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GRADUATE COLLEGE

AN IN VITRO OUTGROWTH ORIENTING FACTOR: STUDIES CONCERNING IT AND ITS RELATION TO THE REPORTED GROWTH STIMULATING PROTEINS FROM THE SUBMAXILLARY GLAND OF MICE

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

SISTER EILEEN MARIE BAST, S.S.N.D.

Norman, Oklahoma

1966

AN *IN VITRO* OUTGROWTH ORIENTING FACTOR: STUDIES CONCERNING IT AND ITS RELATION TO THE REPORTED GROWTH STIMULATION PROTEINS FROM THE SUBMAXILLARY GLAND OF MICE

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AN *IN VITRO* OUTGROWTH ORIENTING FACTOR: STUDIES CONCERNING IT AND ITS RELATION TO THE REPORTED GROWTH STIMULATING PROTEINS FROM THE SUBMAXILLARY GLAND OF MICE

CHAPTER I

INTRODUCTION

Some effects of certain proteins extracted from the submaxillary glands of mice upon cell growth *in vitro* have been described. These are nerve growth stimulation (Bueker, *et al.*, 1959; Levi-Montalcini and Cohen, 1960), increased division and subsequent keratinization of cells from the epidermal layer of dorsal skin and the eyelid (Cohen, 1965), enhancement of the growth of lung mesenchymal tissues and dedifferentiation of skeletal muscle and cartilage (Attardi, *et al.*, 1965), and stimulation and orientation of cellular outgrowth between chick cardiac explants (Bast and Mills, 1963). The nerve growth protein was purified and described by Cohen (1960). The epidermal outgrowth protein was also isolated and described by Cohen (1962). The macromolecular fraction which enhances growth of mesenchymal tissues and causes dedifferentiation of skeletal muscle has been only partially purified and described (Attardi, *et al.*, 1965). Outgrowth stimulation and orientation of cells from cardiac explants was produced by a crude, water soluble extract from the submaxillary glands, by the extract purified by ethanol

precipitation, and by the alcohol fraction after it was further purified by precipitation with ammonium sulfate. No description of the orienting factor was reported (Bast and Mills, 1963).

The studies of nerve growth stimulation in vitro and in vivo were conducted mainly with a highly purified fraction of the water soluble extract from the mouse submaxillary glands (Levi-Montalcini and Booker, 1960a; Levi-Montalcini and Booker, 1960b; Levi-Montalcini and Cohen, 1960; Cohen, 1960). The purification processes involved ethanol precipitation; ammonium sulfate precipitation; DEAE cellulose anion exchange; and three cation exchange columns, the products of which were called the CM-1 fraction, the CM-2 fraction, and the CM-3 fraction. When the least pure of the ion exchange purified proteins, CM-1 fraction, was administered to newborn mice, there was a loss of body weight and stunted growth, a failure of hair growth, a precocious opening of the eyelids, and a precocious eruption of the upper and lower incisors. These effects were not present in the mice treated with the more purified CM-3 fraction. In 1962 Cohen isolated and characterized a heat stable protein from the CM-2 fraction which elicited precocious opening of the eyelids and eruption of the teeth in newborn mice, but this protein did not produce stunting or inhibition of growth. These latter two effects were abolished by heating the submaxillary extract. Cohen (1965) used the heat-stable protein for growth enhancement of embryonic chick dorsal skin explants and sheets of epidermal cells removed from the dermis of the eyelid with trypsin.

These studies have indicated that there are at least two proteins in the mouse submaxillary gland that produce effects upon cellular growth in vitro. The outgrowth orienting factor could easily

be yet another protein which remains in the material retained from the first three purification procedures, i.e., the preparation of the crude homogenate, the ethanol precipitation, and the ammonium sulfate precipitation.

The nerve growth protein from the submaxillary glands of mice has been repeatedly reported to be specific for enhancement of the growth of sensory and sympathetic ganglia with no detected effects on other sectors of the nervous system or other organs (Levi-Montalcini and Booker, 1960a; Levi-Montalcini and Booker, 1960b; Levi-Montalcini and Cohen, 1960; Cohen, 1960). In vivo specificity of the nerve growth factor for sensory and sympathetic ganglia was demonstrated by the injection of the purified protein from the mouse submaxillary glands into newborn mice or chick embryos (Levi-Montalcini and Cohen, 1960). Mitotic counts, volume measurements of cells, photomicrographs of whole mounts of the sympathetic thoracic chain ganglia, and photomicrographs of histological sections of stellate ganglia and superior cervical ganglia were given for the non-injected and injected mice. However, these authors did not report any details in method or results, but merely stated that no effects were detected in other sectors of the nervous system or in other organs. No reports of in vitro studies concerning tissue explants other than sensory or sympathetic tissue could be found.

Cohen (1960) injected the purified nerve growth factor from mouse submaxillary glands into rabbits. He then isolated the gamma globulin fraction which contained antibodies from the serum. The antibody fraction was then injected into newborn mice and almost total destruction of neurons in the sympathetic chain resulted but no effects in

"other tissues" were observed. Information on which "other tissues" and on how these "other tissues" were analyzed was not found. No in vitro studies using tissues other than spinal and sympathetic ganglia have been reported.

The epidermal growth factor, also present in the CM-1 fraction, has been reported to be specific for enhancing the growth of epidermal tissue (Attardi, et al., 1965; Cohen, 1965). The belief held by Cohen and Elliott (1963) that the epidermal growth factor is specific seems to be based upon the observation that when newborn mice and rats and juvenile mice (12-20 days of age) were injected with the purified protein an enhanced keratinization of the foot epidermis, tail epidermis and dorsal skin epidermis resulted, with no obvious enhancement of the growth of the dermis. The paper did not state whether other tissues in the mice besides the skin from the foot, tail, or back were examined or how they were analyzed. Only dermal and epidermal tissues have been compared in vitro. The fact that the epidermal growth factor enhances in vitro growth of sheets of epidermal cells, even when they were removed from the dermis with trypsin, does not conflict with the idea that the growth-stimulating protein is specific. On the other hand, it does not offer evidence for the specificity of the protein's effect as Cohen (1965) has implied. Therefore, no conclusive evidence has been reported which rules out the possibility that either the nerve growth factor or the epidermal growth factor is the same as the in vitro cardiac outgrowth orienting factor.

The nerve growth factor has been shown to be present in a variety of biological materials, i.e., mouse sarcoma 180, snake venoms, mouse submaxillary glands (Levi-Montalcini and Cohen, 1960), and the combined

axial structures (spinal ganglia, spinal cord, notochord and somite derivatives) of 7-8 day chick embryos (Bueker, et al., 1960). Low levels of nerve growth-promoting activity has been reported from cell-free extracts of mouse kidney, thymus, spleen, placenta, heart and voluntary muscles by Bueker, et al., (1960), and in the serum of adult and weanling mice by Levi-Montalcini and Booker (1960a). The epidermal growth factor has been prepared only from the submaxillary glands of adult male mice. Extracts of adult female mouse submaxillary glands, bull and cow submaxillary glands, and sheep submaxillary glands do not produce the characteristic in vivo effects of the epidermal growth factor--the acceleration of incisor eruption and opening of the eyelid in new-born mice (Cohen, 1962). The macromolecular fraction which enhances the growth of mesenchymal tissues and causes dedifferentiation of skeletal muscle has only been obtained from the submaxillary gland of mice. Neither effect was obtained with extracts of mouse thymus, liver, kidney, and pancreas (Attardi, et al., 1965).

In 1959 Porath and Flodin separated protein molecules by passing a solution through a column packed with cross-linked dextran gel particles (commercially known as Sephadex). Although the elution volume for a molecule of specific molecular weight varies with the density and volume of the gel in the column, an excellent linear correlation between the common logarithm of the molecular weight and the ratio of the elution volume to the void volume has been reported by various workers. Whitaker (1963) used Sephadex G-75 gel for the molecular weight range of 13,000-40,200 and Sephadex G-100 gel for 13,000-76,000. Wieland, Duesberg and Determann (1963) used Sephadex G-200 gel for the molecular weight range

of 13,000-150,000. Andrews (1964) used Sephadex G-75 gel for the molecular weight range of 3,500-67,000 and Sephadex G-100 gel for 3,500-160,000. Leach and O'Shea (1965) used Sephadex G-200 gel for molecular weights up to 225,000.

The molecular weight of the nerve growth protein has been estimated as 44,000 from ultracentrifugation (Cohen, 1960). The molecular weight of the epidermal growth promoting protein has been reported to be 14,638 on the basis of amino acid composition (Cohen, 1962). The gel filtration molecular weight estimations for these two compounds have not been reported.

Deviation from the radial spreading of cells from an explant, termed oriented outgrowth in this paper, has been explained by Weiss (1934, 1941, 1952) by "contact guidance." According to Weiss (1934) patterned outgrowth is due only to the physical structure of the ground substance. In the case of a plasma clot this would be the orientation of the fibrinogen fibers in the clot. Cells growing out of an explant move along the fibers of the clot and therefore reflect the pattern of the fibers. Not only specificity of the in vitro cardiac outgrowth orienting factor, but also "contact guidance" as a possible explanation of the effect of the submaxillary extract upon the cellular outgrowth pattern between explants, could be further tested by using different types of tissues in the cultures. Sarcoma cells (Abercrombie, 1961), kidney epithelium (Weiss and Taylor, 1956), fibroblasts (Abercrombie, et al., 1957) and nerve tissue outgrowth (Weiss, 1934) have been observed to undergo contact guidance.

Further study, especially concerning the relationship between the nerve growth factor and the outgrowth orienting factor seemed necessary. The outgrowth pattern studied in a number of different kinds of tissue culture medium and conditions would give further assurance that the extract was responsible for the oriented growth. The nerve growth factor is reported as being highly specific for nerve cells. This response would be compared by subjecting several different types of tissue to the outgrowth orienting factor. Extracts of two other tissues, skeletal muscle and thymus, would also contribute toward the relationship as some growth promoting proteins from the submaxillary gland are reported to be limited in origin. Early crude extracts which contained the outgrowth orienting factor indicated that it is in the same class of protein as the nerve growth factor. Upon further purification, a description of characteristics such as molecular weight, absorption spectra, lability to pH and temperature changes, and solubility could be compared to the reported characteristics of other mouse salivary gland growth factors.

CHAPTER II

MATERIALS AND METHODS

The submaxillary salivary glands were obtained from adult male mice which originated from Jackson Laboratories and have been maintained by the Department of Zoology at the University of Oklahoma. The mice were anesthetized with chloroform and the excised submaxillary glands were placed in sterile 0.7% saline solution at 0°C for approximately 4 hours. All the tissues were handled as aseptically as was practically possible. Connective tissue and sublingual glands were removed and discarded before the submaxillary glands were frozen and stored at -11°C in the freezing compartment of a refrigerator. Various amounts of submaxillary tissue (wet weight) were collected and pooled on the following separate occasions: 6 g in June, 1963; 12.5 g in July, 1964; 2 g in October, 1965; and 6.7 g in January, 1966. Adult male mouse skeletal muscle (thigh), 1 g in July, 1964, and 4 g in October, 1965, and thymus from male mice 2-6 weeks old, 1 g in July, 1964, and 0.6 g in February, 1966, were collected. The various types of tissue were pooled and handled separately, in the same manner as the submaxillary glands.

At the beginning of the extraction process the tissue was thawed and placed in a chilled semimicro, Waring blendor Monel metal jar and 8.25 ml of cold, glass-distiled, sterile water were added for each gram

(wet weight) of tissue (Cohen, 1960). After homogenizing the tissue for 2 min in the Waring blendor, maceration was completed in a glass tissue homogenizer (Kontes, Size C) which was kept in an ice bath. The homogenates were centrifuged (Servall Refrigerated Automatic Centrifuge, Model RC-2; rotor head radius, 4.25") at 15,000 x g (11,250 rpm) for 10 min at a temperature of 0-3°C. All centrifugations were performed with this centrifuge and head and at the same temperature, time, and force unless otherwise indicated. After centrifugation the supernatant was decanted and saved. All residues were resuspended in 2.75 ml of glass-distilled, sterilized water for each gram of original tissue (wet weight) and discarded. Once, with the 6 g sample of submaxillary tissue (June, 1963), these first two centrifugations were at a force of 27,000 x g (15,000 rpm).

The nucleoproteins were precipitated from the supernatant by a modification of Cohen's method (1960). One ml of streptomycin sulfate solution, 0.2 M, was added to each 9 ml of the pooled supernatant solution and the pH immediately adjusted to 6.9-7.1 with NaOH. Once Cohen's procedure was followed exactly. With the 6 g sample of the submaxillary tissue (June, 1963) the pH of the streptomycin sulfate solution was adjusted to 7.8 with NaOH before its addition to the pooled supernatant solution. The mixture was placed in the refrigerator overnight and then centrifuged. The supernatant was stored at -11°C. The material prepared in this fashion will be called crude homogenate throughout this paper. It was used as the starting material for the alcohol purification, the ammonium sulfate purification, and for most of the separations on the gel chromatography columns. Before its application on the gel chromatography

columns, the crude homogenate was centrifuged for 5 min or filtered through a cellulose membrane filter (Millipore, pore size 0.45 μ; Type Code HA).

