAND. The procedure for the isolation is summarized in figure 1. The submaxillary glands from 45 male mice yielded approximately 15-20 mg of fairly pure 7sNGF. The protein patterns for the isolation are shown in figure 3.

ISOLATION OF NERVE GROWTH FACTOR FROM SNAKE VENOM. The procedure used to isolate NGF from snake venom is summarized in figure 2. 1 gram of lyophilized snake venom yielded approximately 2 mg of NGF. The protein patterns for this isolation are shown in figure 4. As evident from the protein pattern from the DEAE-Cellulose column, the 0.05 M NaCl wash failed to elute the NGF off the column in a large peak, so a 1.0 M NaCl wash was used. This 1.0 M NaCl fraction contained the NGF activity and about 2 mg of protein.

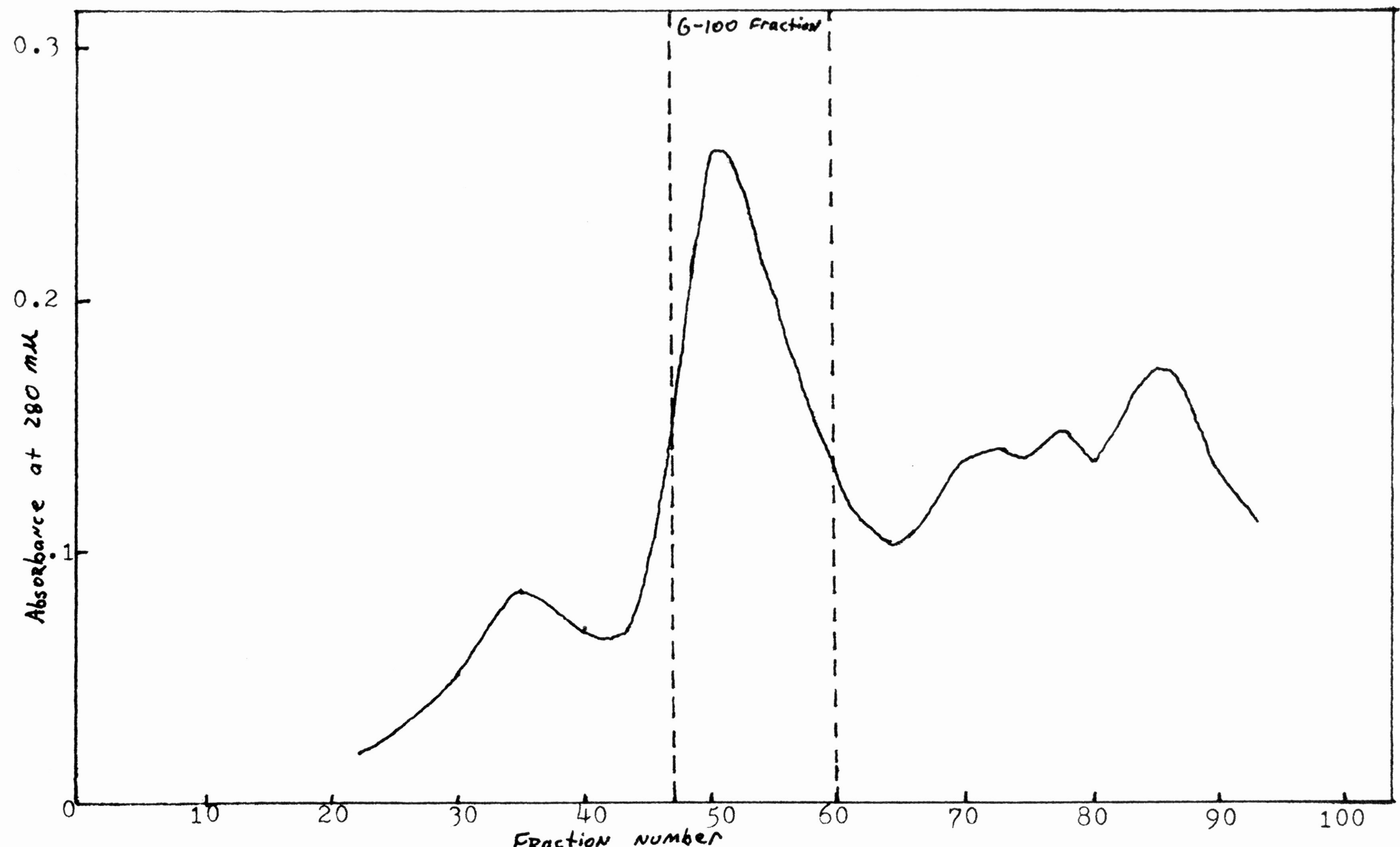

FIGURE 3a. Protein pattern for gel filtration of supernatant on Sephadex G-100. Fractions contained 3 ml.

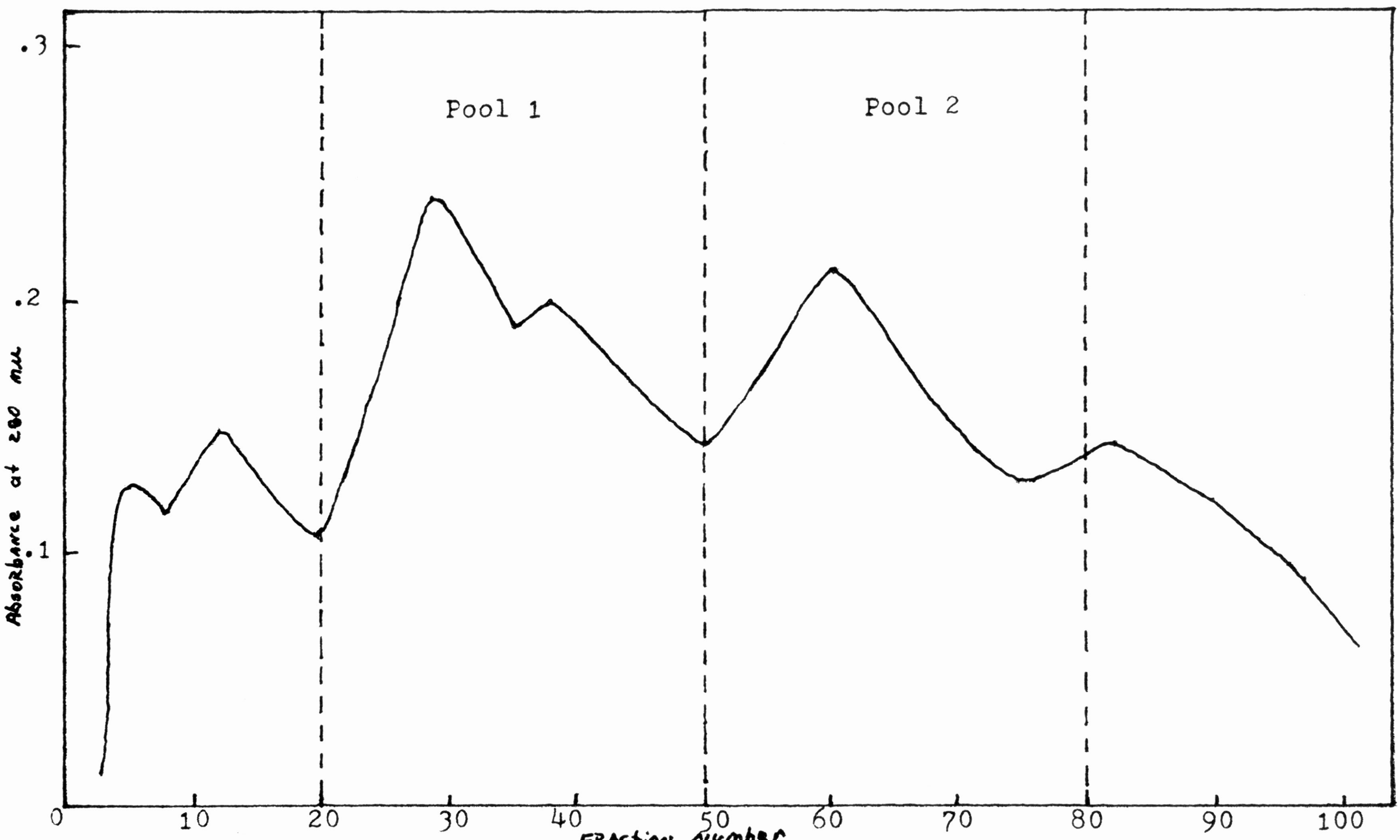

FIGURE 3b. Protein pattern for gel filtration of DEAE fraction on Sephadex G-150. Fractions contained 3 ml.