Some of the proteins were removed by precipitation with alcohol following Cohen's method of purification of the nerve growth stimulating factor (Cohen, 1960). In this purification step each ml of the crude homogenate which was used was combined with 0.07 ml of absolute ethanol. The mixture was placed in the cold centrifuge (0°C) and the precipitation reaction was allowed to proceed for 15 min before the mixture was centrifuged. The precipitate was discarded and 0.33 ml of absolute ethanol was added for each ml of the original solution. The second alcohol treatment for 45 min at 0°C was followed by centrifugation; the supernatant was discarded and the active fraction was taken up in 0.1 ml of water for each ml of crude homogenate. This material was filtered through a cellulose membrane filter before it was used for electrophoretic and gel column fractionation analysis.

Cohen's method (1960) of ammonium sulfate purification was used. The pH of a saturated ammonium sulfate solution was adjusted to 7.4 with ammonia. Two-thirds ml of the saturated ammonium sulfate solution was added to each ml of the crude homogenate used in this purification step. The mixture was placed in the cold centrifuge (0°C) and allowed to stand for 15 min before the mixture was centrifuged. The precipitate was discarded and the supernatant was combined with an equal volume of saturated $(NH_4)_2SO_4$ solution. The second precipitation reaction proceeded for 15 min at 0°C and the mixture was centrifuged. The supernatant was discarded. The precipitate was dissolved in 1 ml of glass-distilled

water. This material was analyzed by fractionation on a gel chromatography column.

Four types of cross-linked dextran gels (Porath and Flodin, 1959), commercially known as Sephadex G-25, G-75, G-100, and G-200, were used as packing material in the chromatography columns. The dry Sephadex powder was suspended in an excess of liquid ((0.03 M sodium phosphate buffer, pH 6.5; 0.005 M Tris (hydroxymethyl aminomethane) HCl buffer, pH 7.2; or glass-distilled water)) and allowed to swell. The swelling times were at least: G-25, 1 day; G-75, 4 hr; G-100, 1 day; and G-200, 4 days. Repeated sedimentation and decantation removed fine particles.

Two styles of chromatography columns were used. Both were glass. One column had an inside diameter of 1.8 cm and length of 51 cm. The lower end tapered from 1.8 to 0.5 cm in 1.25 cm. The exit of this column was a glass tube 0.5 x 2 cm. A 0.5 x 15 cm piece of polyethylene tubing was tightly fitted into the 0.5 cm bottom of the column with a ring of the appropriate size and thickness of tubing. A clamp on the polyethylene tubing was used to prevent buffer flow during periods when the column was not in use. In order to achieve better resolution the lengths of the columnar exit and of the polyethylene tubing were reduced in later experiments to approximately 0.75 cm and 4-6 cm respectively. This column will be referred to as Column I.

The other chromatography column had an inside diameter of 2.2 cm and was 80 cm high. A sintered glass filter (extra coarse pore size) was fixed in the lower end. A 1 mm bore ground glass stopcock was fitted into the bottom of the column by centering it in a hole in a #3 neoprene stopper pressed approximately 2 mm from the sintered glass filter.

The column was cooled by a water jacket, an outside glass tube whose diameter was 3.5 cm. This column will be referred to as Column II.

Column I was prepared for use in the following way. The columnar exit of the column was tightly packed with glass wool. The tapered portion of the column was filled with approximately 1 cm of glass beads.[1] The column was filled with 20-30 cm of buffer. Vertical alignment of the column was established with a plumb line. A funnel reservoir was attached to the top of the column and the homogeneous slurry of the swollen Sephadex gel particles of the desired porosity was added. After about 10 cm of the gel had settled on the beads the clamp was opened and the gel packed as buffer flowed through the column. Excess gel was removed from the funnel reservoir with a pipette. The height of the gel bed varied from 42.5 cm to 46 cm. When this column was packed with Sephadex G-200 gel a slurry of swollen Sephadex G-25 gel, which settled to form a layer of approximately 0.75 cm, covered the glass beads before the gel of fine particle size was packed.

Column II was prepared by covering the sintered glass filter with beads to form a 2-3 mm layer, adding a slurry of swollen Sephadex G-25 gel which settled to form a 3-4 mm layer, filling the column with 25-50 cm of buffer, and adding the homogeneous slurry of swollen Sephadex G-200 gel into the column and the attached funnel reservoir. After about 10 cm of the gel had settled the stopcock was opened and the remainder of the column was packed under buffer flow. Water from a refrigerated bath (Fisher, Isotherm) was circulated through the water jacket on this column.

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[1] #12 glass glow beads; the type used in art work, i.e., sign making.

The temperature of the water in the reservoir of this refrigerated bath was $10^{\circ} \pm 1^{\circ}C$.

The columns packed with the G-100 gel and G-200 gel were stabilized by washing with buffer solution for at least 5 hr. A filter paper disc, the diameter of the column, was placed on the top of the gel in the smaller column to prevent disturbing the gel bed during the application of the sample and buffer, but no paper disc was necessary on the gel in the larger column. Before application of the sample most of the buffer above the gel bed was removed with a pipette, then the stopcock or clamp at the bottom was opened and the last few ml of buffer were allowed to soak into the gel bed. When the fluid level reached the gel surface the sample was applied.

The volume of buffer outside the gel grains in the column bed, also known as the void volume (V_o), was determined by applying a 0.5 ml, 1 ml, or 2 ml sample of a 0.2% solution of a dextran polymer with an average molecular weight of 2×10^6, Blue Dextran, (Pharmacia Fine Chemicals, Inc.) to the chromatography columns. The volume of buffer required to elute this substance, which is excluded by the gel granules, is the void volume. The effluent was collected in vials and the volumes of the collected fractions from the time of the application of the sample were determined by weight to the nearest 0.01g(Torsion balance, Model DLT2). One ml was assumed to weight 1 g at room temperature. Blue Dextran absorbs light strongly at a wave length of 625 mμ (Technical brochure, "Blue Dextran 2000", Pharmacia Fine Chemicals, Inc.) and the light absorbance of the effluent was measured at this wave length on the Beckman DU Spectrophotometer. The amount of light absorbed was plotted against

the total effluent volume. The amount of light absorbed by the effluent increases as the dye emerges from the gel bed and decreases as the dye is washed from the gel. The increased absorbance when the dye was present in the effluent resulted in a peak on the graph and also indicated the volume of effluent that contained the dye. The elution volume of the sample of Blue Dextran was determined from a graph by extrapolating both sides of the solute peak to an apex and reading the elution volume of this peak to the nearest 0.1 ml from the x axis under this point. The total volume of the sample of Blue Dextran applied to the gel bed was used to estimate the dilution factor of the gel bed. The visible movement of the Blue Dextran through the column bed was a means of evaluating the uniformity of the packing of the gel and the column design.

The elution volume (V_e) of a sample which contained protein was determined in the same way as the void volume (V_o) except that the protein in the effluent was determined by measuring the amount of light absorbed at 280 mμ (Goldfarb, et al., 1951). Again, the amount of light absorbed by the effluent increases as the protein material emerges from the gel bed and decreases as the protein is washed from the gel. A linear relation exists between the ratio of elution volume to void volume and the common logarithm of the molecular weight of the material washed from the gel, V_e/V_o = log mol. wt. (Whitaker, 1963). The smaller chromatography column (Column I) was calibrated with a 0.5 ml sample which contained either 10 mg of reconstituted, lyophilized trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio), molecular weight 23,800 (Cunningham, 1954), or 12.5 mg of bovine serum albumin (Mann Research Laboratories, Inc., New York, New York), molecular weight 67,000 (Loeb and Scheraga, 1956).

Large sample volumes of 7-10 ml were applied to the columns of G-25 or G-75 gel. Smaller sample volumes of 0.5, 1 or 2 ml were used on columns of G-100 or G-200 gel. After the sample entered the gel, buffer was added. Attached to the top of the column was a reservoir of buffer so that there would be a hydrostatic pressure of approximately 20-30 cm of water on the gel bed in Column I or 10-12 cm of water on the gel bed in Column II. Samples of approximately 3 ml were collected. These samples were weighed whenever void volume determinations of the gel bed were made. The samples were stored in 5 ml glass vials at -11°C. Before the purified extract was used in culturing it was sterilized by filtration through a cellulose membrane.

Fractionation was monitored by measuring the absorption of light in the ultraviolet range (usually at a wave length of 280 mμ, but 290-310 mμ were also used where this is indicated in the results) with a Beckman Model DB Spectrophotometer with a constant flow-through, silica absorption cell and continuous recorder; or by reading the amount of light absorbed by the collected samples at a wave length of 280 mμ on a Beckman Model DU Spectrophotometer with photomultiplier attachment and the hydrogen lamp. Silica absorption cells (Beckman) which transmit light in the wave length of 220 to 2500 mμ were used for both the reference and the sample solutions. Readings were corrected for interference by ultraviolet absorbing material, spontaneously released from the dextran gels (Ackers, 1964), by setting transmission at 100% with the buffer from the washed down column. The 100% transmission level was set with unused buffer when G-25 gel, G-75 gel, and twice when G-100 gel (8/26/65 and 9/8/65) was used.

The absorption spectrum for some of the fractions from the gel separations was determined. Measurements were generally made for wave lengths separated by 1 mμ in the wave length range of 270 to 285 mμ, every 5 mμ from λ = 245 to 270 mμ and from 285 to 310 mμ, and every 10 mμ from λ = 310 to 330 mμ. Measurements always proceeded from the shorter to the longer wave lengths. The light absorption measurements are reported in terms of per cent transmission and optical density. The per cent transmission was read from the Beckman Spectrophotometer; the optical density was determined from a table (Hodgman, 1960).

The crude homogenates of the male and female mouse submaxillary glands used by Bast and Mills (1963) were compared by paper chromatography with the protein material in the first peak (as judged by light absorption at λ = 280 mμ) of the effluent packed with G-25 gel. The chromatographic solvent, butanol-water-acetic acid, was prepared by mixing 4 parts of n-butanol, 5 parts of metal-free water, and 1 part of glacial acetic acid. The lower phase from this mixture was put into a glass container in the bottom of the 41 x 75 x 63 cm chromatographic chamber. The upper phase of this emulsion was used as the developing phase in the descending chromatographs. Samples of 5 lambda were placed 5 cm from the edge of the chromatographic paper.

After developing, the chromatograms were dried, placed in methanol for 5 min and stained for 10 min in a bromophenol blue dye solution, prepared by combining 2 ml of glacial acetic acid, 1 g of mercuric chloride, and 0.05 g of bromophenol blue powder in a volumetric flask and bringing the volume to 100 ml with glass-distilled water. Three six-minute rinses in 5% glacial acetic acid followed. The dried

chromatogram was exposed to ammonia. This procedure detects large protein molecules. Another dried chromatogram was sprayed with ninhydrin (0.3% ninhydrin in 95% ethanol), a detector for amino acids and small peptides.

Some of the protein fractions were concentrated by adding of 133 mg of dry Sephadex G-25 gel (Medium) to each ml of sample solution (Flodin, et al., 1960, modified). After addition of the G-25 gel it was allowed to swell for at least 45 min and was removed either by suction filtration on a Buchner funnel (1.5 cm inside diameter), using filter paper cut to the appropriate size (Whatman, No. 1), or by filtering the sample through a cellulose membrane filter. This procedure was sometimes repeated as many as four times, depending upon the final concentration desired before electrophoretic studies were made. Aliquots of some of the concentrated submaxillary crude homogenate were also fractionated on chromatography columns of the G-100 and the G-200 gels.

Prior to lyophilization 15 ml aliquots of the protein material represented by the first peak obtained by purifying the submaxillary crude homogenate on Sephadex G-25 gel (Table I, 12/65) and material represented by Area D_2 (Fig. 5d) were desalted on chromatography Column I packed with Sephadex G-25 gel which had been washed with glass distilled water. Porath and Flodin (1959) originally introduced this technique of gel filtration for desalting protein solutions. The desalted protein solutions, pH 6.9 or 7.0 were stored frozen at -11°C for 1-3 days before lyophilization. Approximately 30 ml of the protein solution were frozen in each conical lyophilization flask, then lyophilized according to standard procedures.

Electrophoresis of variously purified protein fractions was carried out on 1" x 4 1/2" - 6 3/4" cellulose polyacetate strips (Gelman Instrument Co. or Millipore Filter Corp.) in a modified Beckman Model R Electrophoresis Cell as described by Dillard (1966). The cell was filled with barbiturate buffer, ionic strength 0.075, pH 8.5. The electrophoresis strips were pretreated by floating them on buffer in a plastic tray. The cellulose polyacetate strips from Gelman Instrument Co. were pretreated for approximately 10 min; the cellulose polyacetate strips from Millipore Filter Corp. were pretreated for approximately 3 min. The wet strips were placed on the extended folding rack and this rack was placed in the cell. The lid was sealed onto the cell with tape and the atmosphere was equilibrated for at least 15 min. A 20 or 30 lambda sample was applied to the strip with a sample applicator (Beckman #320005). A current of 6-10 milliamperes, applied for 35 min to 3 1/4 hours, was supplied from a regulated power supply (Reco, Model E-800-2).

The cellulose polyacetate strips were removed immediately after electrophoretic separation. They were dried between sheets of blotter paper at 37°C and stained for approximately 15 min in Napthol Blue Black (Colour Index No. 246; Conn, 1953), 1 mg/ml in 10% acetic acid. After 2-3 rinses in a 5% water solution of glacial acetic acid the cellulose polyacetate strips were made transparent in the following way.

Either dried or drained strips were placed in a 95% ethanol rinse for 1-3 min. A glass plate was placed under the strip to prevent stretching of the strip while handling it. The strip on the glass plate was placed in the clearing solution of 25% glacial acetic acid and 75% ethanol (95%) for 1-3 min. The excess liquid was removed by pulling a rubber

squeegee gently and evenly over the whole length of the strip. The strip was peeled off the glass plate after it was dried in an oven at 80-110°C for approximately 30 min or air dried overnight.

The electrophoretic strips were analyzed with a scanning densitometer (Photovolt Scanner, Model 52C; Photovolt Densitometer, Model 501a) that had a 2 mm aperture. The instrument was adjusted to zero optical density at the place of least light absorbance on the electrophoretic strip. The strips were scanned from the anodal end beginning at a position 16 mm from the origin. Optical density readings were recorded at each mm as the electrophoretic strip was moved from the 16 mm position, through the origin, to a position 55 mm in the cathodal direction.

The various fractions from the gel purifications of the submaxillary gland muscle, and thymus homogenates were assayed for their effect on cells grown in tissue cultures. Cultures were grown in Maximow deep depression slides or 30 ml plastic tissue culture flasks (Falcon Plastics). The culture medium usually consisted of 20% embryo extract (Bast and Mills, 1963) and 80% Simms (1942) solution. A few times several other media were used. One consisted of 5 drops of horse serum (Difco Laboratories) and 2 ml Simms solution. Another was 5 drops of agamma horse serum (Hyland Laboratories) and 2 ml Simms solution. A third consisted of 10% Tissue Culture Medium #199 (Morgan, et al., 1950), 70% Hanks solution (Hanks and Wallace, 1949), and 20% agamma horse serum. Buffered (4.5% sodium citrate) penicillin G (Eli Lilly and Co.), 200 units/ml of medium, was the antibiotic usually used, but once mycostatin (E. R. Squibb and Sons) in the amount of 200 units/ml of

medium and once in the amount of 100 units/ml of medium was used. When necessary the pH of the media which contained Simms solution was adjusted with 1N NaOH or with CO_2. The pH of the medium which contained Hanks solution was adjusted with 7.5% or 10% Na_2CO_3 or with CO_2. The measured pH of the medium was 7.3-7.5 when it was used (Beckman Model 76 expanded scale pH meter).

Monolayers of cells were grown from explants on the surface of the plastic culture flasks or the cover glasses of the Maximow depression slides in a medium which consisted of 10% T. C. Medium # 199, 70% Hanks and 20% agamma horse serum. Cells were grown in plasma clots in all the media described. The plasma clots were prepared by combining equal amounts of embryo extract and reconstituted, lyophilized chick plasma (Difco) with approximately 20-30 tissue explants. These three components were immediately stirred with a stainless steel insect pin or hypodermic needle and allowed to form a clot which adhered to the cover glass. The tissue explants, approximately 0.125 mm^3, were obtained by dicing selected tissue from White Leghorn chick embryos. Usually the embryos were 7-9 days old. The tissues selected were chick heart, kidney, liver, gut, posterior limb, or spinal cord. Embryonic mouse heart tissue, 14 and 15 day embryonic chick spinal cord tissue, and 14 day embryonic chick pancreas tissue were also cultured.

After the clots had formed the cover glass was inverted over the depression slide and sealed with paraffin. The completed culture slides were labeled, placed in wooden racks, and incubated (cover glass down) at 37°C. When the cultures were incubated longer than 24 hours the slides were inverted at the beginning of the second day.

Culturing operations were performed in a 61 x 153 x 55 cm dry box which had been sterilized by washing the interior with a surface active bactericidal and mycocidal agent (Cetylcide) and exposing it to UV light for at least one hour. A slanted, 150 x 31 cm plate of glass on the front permitted a view of one's work. A 16 cm opening across the bottom of the front provided an entrance for hands and arms. Hands and arms were scrubbed with soap and water, rinsed with a bactericide and dried on a sterile towel before placing them in the cabinet. A continuous, regulated flow of filtered air into the dry box maintained a positive air pressure and reduced the possibility of bacterial or mold spores entering the cabinet. When needed, a regulated flow of CO_2 into the dry box was available. A binocular, wide-field, dissecting microscope (Bausch and Lomb "StereoZoom"), with eye pieces projecting through an opening in the glass front of the dry box, and two variable intensity dissecting lamps (Bausch and Lomb) were also available inside the dry box for aid in dissections and dicing of the tissue.

Fine dissecting instruments, microsyringes, and the Millipore filter holders and filter discs were sterilized by immersion in 70% alcohol for at least 15 min. Glassware was sterilized in a steam autoclave for 15-30 min at 15-20 lbs/in^2 pressure and 121°C, then heat dried. Dishes were routinely washed in a 1% solution of a non-toxic neutral liquid detergent (7X). They were rinsed with running tap water, distilled water, and three changes of glass distilled water, then air dried.

At each culturing session the materials, the method of preparation, and the time of incubation before observing and photographing the outgrowth

from the explants were the same for the control cultures and for each group of experimental slides. Usually the proteins extracted from the mouse tissues which were added to the experimental culture slides were dissolved in 15 µl of sodium phosphate buffer (pH 6.5), but 1.5, 5, 10, 11, 45, 60 and 150 µl of extract were also used. The kinds and the amount of the mouse tissue proteins in the volumes added varied with different purification procedures and different stages of the same purification procedure.

The weight of the extracted mouse submaxillary protein in one ml of the extract represented by Area A (Fig. 1a) of the Sephadex G-25 gel fractionation from November, 1963, was 3 mg. This was determined by washing 2 pieces of dialzying tubing in water, drying them, placing a 1 ml aliquot of the protein extract into one of the pieces of tubing, dialyzing against water at 3°C for one week with frequent changes of glass-distilled water (the empty piece of dialyzing tubing was in the same container), drying them in the air for one week followed by drying in a desiccator for 2 weeks, and weighing them on an analytical balance (Mettler, Type H-6). Two sets of five weighing each were taken and averaged. After the weighing the dialyzing membranes were softened in water. The one which contained the protein extract was slit open and washed. Both samples of dialyzing membrane were washed and dried as before. Each piece of dialyzing membrane was weighed 5 times and the weights averaged. The piece of dialyzing tubing which did not contain the protein extract served as a control. The length of time each piece of tubing was removed from the desiccator for weighing was approximately the same in all instances.

In general the pattern of outgrowth was determined as radial or oriented by inspection of the culture slides in all the cultures studied in 1964 and 1965. These cultures were almost always photographed. A quantitative assessment of the extent of oriented outgrowth as influenced by the amount of submaxillary protein added to the experimental culture or water added to the control cultures was attempted. This study was done in 1966 by an analysis on photomicrographs of the outgrowth between cardiac explants.

A stock solution of the lyophilized, desalted mouse submaxillary extract, represented by Area A of the G-25 fractionation (Fig. 1a) was prepared by dissolving 5 mg in 5 ml of glass-distilled water. Eleven, 20, 27, or 45 µg of protein in 11, 20, 27, or 45 µl of water were added to the 2.5 ml of medium in each of the experimental culture slides. An aliquot of the protein stock solution was diluted with an equal volume of water. Forty-eight µl (24 µg) were also used in experimental culture slides.

In the quantitative analysis of the photomicrographs a line was drawn connecting the midpoint of the widest part of each pair of explants on the print. This line is called the midline. Lines parallel to and approximately one cm from it were constructed on both sides. The cells in this midregion strip, one cm on each side of the midline, were not analyzed. Lines parallel to the midline and 3 cm from it were drawn on either side of the midregion. The cells in these two strips, each 2 cm wide, were analyzed.

A line was drawn through the long axis of the cell body of the bipolar cells. The angle between this line and the midline was measured.

The part of the cell nearer the explant was called the proximal end. When the proximal process of the cell was nearer the midline, the angle was designated as positive; when the distal end was nearer the midline the angle was designated as negative. If the axis of the cell were parallel with the midline, its angle was zero. Any cell suspected of being an amoebocyte was disregarded. For each photomicrograph the sum of these angles was divided by the number of angles measured and this average number of degrees was plotted against the amount of submaxillary protein added to the culture slide. The algebraic sum tended to be closer to zero the more the fibrocytes tended to be parallel to each other. The average angle for all of the cells grown in the presence of a particular amount of submaxillary protein was also calculated and plotted. These averages determined the shape and placement of the curve in Fig. 12. All explants in all the cultures which were closer together than 0.70 mm, far enough apart to have an outgrowth region between them, and not close enough to a third explant to have the outgrowth region visibly affected by this third explant were photographed. Twice the outgrowth region between explants which were 0.71 and 0.75 mm apart were photographed on separate negatives and the outgrowth regions analyzed as described when both explants were close enough to be photographed at the same time.

Control cultures were prepared for this variation-in-concentration study by adding 11, 20, 27, or 45 µl of water to the 2.5 ml of medium in each culture slide. Photomicrographs were taken and analyzed, and the data plotted, as described for the experimental cultures.

Lability of the outgrowth orienting protein was tested by heating aliquots of the protein material in solution to 100°C or by treating the

protein for 1 hr in a 0.1 N HCl or 0.1 N NaOH solution. The activity of the boiled extract, 15 µl per culture slide, was tested. Ten µl of a 1 N solution of acid or base was added to 90 µl of the protein fraction represented by Area A of the G-25 fractionation of November, 1963 (Fig. 1), or the protein fraction represented by Area D_2 of the G-200 gel fractionation (Fig. 5d). After one hr, 15 µl of this material were added to the medium in the experimental slides, or the pH of the treated protein solution was readjusted. The pH of the submaxillary extract treated with NaOH was not readjusted to near neutrality. The measured pH of the culture medium in these slides was 7.6-7.8. Once, when testing the material in Area A of the G-25 fractionation for NaOH lability, 15 µl of 0.1 N NaOH (90 µl of water and 10 µl of 1 N NaOH) were added to 2 culture slides, as a type of control culture. The pH of the submaxillary protein solution treated with 0.1 N HCl was readjusted by adding an equal volume of 0.1 N NaOH. Fifteen µl of this diluted, treated extract was added to the experimental slides.

In examinations of the culture slides a phase contrast and dark field microscope (Bausch and Lomb) was used. Cultures were scanned immediately after preparation. The cultures were observed, descriptive notes taken, and photomicrographs made at 15 or 18 hr, often again at 24 hr, and occasionally at times as long as 48 hr after incubation at 37°C. The phase contrast microscope was equipped with a long working distance condenser and a triocular head. Photomicrographs were taken with a 2 1/4" x 2 1/4" camera (Reflex-Korelle) and with microscope lens combinations of 70 x dark field, 200 x phase contrast, and 420 x phase contrast. Panchromatic film was used (Kodak Verichrome Pan or Rex). Prints were

enlarged 2.1 times. A stage micrometer was photographed with this enlargement system and used to calculate actual magnifications.

The term "outgrowth" is used in this paper to denote the cells which seem to be growing adjacent to an explant. No distinction was made between cellular hyperplasia and cellular migration from the explant.

CHAPTER III

RESULTS

Purification of the Male Mouse Submaxillary Crude Homogenate

Excess streptomycin sulfate from the precipitation of the nucleoproteins was removed from three 10 ml aliquots of the male mouse submaxillary crude homogenate (MMSCH) by gel filtration on Sephadex G-25 gel (Table I). Sodium phosphate buffer, 0.02 M and pH 6.5, was used. In these purifications the variations in the amount of ultraviolet light absorbed by the first 72 or 79 ml of effluent indicated the presence of two different groups of protein material of differing molecular weights. These are designated as Areas A and B on Fig. 1a. The absorption spectra of the materials represented by Area A from the purification in November, 1963, and the comparable peak from the purification in July, 1964, showed maximum absorption at wave lengths of 276 to 283 mμ and minimum absorption at 250 mμ (Table II). Analysis of the material represented by Area A on paper chromatography showed no migration in a butanol-water-acetic acid solvent when the chromatogram was stained with ninhydrin or with bromophenol blue. Chromatographic comparison of this material represented by Area A with the male mouse submaxillary crude homogenate and the female mouse submaxillary crude homogenate used by Bast and Mills (1963), stained with ninhydrin, showed more spots and darker spots on the paper chromatogram from the sample of male mouse submaxillary crude homogenate than

TABLE I

PURIFICATION OF MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE ON SEPHADEX G-25

DATE OF PURIFICATION	11/63			7/64			12/65		
Gel bed dimensions--cm (diameter x height)	1.8 x 42.0			1.8 x 42.0			1.8 x 47.5		
MMSCH Preparation date	6/63			7/64			7/64 and 10/65,pooled		
Spectrophotometer Model	DB			DB			DU		
Absorbed light recording method	Continuous			Continuous			Aliquot Readings		
Wave length--mμ	280			Varied			280		
Flow rate	--			0.7 ml/min			1.1 ml/min		
Readings recorded	ml	% T	O. D.	ml	mu	% T	O. D.	ml	% T	O. D.
	35.5	100	0.000	36.0	280	100	0.000	38	100	0.000
	36.5	58	0.237	37.0	280	70	0.155	41	45	0.347
	37.5	3	1.523	40.0	280	0		56	00	
	38.0	99	2.000	53.0	300	0		59	47	0.328
	58.0	99	2.000	58.5	300	0		61	47	0.438
	58.5	23	0.638	61.0	310	60	0.222	64	42	0.377
	59.5	31	0.509	62.0	310	48	0.319	67	19	0.721
	60.5	25	0.602	62.5	310	42	0.377	70	8	1.097
	61.5	19	0.721	63.0	310	45	0.347	73	28	0.553
	63.5	32	0.495	66.0	310	60	0.222	76	34	0.369
	72.0	33	0.482	79.0	310	61	0.215	79	38	0.420

ml --Effluent volume
mu --Wave length
% T --Percent transmission
O. D.--Optical density