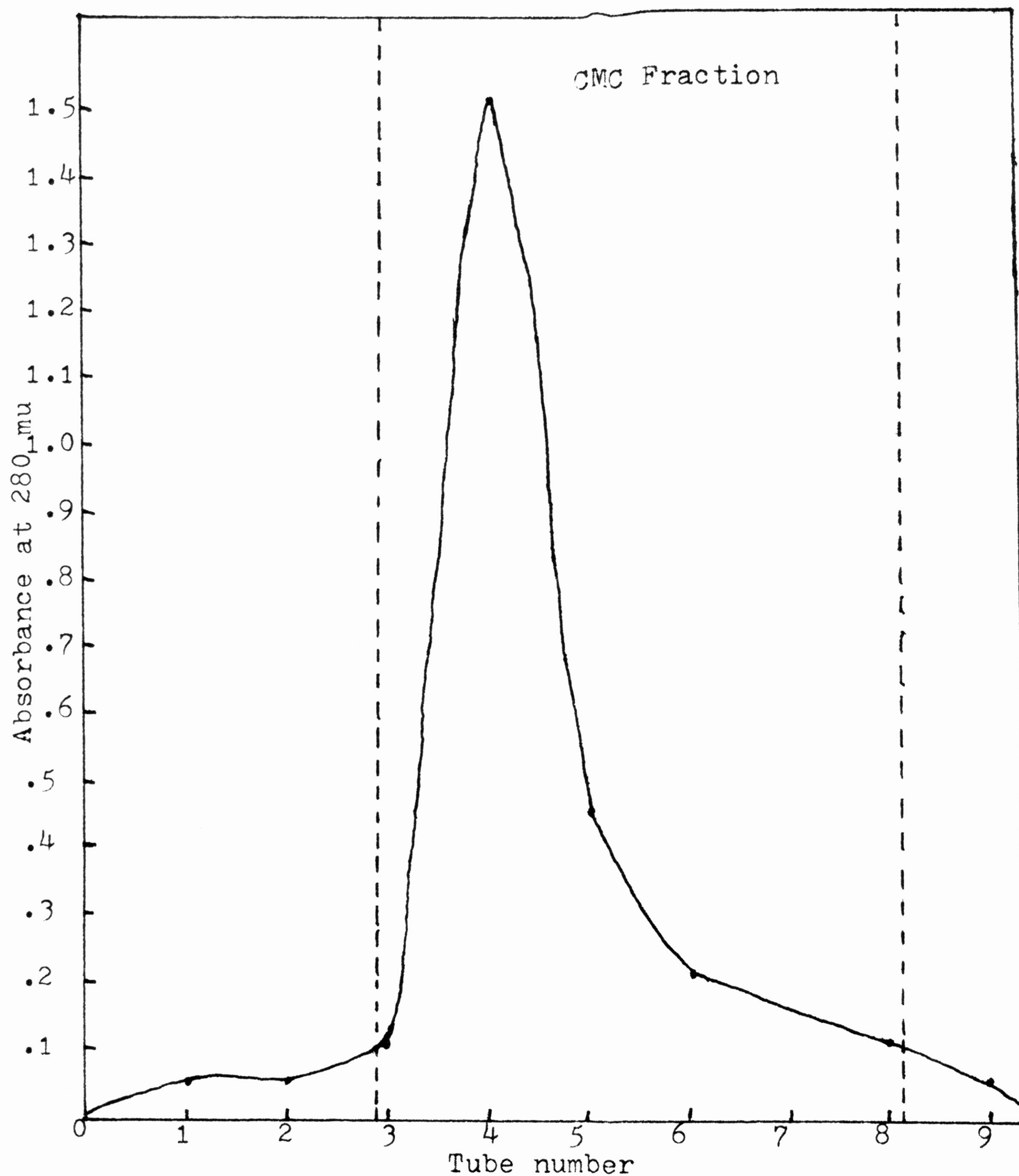

FIGURE 4a. Protein pattern for chromatography of DEAE eluant on CM-Cellulose column. (5 ml Fractions)