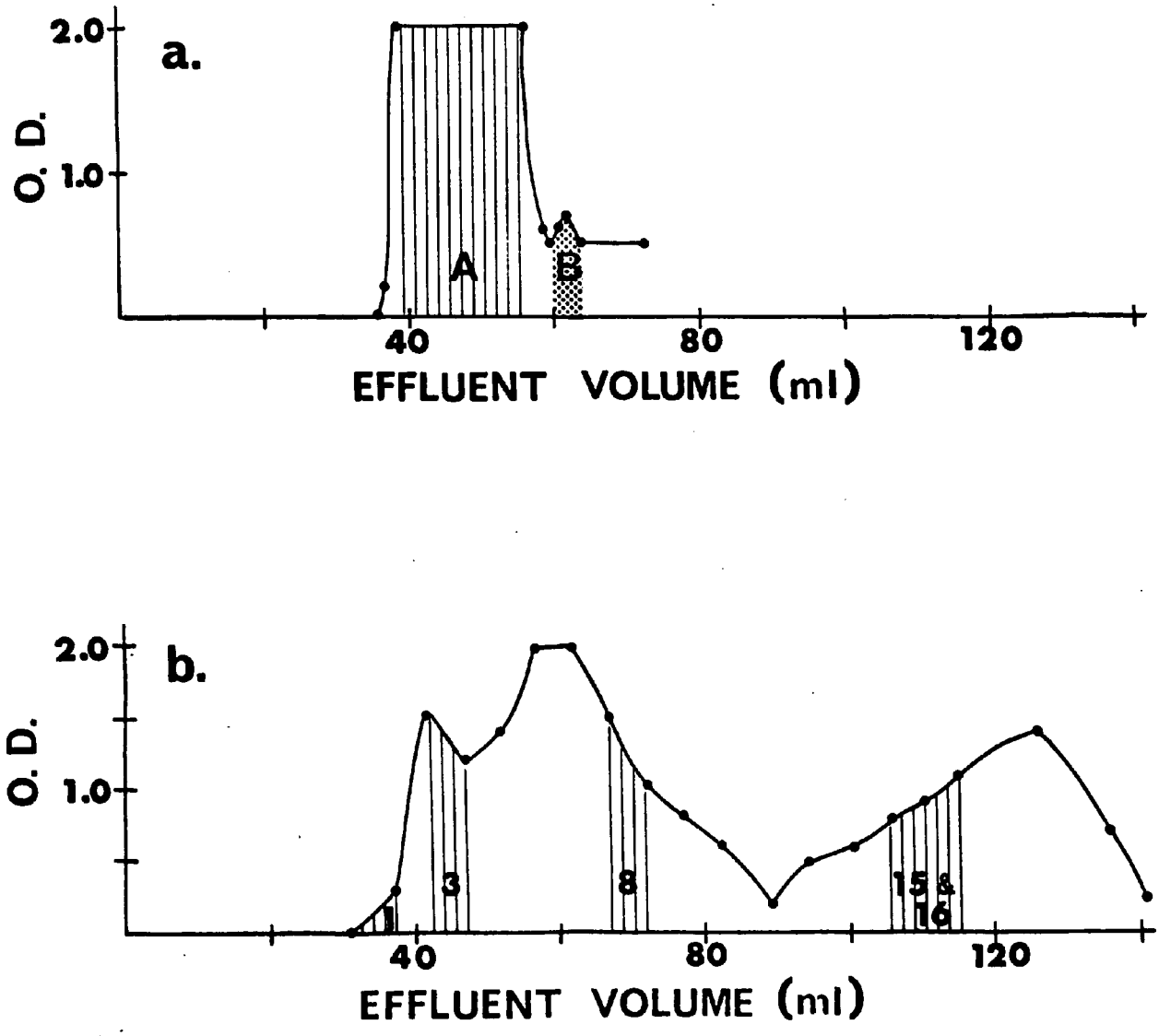


FIGURE 1: Fractionation of Male Mouse Submaxillary Crude Homogenate on G-25 Gel and G-75 Gel. A 0.02 M sodium phosphate buffer, pH 6.5 was used.

a. Fractionation of a 10 ml sample on G-25 gel in Column I. Date: 11/63. Height of gel: 42.0 cm.

b. Fractionation of a 10 ml sample on G-75 gel in Column I. Flow rate: 1.0 ml/min. Height of gel: 43.0 cm.

TABLE II

ABSORPTION SPECTRA FOR MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE

REPRESENTED BY AREA A, FIG. 1a

WAVE LENGTH (mμ)	November, 1963		July, 1964	
	% Transmission	Optical Density	% Transmission	Optical Density
245	92	0.036	42	0.377
250	93	0.032	51	0.292
255	91	0.041	50	0.301
260	89	0.051	46	0.337
265	85	0.071	40	0.398
270	85	0.071	36	0.444
271	--	--	36	0.444
272	84	0.076	35	0.456
273	--	--	35	0.456
274	84	0.076	34	0.469
275	84	0.076	34	0.469
276	83	0.081	33	0.482
277	83	0.081	33	0.482
278	83	0.081	33	0.482
279	83	0.081	33	0.482
280	83	0.081	33	0.482
281	83	0.081	33	0.482
282	83	0.081	33	0.482
283	83	0.081	33	0.482
284	84	0.076	34	0.469
285	85	0.071	35	0.456
290	88	0.056	43	0.367
295	92	0.036	57	0.244
300	95	0.022	71	0.149
305	96	0.018	79	0.102
310	97	0.013	82	0.086
320	98	0.009	85	0.071
330	98	0.009	86	0.067

from the sample of female mouse submaxillary crude homogenate. Some protein material did not move from the origin.

Fractionation of a 10 ml aliquot of MMSCH on Sephadex G-75 (Fig. 1b; Table III) monitored at a wave length of 290 mμ with the Beckman DB Spectrophotometer, equipped with the continuous recorder, showed little separation. More separation was achieved with a smaller sample volume and Sephadex G-100 (Fig. 2a; Table IV). Differences in the height of the gel in Column I and the concentration of the material in the sample applied to the gel resulted in some variations in the height and position of the peaks of absorbed ultraviolet light. The absorption spectra of the material in the effluent fractions numbered 3 and 4 in Fig. 2a (Fig. 2b; Tables V and VI showed maximum ultraviolet light absorption at or near λ = 280 mμ. The absorption spectra of the materials in the fraction numbered 5 showed maximum absorption at λ = 275 mμ (Fig. 2c; Table VI). The absorption spectra of the materials in the fractions numbered 1 and 2 (Fig. 2b; Table V) showed no definite peak of maximum absorption, but rather slight random fluctuations. Increased separation of the material represented by peaks V and VI in Fig. 3a (Table VII) was achieved when the sample was reduced to 0.5 ml.

Ten mg of lyophilized submaxillary protein, represented by Area A of the G-25 fractionation of December 1965 (Table I), had 4 peaks in the first 80 ml of the effluent (Fig. 3b; Table VIII). The four peaks of absorbed ultraviolet light were assumed to represent four groups of protein molecules of different molecular weight. The lyophilized material was dissolved in 0.5 ml of 0.02 M sodium phosphate buffer, pH 6.5, and the same kind of buffer was used to equilibrate the gel. A

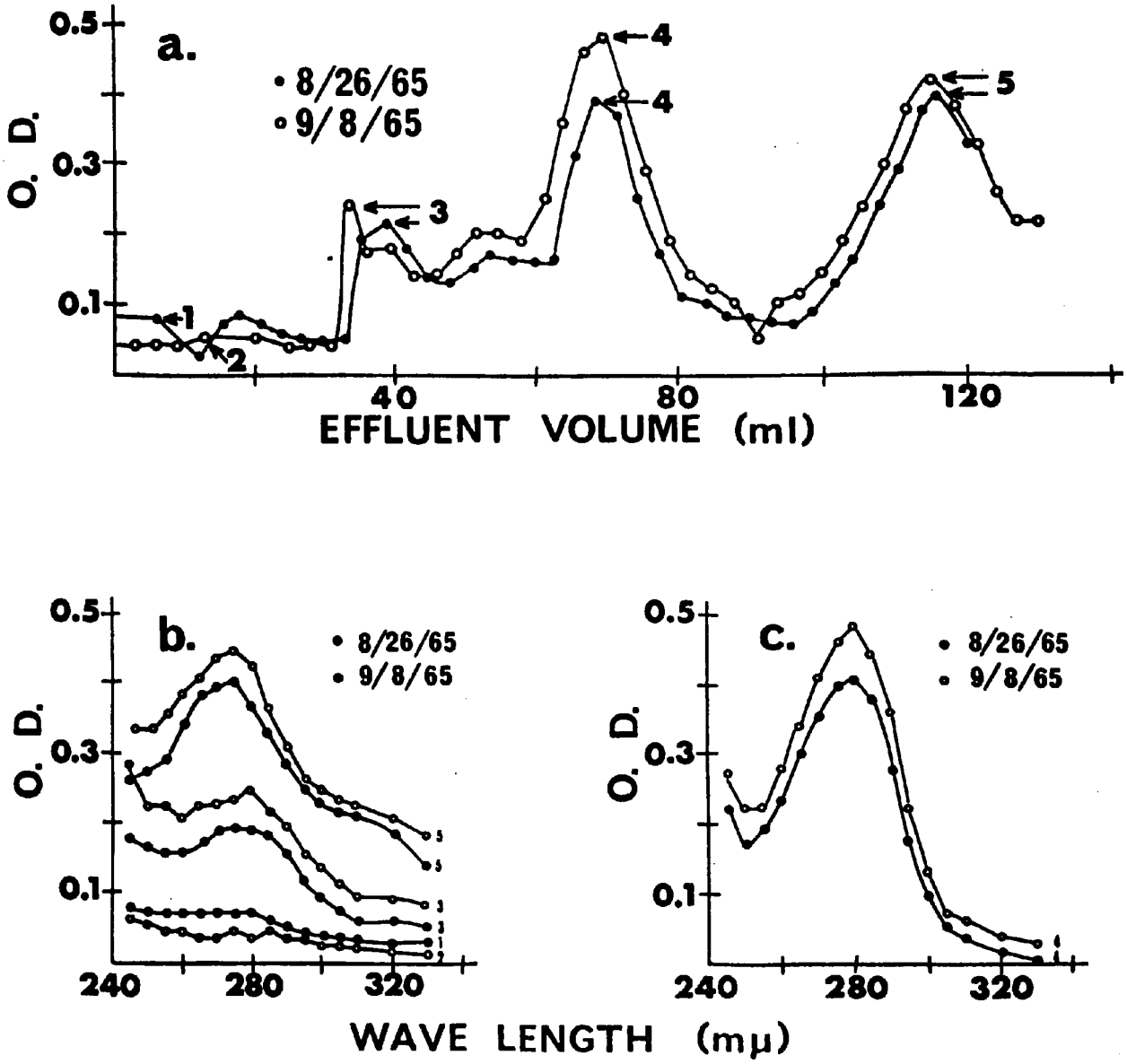


FIGURE 2: Fractionation of Male Mouse Submaxillary Crude Homogenate on G-100 Gel and the Absorption Spectrum of the Indicated Fractions.

a. Fractionation of a 1.2 ml sample on Column I. Buffer: 0.2 M sodium phosphate, pH 6.5. Fractionation on 8/26/65: flow rate of 0.6 ml/min, gel height of 46.5 cm. Fractionation on 9/8/65: flow rate of 0.5 ml/min, gel height of 46.0 cm, sample concentrated once with G-25 gel.

b. Absorption spectrum of the indicated fractions.

c. Absorption spectrum of the indicated fractions.

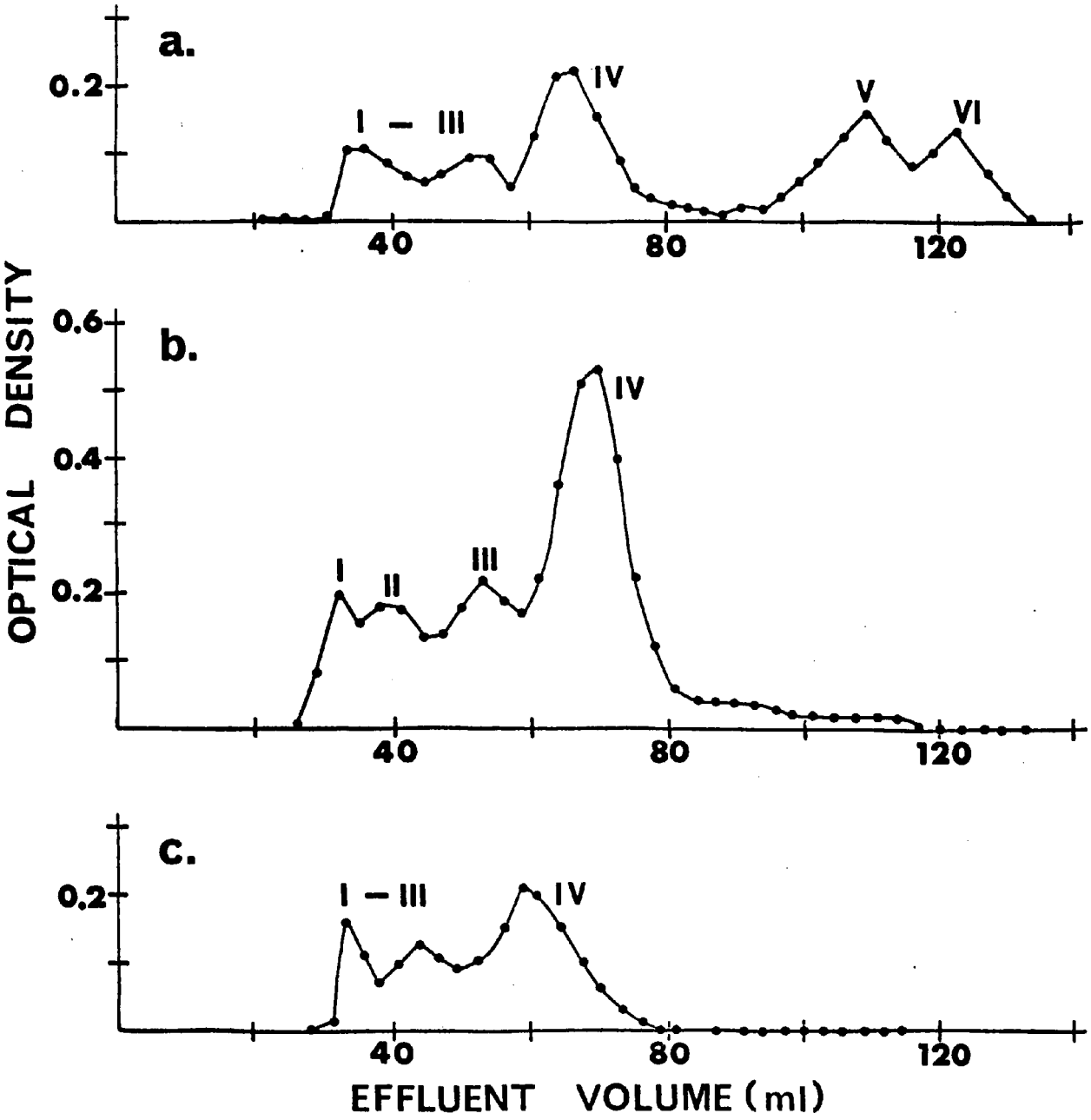


FIGURE 3: Separations of Male Mouse Submaxillary Proteins on G-100 Gel. Column I and 0.5 ml sample volumes were used.

a. Fractionation of MMSCH. Buffer: 0.02 M sodium phosphate, pH 6.5. Flow rate: 0.4 ml/min. Height of gel: 40.5 cm.

b. Fractionation of 10 mg of lyophilized protein represented by Area A of the G-25 fractionation. Buffer: 0.02 M sodium phosphate, pH 6.5. Flow rate: 0.5 ml/min. Height of gel: 45.4 cm.

c. Fractionation of 5 mg of lyophilized protein represented by Area A of the G-25 fractionation. Buffer: 0.005 M Tris HCl, pH 7.2. Flow rate: 0.5 ml/min. Height of gel: 45.3 cm.

similar elution curve (Fig. 3c; Table IX) was obtained from the separation of 5 mg (in 0.5 ml of Tris HCl buffer) of the same material on a column of G-100 gel equilibrated in 0.005 M Tris HCl buffer, pH 7.2, although the first three peaks merged into two in this separation.

Electrophoresis of the protein material in peak IV of the G-100 fractionation (Fig. 2a, sample 4 of 9/8/65) showed, after staining, two dark blue bands which migrated toward the cathode, and a band at the origin. The two cathodal bands corresponded in position with the third and fourth cathodal bands which had been separated electrophoretically from the protein material represented by Area A of the G-25 fractionation. The optical density of the bands were analyzed with the densitometer and these results are diagramed in Fig. 4 (Table X).

The least cross-linked gel, Sephadex G-200, did not increase the separation previously achieved with the G-100 gel when a 1.2 ml sample of MMSCH was fractionated on Column I (Fig. 5a, Table XI). Better separation was obtained when a 2 ml sample was fractionated on Column II (Fig. 5b; Table XII). Recycling of the material represented by Area D in Fig. 5b did not increase the separation of the material even though the volume of each of the ten samples was reduced by concentration with dry G-25 gel and each sample was applied consecutively (Fig. 5c; Table XVIII). Better separation was achieved by reducing the volume of the sample of MMSCH applied to Column II to 1 ml (Fig. 5d; Table XIV). The ten fractions indicated by the vertical lines in Fig. 5d, Area D_2, were pooled, desalted and lyophilized. When a sample was electrophoresed the pattern of the bands was similar in number and spacing to that obtained from the same quantity of material represented by Area A of the G-25 fractionation

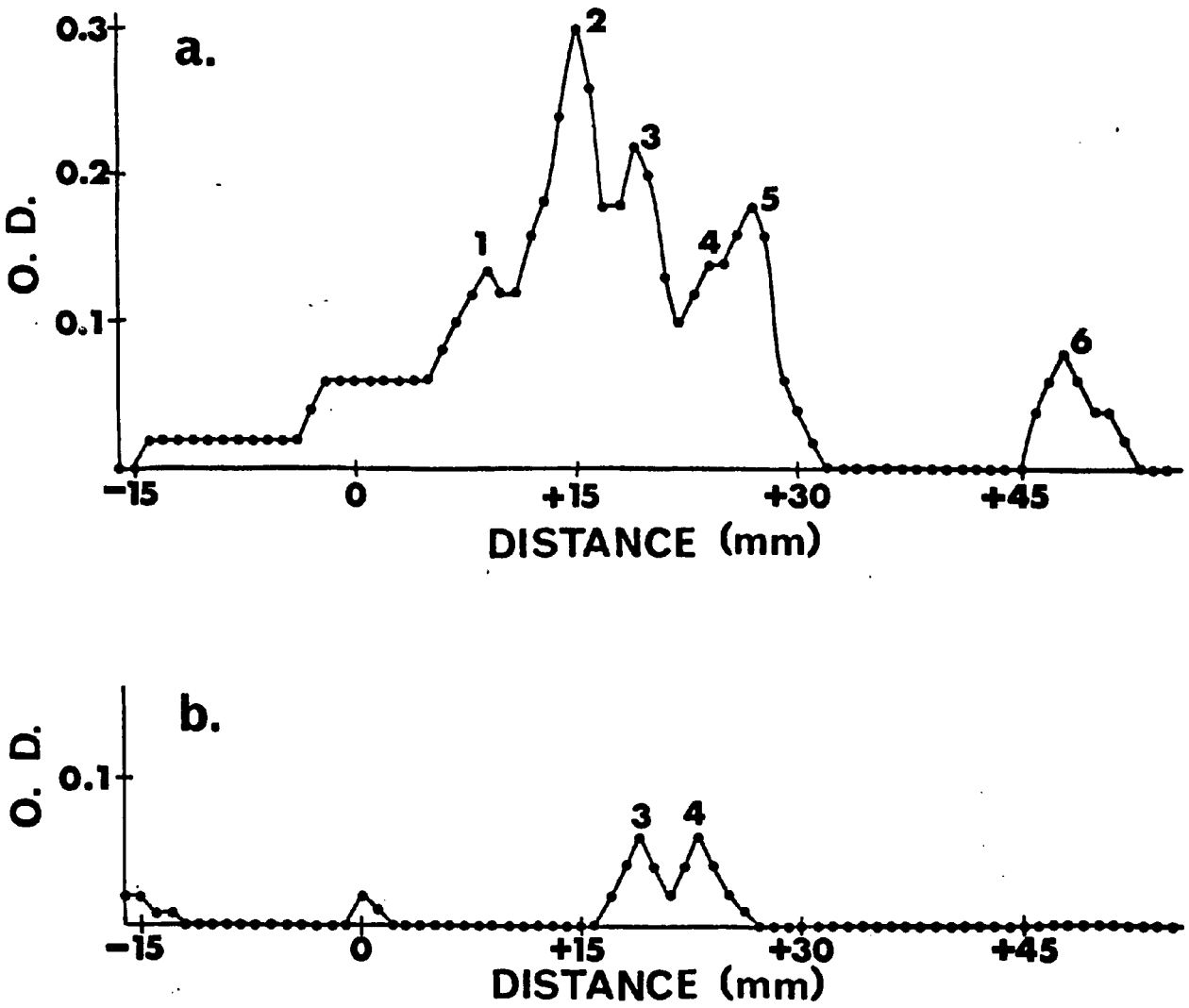


FIGURE 4: Densitometer Analysis of the Electrophoretic Separations of Protein Represented by Area A of the G-25 Gel Fractionation and Peak IV of the G-100 Gel Fractionation. The 30 λ protein samples were applied at the distance indicated as zero. The samples were electrophoresed for 90 minutes at 10 milliamperes with the voltage gradually decreasing from 200 to 100 volts. The anodal distance from the origin is indicated as "-"; the cathodal distance from the origin is indicated as "+". The protein in both fractions had been concentrated twice with dry Sephadex G-25 gel.

a. Protein Represented by Area A of the G-25 Gel Fractionation.
b. Protein Represented by Peak IV of the G-100 Gel Fractionation.

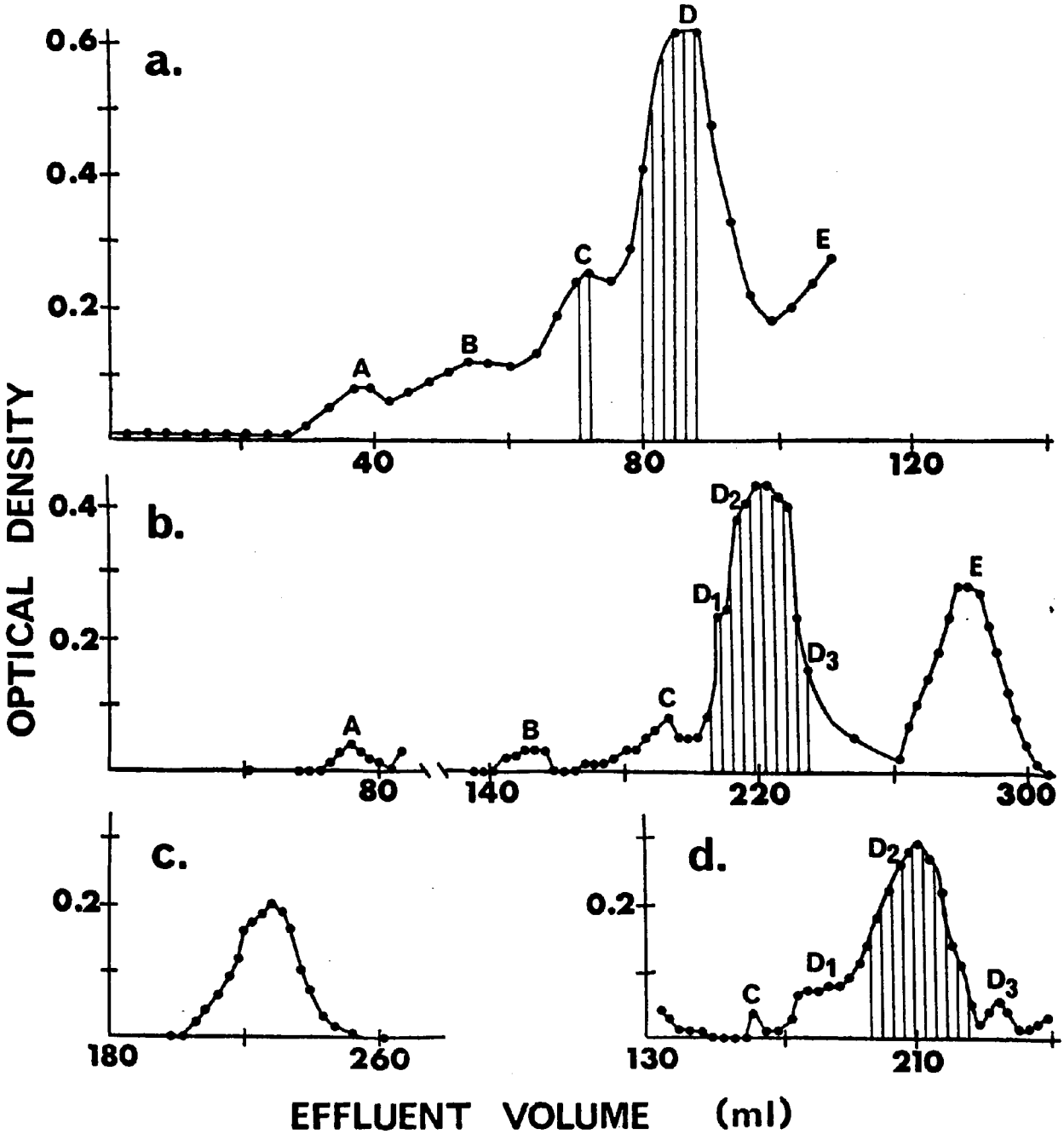


FIGURE 5: Fractionation of Male Mouse Submaxillary Crude Homogenate on G-200 Gel. A 0.02 M sodium phosphate buffer, pH 6.5, was used.

a. Fractionation of a 1.2 ml sample, concentrated once with dry G-25 gel, on Column I. Flow rate, 0.2 ml/min. Height of gel, 45.6 cm.

b. Fractionation of a 2 ml sample, concentrated once with dry G-25 gel, on Column II. Flow rate, 0.1 ml/min. Height of gel, 75 cm.

c. Recycling of Area D on Column II. Flow rate, 0.1 ml/min. Height of gel, 75 cm.

d. Fractionation of a 1 ml sample, concentrated twice with dry G-25 gel, on Column II. Flow rate, 0.2 ml/min. Height of gel, 70.2 cm.