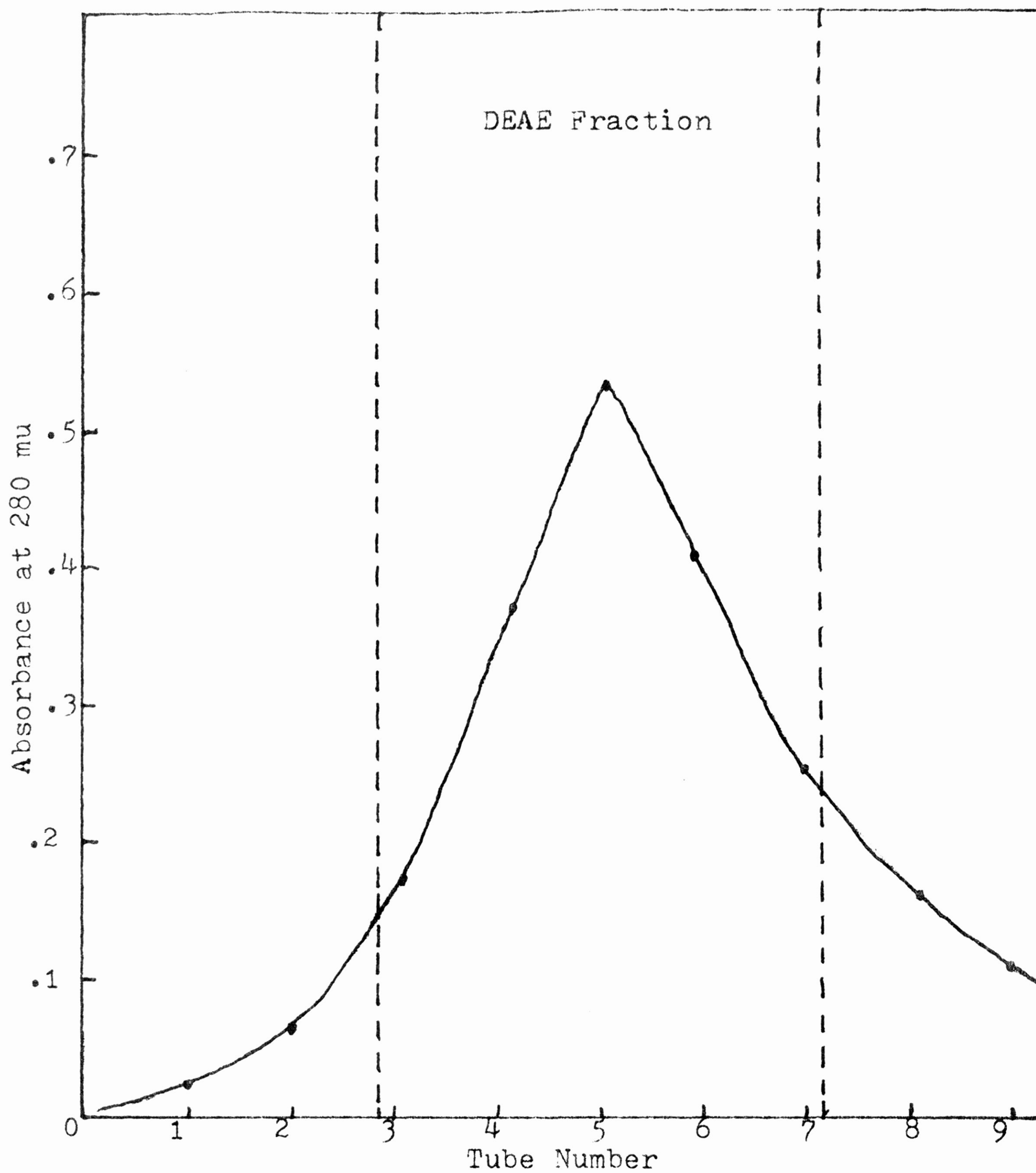

FIGURE 4b. Protein pattern for chromatography of CMC fraction on second DEAE column. (5 ml fractions)