(desalted and lyophilized material of the fractionation of December, 1965, Table I). These bands of differently charged proteins are represented in Fig. 6 (Table XV). The vertical displacement of the line represents the amount of white light absorbed by the densitometer which, in turn, represents the darkness of the band of stained protein on the electrophoretic strip. The relative heightof the peaks that are the same distance from the origin are different in the more purified material (protein represented by Area D_2) and the less purified material (protein represented by Area A). The relative height of cathodal peak 3 and anodal peak 2 of the protein sample represented by D_2 increased and the relative height of cathodal peaks 2 and 5 decreased.

Purification of the MMSCH by precipitation with ethanol did not eliminate the peaks which were present in the first 135 ml of effluent from the fractionation on Sephadex G-100 gel, but it did decrease the relative height of peak IV and increased the relative height of peak V (Fig. 7; Tables VII and XVI). Electrophoretic comparison of the proteins in the material represented by Area A of the G-25 fractionation and those present in the material from the alcohol purification showed that bands 2 and 6, especially band 6, were being concentrated by the alcohol purification. Strips were analyzed with the densitometer. Figure 8 shows the amount of light absorbed by the densitometer as peaks (Tables XVII-XX). Due to the short length of the electrophoretic strip the protein material which migrated as band 6 (peak 6, Fig. 8a) was no longer on the strip when the material was electrophoresed for 90 min. The material electrophoresed for 90 min showed better separation of the bands nearer the origin than that electrophoresed for 35 min.

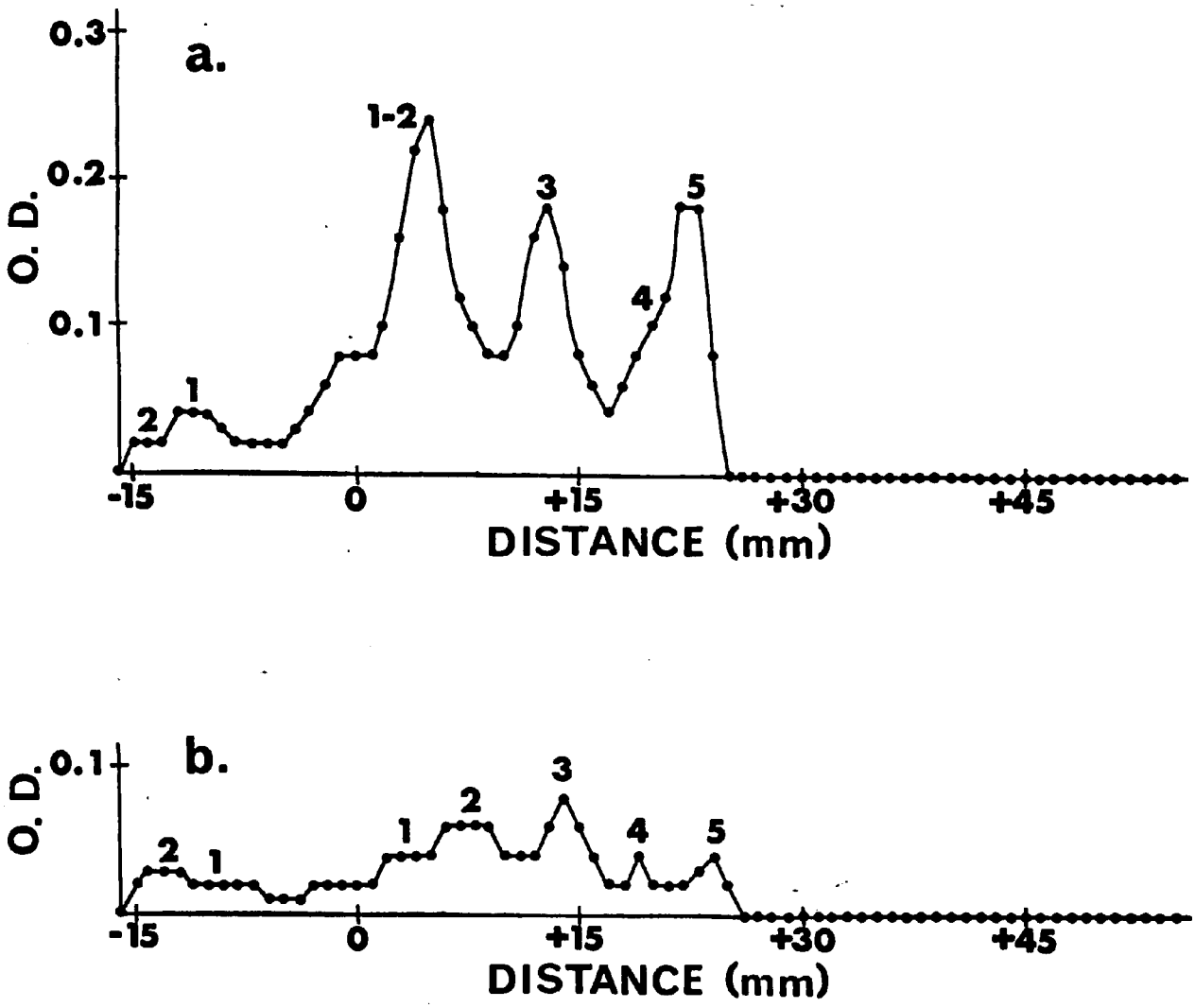


FIGURE 6: Densitometer Analysis of the Electrophoretic Separations of Protein Represented by Area A of the G-25 Gel Fractionation and Peak D_2 of the G-200 Gel Fractionation. The 20 λ samples, each containing 0.4 mg of reconstituted, lyophilized protein, were applied at the distance indicated as zero. The samples were electrophoresed for 1 3/4 hours at 7-10 milliamperes and 200-225 volts. The anodal distance from the origin is indicated as "-"; the cathodal distance from the origin is indicated as "+".

a. Protein Represented by Area A of the G-25 Gel Fractionation.
b. Protein Represented by Peak D_2 of the G-200 Gel Fractionation.

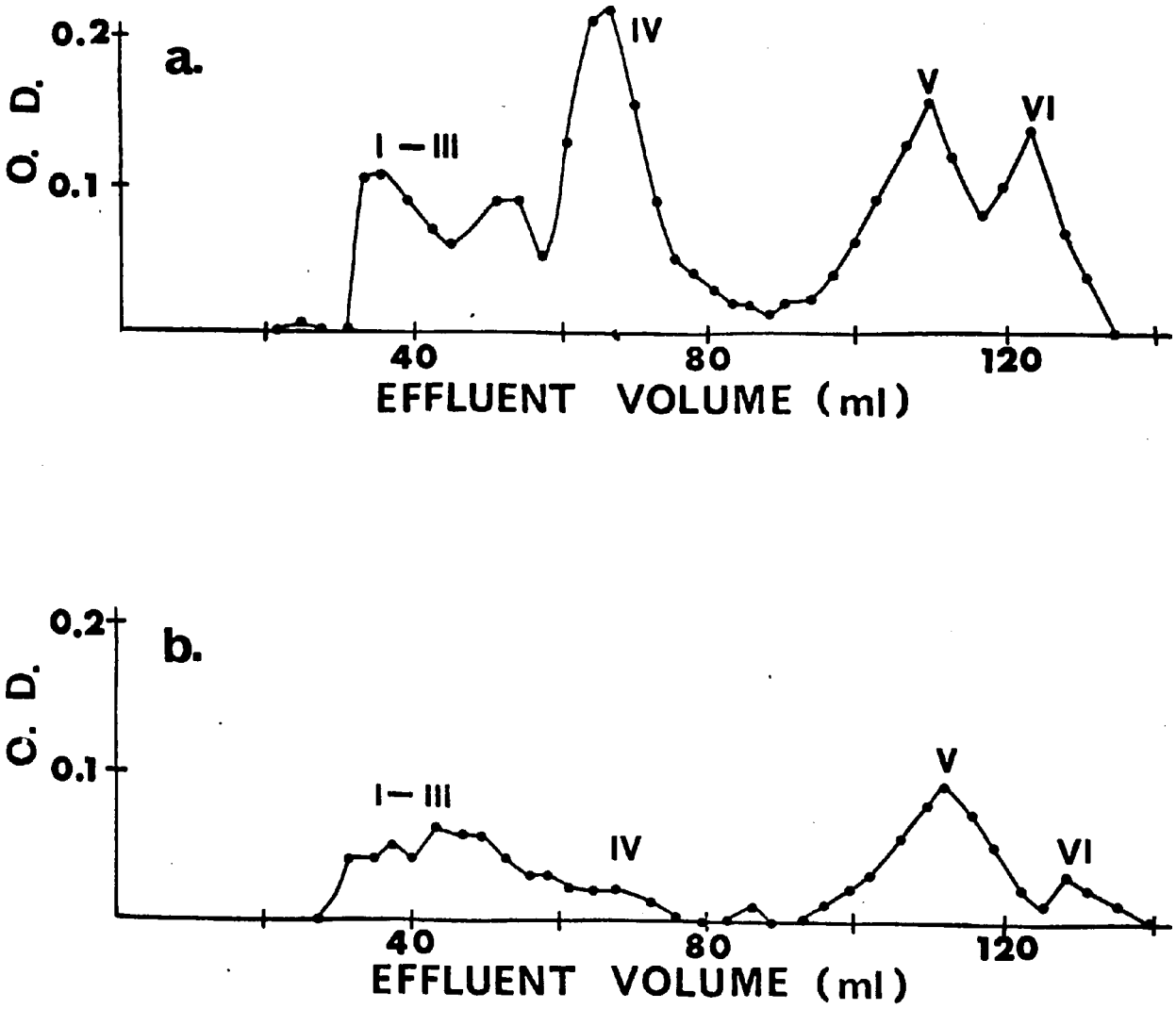


FIGURE 7: Comparison of the Elution Curve of the Male Mouse Submaxillary Crude Homogenate with the Elution Curve of the Crude Homogenate Purified by Ethanol Precipitation. Column I; 0.02 M sodium phosphate buffer, pH 6.5; and 0.5 ml sample volumes were used.

a. Fractionation of MMSCH. Flow rate: 0.4 ml/min. Height of gel: 40.5 cm.

b. Fractionation of ethanol purified MMSCH. Flow rate: 0.3 ml/min. Height of gel: 44.8

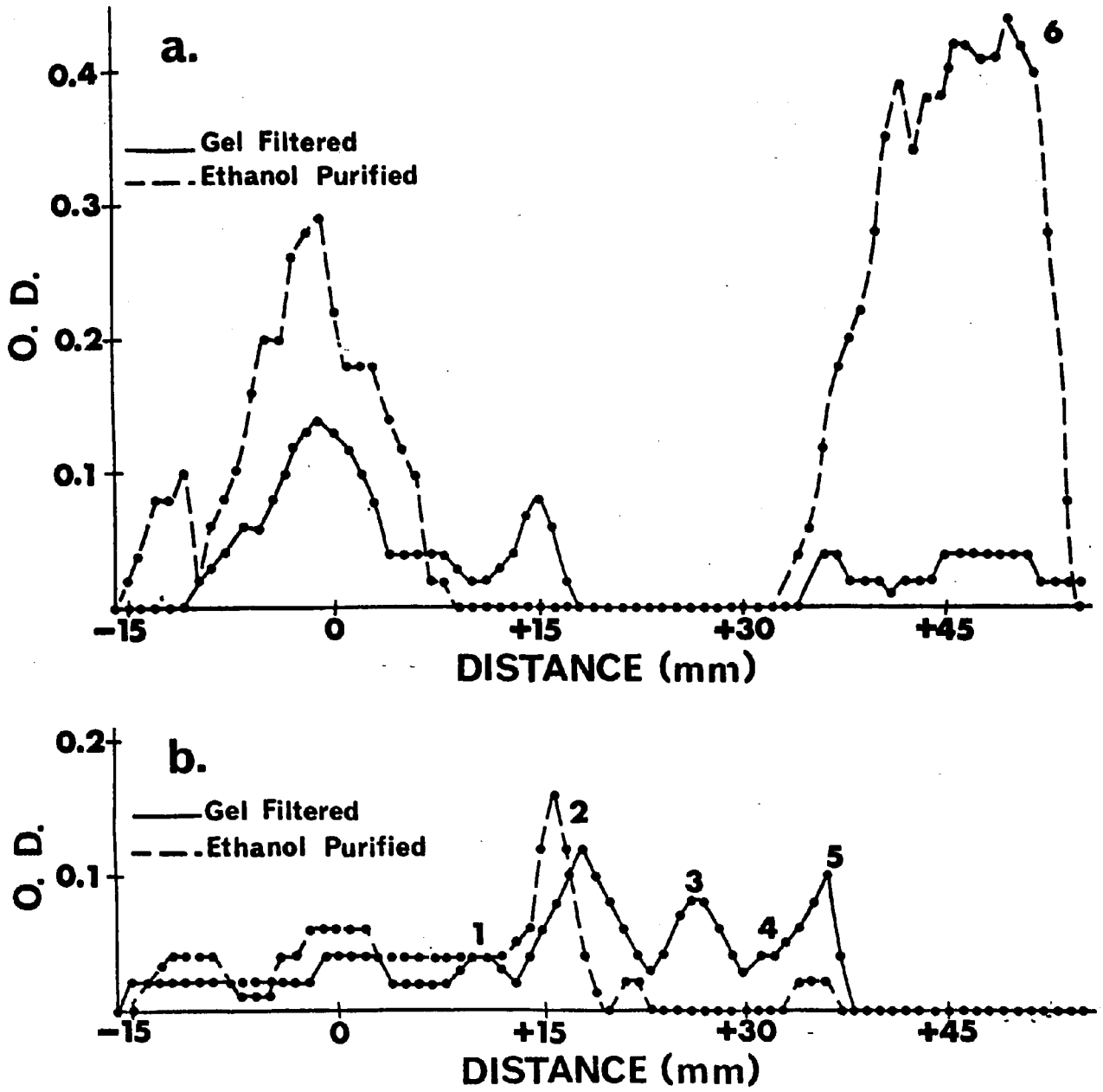


FIGURE 8: Densitometer Analysis of the Electrophoretic Separations of the Proteins in the Male Mouse Submaxillary Crude Homogenate Purified by Gel Filtration or Ethanol Precipitation. A 30 λ sample of the effluent represented by Area A of the G-25 gel fractionation (concentrated twice with dry Sephadex) or a 20 λ sample of the ethanol purified extract was applied at the distance indicated as zero. The anodal distance from the origin is indicated as "−"; the cathodal distance from the origin is indicated as "+".

a. Sample Electrophoresed for 35 minutes at 7 milliamperes and 175 volts.

b. Sample Electrophoresed for 90 minutes at 7 milliamperes with the voltage gradually decreasing from 200 to 150 volts.

The elution curve of the MMSCH purified by precipitating the protein with 40% to 70% saturation of ammonium sulfate showed four distinct peaks in the first 80 ml of effluent when 0.02 M sodium phosphate buffer, pH 6.5 was used (Fig. 9a; Table XXI). The first three peaks merged into two when the column of Sephadex G-100 was equilibrated with 0.005 M Tris HCl buffer, pH 7.2 (Fig. 9b; Table XXII). The protein material represented by peaks V and VI were not eliminated but were relatively decreased.

Fractionation of Other Male Mouse Tissue Crude Homogenates

The excess streptomycin sulfate used to precipitate the nucleoproteins was removed from a 7 ml aliquot of the male mouse muscle crude homogenate (MMMCH) by filtration on Sephadex G-25 gel (Fig. 10a; Table XXIII). This purification monitored by the Beckman DB Spectrophotometer with the constant flow-throughcell and recorder, showed maximum light absorption over a 15 ml effluent volume. This volume is represented as Area A and is divided into two fractions, A_1 and A_2 in Fig. 10a. Area A was extrapolated to 0% transmission as the wave length was changed to 300 mμ in the plateau area in an attempt to detect separation of this material. The absorption spectrum of the material represented by A_2 showed maximum absorption at wave lengths of 273-280 mμ and minimum absorption at 250 mμ (Table XXIV). Further separation of the proteins in the MMMCH was obtained by the fractionation of a one ml sample on Sephadex G-100 (Fig. 10b; Table XXV).

Two peaks were also obtained in the first 80 ml of effluent from a 7 ml aliquot of male mouse thymus crude homogenate (MMTCH) fractionated on Sephadex G-25 (Fig. 11a; Table XXIII). This fractionation

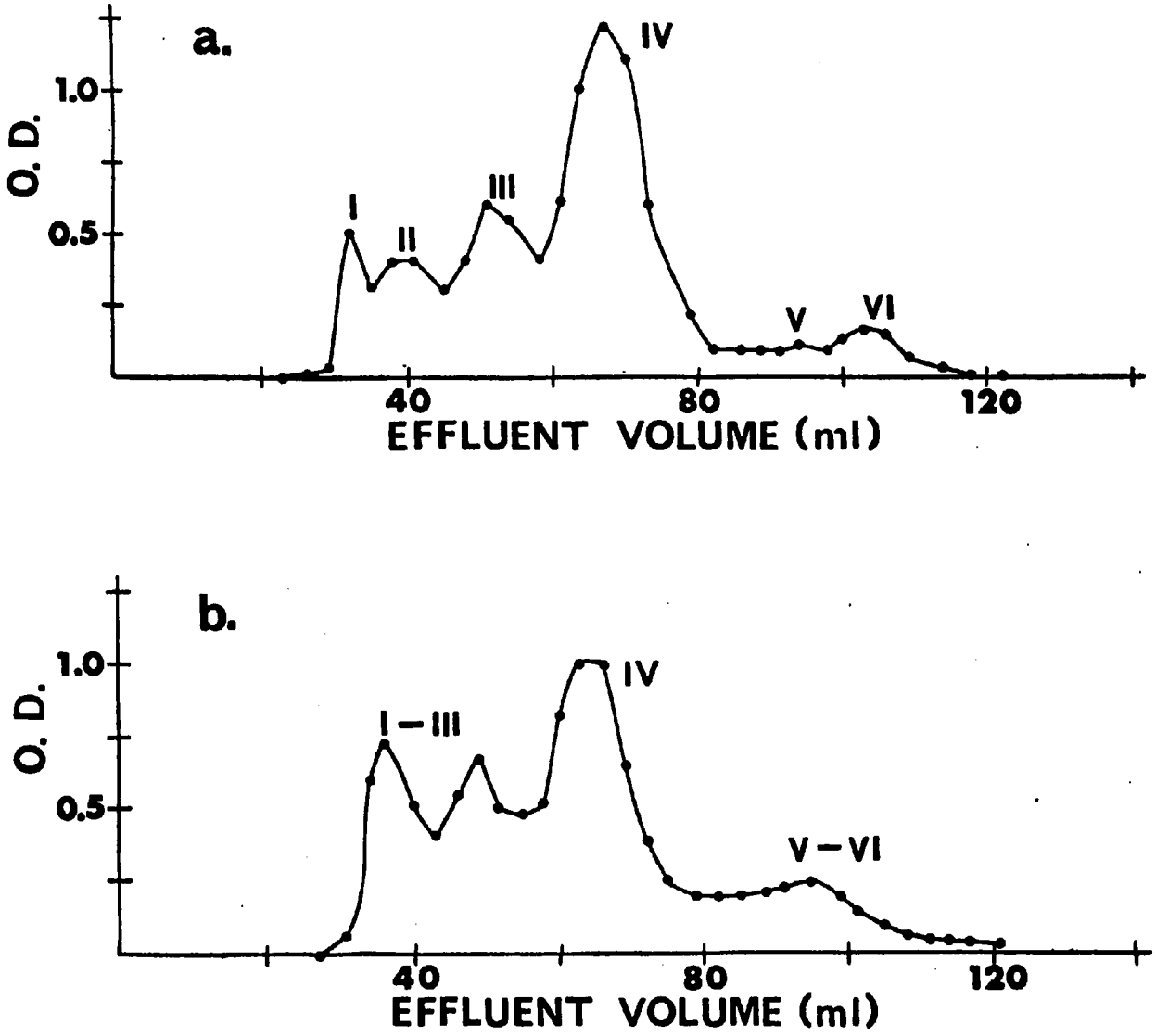


FIGURE 9: Fractionation of Male Mouse Submaxillary Crude Homogenate Purified by Precipitation with Ammonium Sulfate. G-100 gel and Column I were used.

a. Fractionation of a 0.5 ml sample. Buffer: 0.02 M sodium phosphate, pH 6.5. Flow rate: 0.3 ml/min. Gel height: 45.6 cm.

b. Fractionation of a 0.5 ml sample. Buffer: 0.005 M Tris HCl, pH 7.2. Flow rate: 0.5 ml/min. Gel height: 45.1 cm.

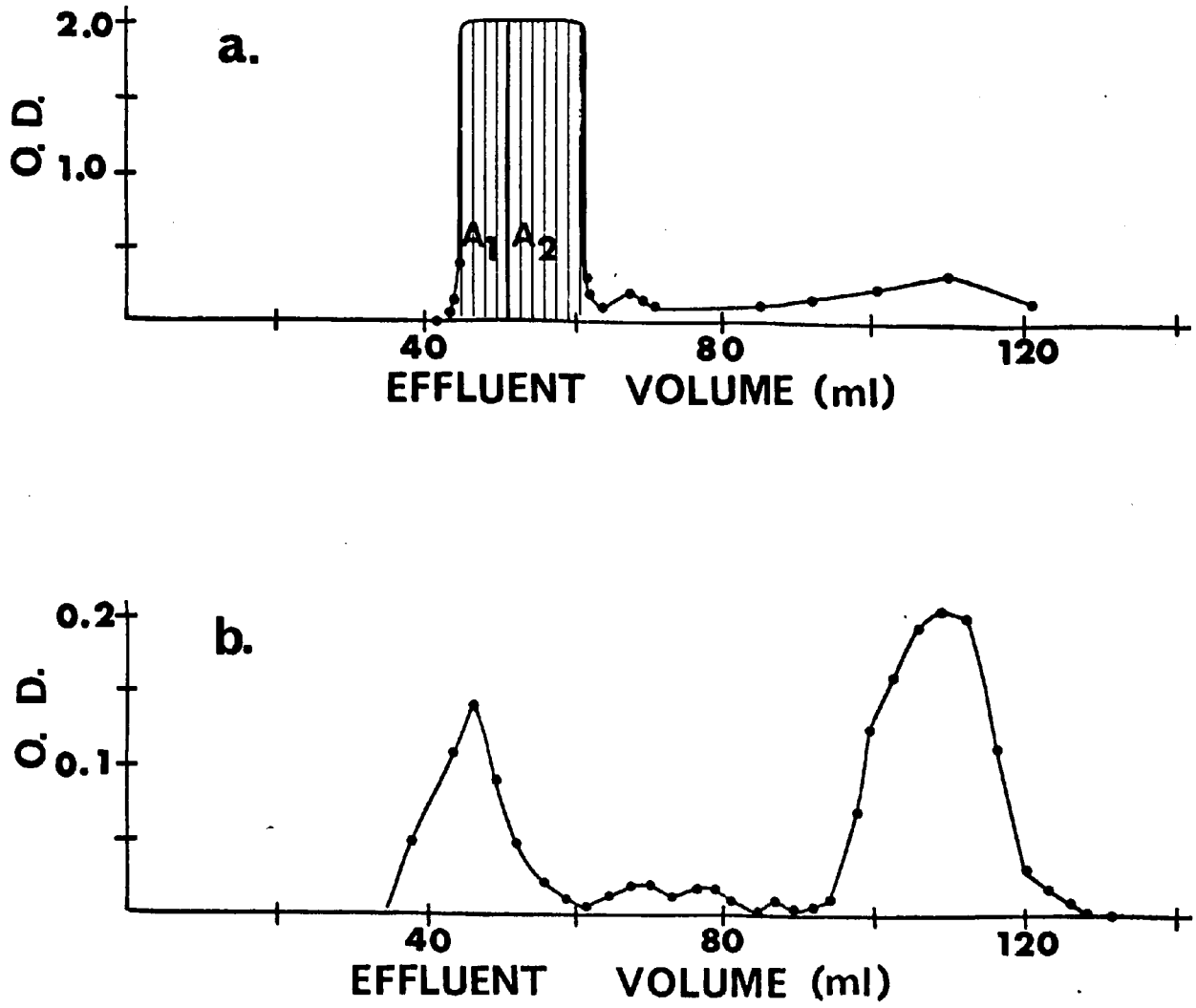


FIGURE 10: Fractionation of Male Mouse Muscle Crude Homogenate. Column I and 0.02 M sodium phosphate buffer, pH 6.5, were used.

a. Fractionation of a 7 ml sample on G-25 gel. Flow rate: 1.6 ml/min. Height of gel: 48 cm.

b. Fractionation of a 1.0 ml sample on G-100 gel. Flow rate: 0.3 ml/min. Height of gel: 46 cm.