ISOELECTRIC FOCUSING. Electrophoresis on isoelectric focusing gels showed the major bands corresponding to the alpha, beta and gamma subunits of the 7sNGF complex for the mouse protein. There were also present a few contaminating proteins, but the purity was adequate. The snake protein, when applied to isoelectric focusing gels, showed one major band and several smaller bands. The gel was cut up into 3 sections and the protein was extracted from the sections with 0.05 M phosphate buffer, pH 7.4. Each sample was assayed separatly. The sample exhibiting the NGF activity corresponded to the gel section containing the major protein band.

DETERMINATION OF ISOELECTRIC POINT. Isoelectric Focusing electrophoresis was used for the dtermination of the isoelectric point of the snake NGF. The protein standards used were Bovine Serum Albumin, Ovalbumen, Cytochrome C, and Beta-Lactoglobulin. Using this method, the pI was determined to be between pH 8.3 and 8.5. (Figure 5a)

COMPARISON OF BIOLOGICAL ACTIVITY. The assay using the dorsal root ganglia from 8-day old chick embryos indicated that the mouse NGF had a maximum activity between 4-35 ng/ml, whereas the snake NGF showed maximum activity between 60-550 ng/ml.

DETERMINATION OF MOLECULAR WEIHGT. SDS gel electrophoresis was used to determine the minimum molecular

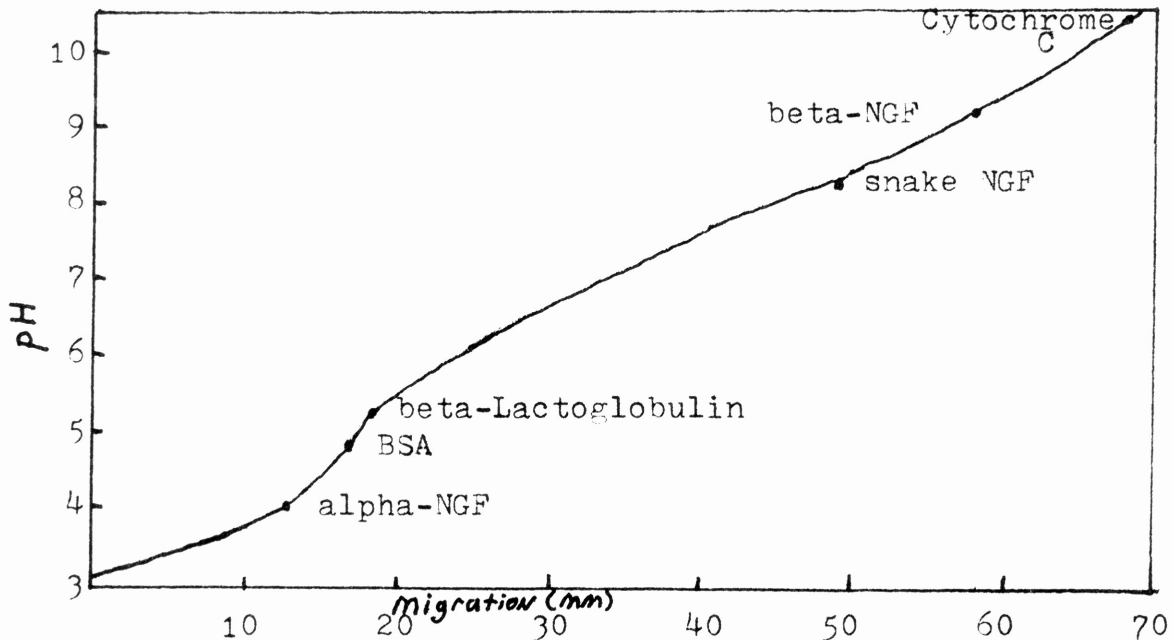

Fig. 5a. Determination of isoelectric point using isoelectric focusing electrophoresis.

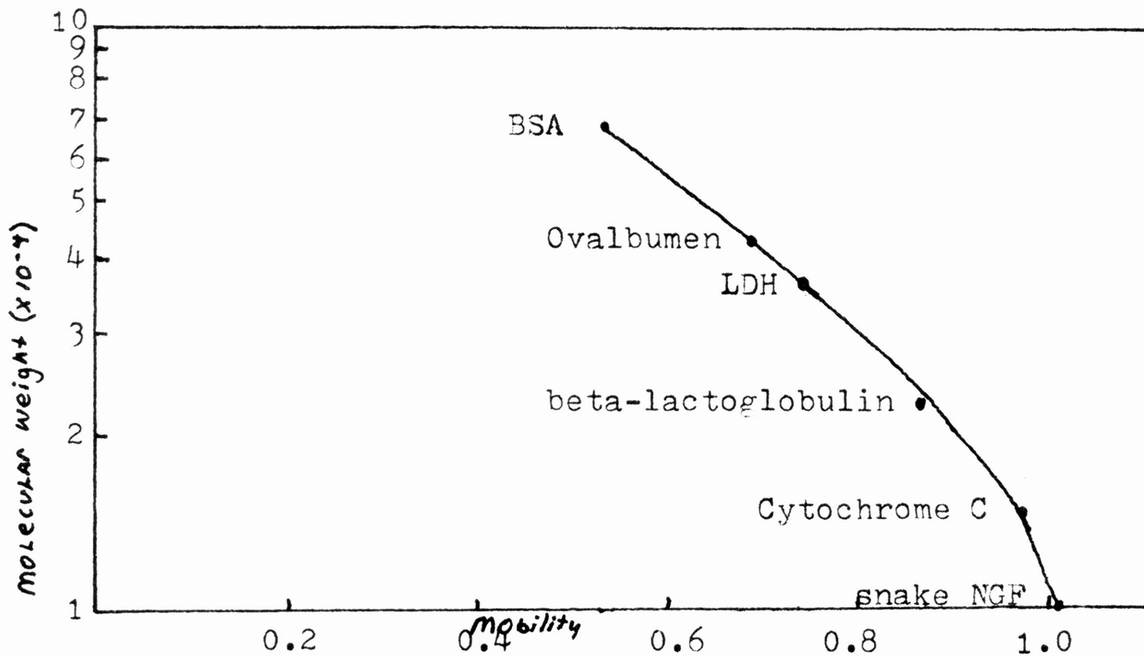

Fig. 5b. Determination of minimum molecular weight using SDS gel electrophoresis.

weight of the snake NGF. The protein standards used for this experiment were Bovine Serum Albumin, Ovalbumin, Lactate Dehydrogenase, Beta-Lactoglobulin, and Cytochrome C. The minimum molecular weight of the snake NGF was determined to be approximately 10,000 daltons. (Figure 5b).

DISCUSSION

The results obtained in this study show both similarities and differences between mouse 7sNGF and snake NGF. The greatest contrast was noted in the level of biological activity possessed by the two NGF proteins. Although both exhibited the same biological effect on the dorsal root ganglia, i.e. the stimulation of extensive neurite outgrowth, the snake NGF required a concentration 10-15 times greater to achieve the same effect as the mouse NGF. This is not surprising however, as other workers (Banks, et. al., 1968; Angeletti, 1970) have observed similar results from other snake NGF proteins.

With respect to molecular weight and isoelectric point, the two NGF proteins appear to be much more similar. The pI of 8.3-8.5 for the snake NGF roughly corresponds to the 9.2 value for the beta-subunit of 7sNGF from the mouse. In fact, nearly all NGF proteins isolated and characterized thus far have been basic proteins with pI's between pH 8.0-10.5. It may be that this is an important factor in the biological activity of the NGF proteins.

The molecular weights of the monomers of beta-NGF have been shown to be approximately 13,600 daltons by Young, et.al.,(1976). If the snake NGF exists as a complex of several monomers also, then 10,000 daltons is a reasonable value as a minimum molecular weight.

There is much more work to be done with the NGF from this species in order to more completely characterize it and perhaps to elucidate its mechanism of action on its target cells. Studies on Nerve Growth Factors from other vertebrates will hopefully discover the evolutionary relationships between them , and possibly link them to the insulin molecule. It is my hope that this work will contribute something to that effort.

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