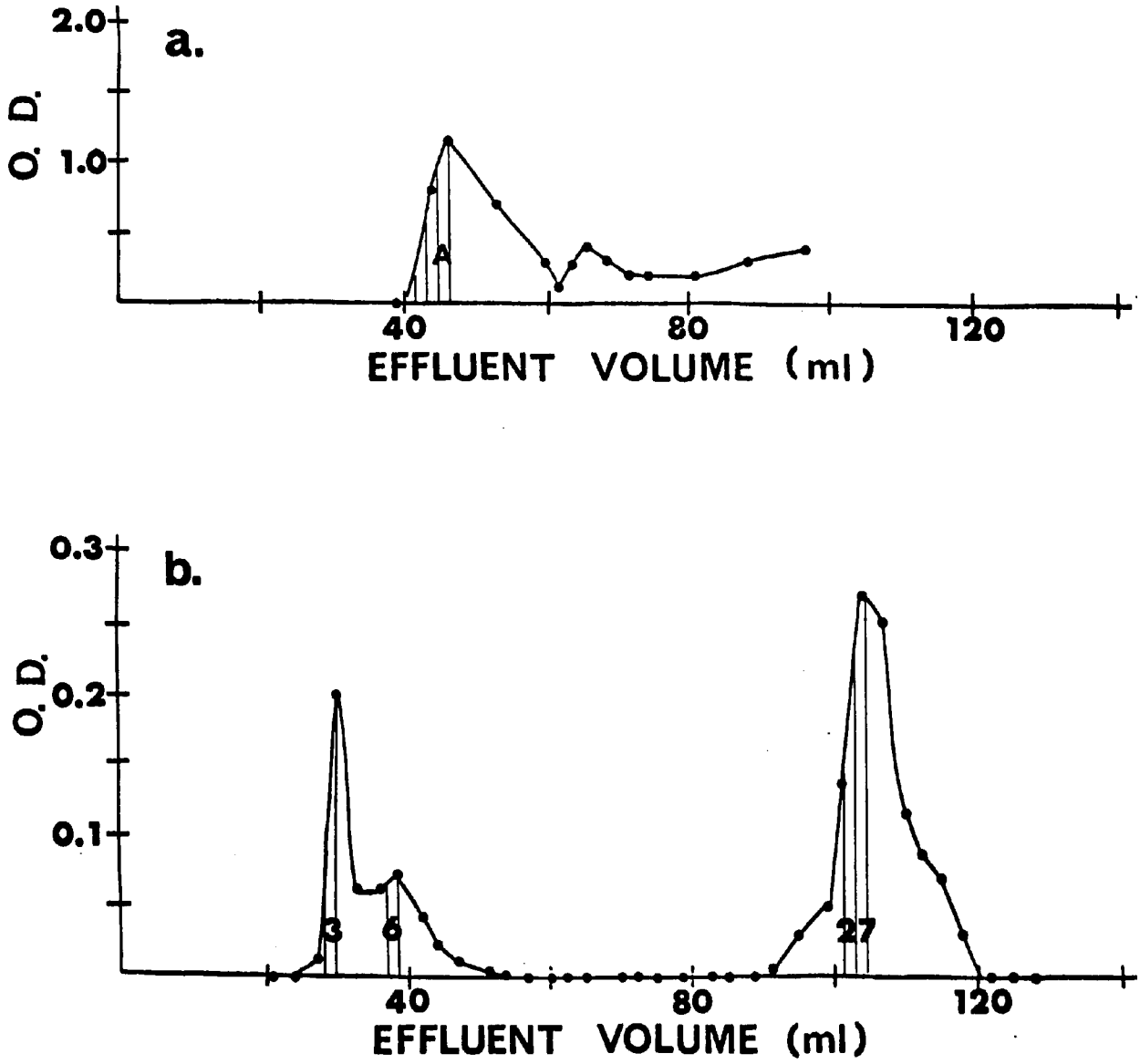


FIGURE 11: Fractionation of Male Mouse Thymus Crude Homogenate. Column I and 0.02 M sodium phosphate buffer, pH 6.5, were used.
a. Fractionation of a 7 ml sample on G-25 gel. Flow rate: 1.0 ml/min. Height of gel: 42 cm.
b. Fractionation of a 0.5 ml sample on G-100 gel. Flow rate: 0.4 ml/min. Height of gel: 45.2 cm.

was monitored by the Beckman DB Spectrophotometer with the constant flow-through cell and recorder. Further separation of the proteins in the MMTCH was achieved on Sephadex G-100 (Fig. 11b; Table XXVI).

Assay of the Purified Fractions

All fractions of the tissue extracts from the various purifications on the gel columns which were suspected of containing the *in vitro* outgrowth orienting activity were assayed. In the Sephadex G-25 and G-75 purifications the outgrowth orienting activity was present in the first fractions which absorbed ultraviolet light. Oriented outgrowth was observed in the cultures to which the fraction represented by peaks 4 and IV from the Sephadex G-100 columns or peak D on the Sephadex G-200 columns had been added. The orienting activity was retained in D_2 when the material in peak D was fractionated into three groups of molecules of differing molecular weight (Plate I; Table XXVII).

The orienting activity was also mediated by the extract of the thymus gland from sexually immature male mice (Plate II, 1b, 2b; Table XXVIII).

Effect of Varying the Concentration of the Submaxillary Protein

The concentration of the protein in the effluent represented by Area A of the fractionation on the Sephadex G-25 (Fig. 1a) was 3 mg/ml (Table XXIX). Therefore, there were 45 µg of protein in the 15 µl which were added to the 2.5 ml of medium in the culture slides to produce the patterned outgrowth effect. When the amount of the outgrowth orienting protein, the lyophilized material represented by Area A of the G-25 fractionation, added to the cultures was varied, little or no change in

PLATE I

THE EFFECT OF SOME SUBMAXILLARY FRACTIONS UPON OUTGROWTH PATTERN

1a Dark field photmicrograph of radial outgrowth between several chick cardiac explants in a control culture. Incubation time: 27 hr. Culture medium: Simms. Additive: None. Explants beating. Culture date: 7/17/64.

1b Dark field photomicrograph of oriented outgrowth between several chick cardiac explants cultured with submaxillary protein. Incubation time: 27 hr. Culture medium: Simms. Additive: 15 µl of MMSCH, Area A of the G-25 fractionation (Fig. 1a). Explants beating. Culture date: 7/17/64.

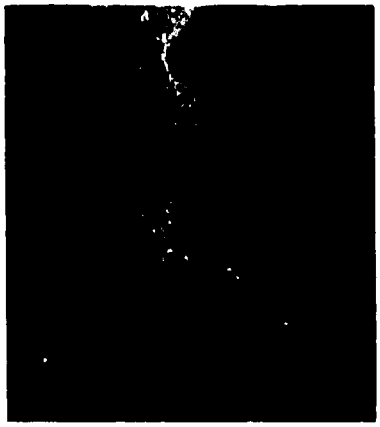
2a Phase contrast photomicrograph of radial outgrowth between chick cardiac explants cultures with protein represented by Peak I. Incubation time: 15 hr. Culture medium: Simms. Additive: 30 µl of MMSCH, purified by ammonium sulfate precipitation and fractionated on Sephadex G-100 (Fig. 9a). Explants beating. Culture date: 2/26/66.

2b Phase contrast photomicrograph of oriented outgrowth between chick cardiac explants cultured with protein represented by Peak IV. Incubation time: 15 hr. Culture medium: Simms. Additive: 30 µl of MMSCH, purified by ammonium sulfate precipitation and fractionated on Sephadex G-100 (Fig. 9a). Explants beating. Culture date: 2/26/66.

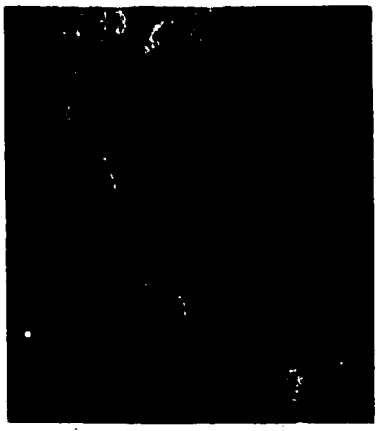
3a Phase contrast photomicrograph of radial outgrowth between chick cardiac explants in a control culture. Incubation time: 15 hr. Culture medium: Simms. Additive: 11 µl of water. Explants beating. Culture date: 2/18/66.

3b Phase contrast photomicrograph of oriented outgrowth between chick cardiac explants cultured with protein represented by Peak D_2. Incubation time: 15 hr. Culture medium: Simms. Additive: 11 µl of water + 44 µg of reconstituted lyophilized protein from the MMSCH purified on Sephadex G-200 (Fig. 5d). Explants beating. Culture date: 2/18/66.

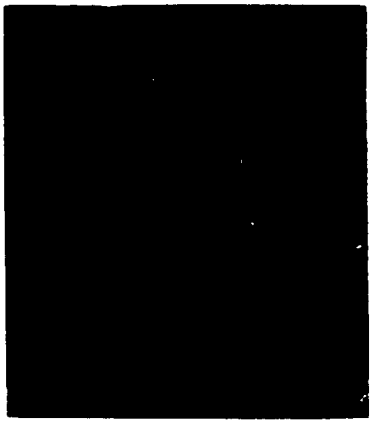
PLATE I



1a



1b



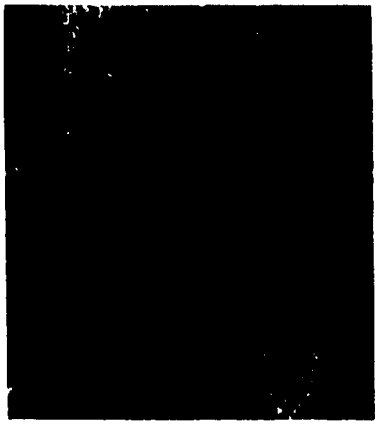
2a



2b



3a



3b

TABLE XXVII

ASSAY OF MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE FRACTIONATIONS

MATERIAL ADDED TO EXPERIMENTAL CARDIAC CULTURES			CULTURE DATE	NO. OF CULTURES OBSERVED	TOTAL NO.	OUTGROWTH PATTERN
Amount (µl)	Sephedex Type	Fraction[1]				
60	G-25	A of 11/63	12/31/63	4	4	No outgrowth
15	G-25	A of 11/63	1/22, 3/31, 4/7/65 and routinely (See Table XLIV)	2, 1, 3		Oriented
1.5	G-25	A of 11/63	11/23/64	2	2	Radial
5	G-25	A of 11/63	11/23/64	2	2	Radial
150	G-25	A of 11/63	11/23/64	2	2	No outgrowth
15	G-25	A of 7/64	7/17,23/64	2, 2	4	Oriented
15	G-25	B of 7/64	7/23/64	2	2	Radial
15	G-25	1	7/16, 17, 23/64	2. 2. 2.	6	Radial
15	G-75	3	7/16, 17, 23/64	2, 1, 2	5	Oriented
15	G-75	8	7/25/64	2	2	Radial
15	G-75	15 & 16	7/25/64	2	2	Radial

[1] See Table I and Figures 1a and 1b.

TABLE XXVII--Continued

MATERIAL ADDED TO EXPERIMENTAL CARDIAC CULTURES			CULTURE DATE	NO. OF CULTURES OBSERVED	TOTAL NO.	OUTGROWTH PATTERN
Amount (µl)[1]	Sephadex Type	Fraction[2]				
15	G-100	1 of 8/65	9/2/65	1	1	Radial
30	G-100	1 of 8/65	9/2, 23/65	1, 1	2	Radial
15	G-100	2 of 9/65	9/2/65	1	1	Radial
30	G-100	2 of 9/65	9/2, 23/65	1, 2	3	Radial
15	G-100	3 of 8/65	9/2/65	1	1	Radial
30	G-100	3 of 8/65	9/2, 23/65	1, 2	3	Radial
15	G-100	4 of 8/65	9/2/65	1	1	Oriented
30	G-100	4 of 8/65	9/2, 16, 23/65	1, 1[3], 2	4	Oriented
45	G-100	4 of 8/65	9/16/65	1[3]	1	Oriented
15	G-100	5 of 8/65	9/2/65	1	1	Radial
30	G-100	5 of 8/65	9/2, 9/23, 10/16/65	1, 2, 2	5	Radial

[1]
[2]Fraction concentrated once with Sephadex G-25.
[2]See Fig. 2a.
[3]Chick Pancreas tissue.

TABLE XXVII--Continued

MATERIAL ADDED TO EXPERIMENTAL CARDIAC CULTURES			CULTURE DATE	NO. OF CULTURES OBSERVED	TOTAL NO.	OUTGROWTH PATTERN
Amount (µl)	Sephadex Type	Fraction				
15	G-100	I of Fig. 9a	2/18/66, 2/28/66	1, 1	2	Radial
30	G-100	I of Fig. 9a	2/18, 2/26/66	1, 1	2	Radial
15	G-100	IV of Fig. 9a	2/18, 2/26/66	1, 1	2	Oriented
30	G-100	IV of Fig. 9a	2/18/2/26/66	1, 1	2	Oriented
15	G-100	I of Fig. 9b	2/26/66	1	1	Radial
15	G-100	IV of Fig. 9b	2/26/66	1	1	Oriented
15[1]	G-200	C of Fig. 5a	9/23, 10/16/65	2, 2	4	Radial
15[1]	G-200	D of Fig. 5a	9/23, 10/16/65	2, 1	3	Oriented
11[2]	G-200	D_2 of Fig. 5d	2/18/66	2	2	Oriented

[1]Effluent concentrated twice with Sephadex G-25.
[2]Effluent desalted and lyophilized; each µl contained 4 µg of the protein.

PLATE II

THE EFFECT OF THYMUS AND MUSCLE EXTRACTS UPON OUTGROWTH PATTERN

1a Phase contrast photomicrograph of radial outgrowth between chick cardiac explants in a control culture. Incubation time: 15 hr. Culture medium: Simms. Additive: None. Explants beating. Culture date: 6/15/64.

1b Phase contrast photomicrograph of oriented outgrowth between cardiac explants cultured with thymus protein. Incubation time: 26 hr. Culture medium: Simms. Additive: 15 µl of MMTCH, Area A of the G-25 fractionation (Fig. 11a). Explants beating. Culture date: 7/16/64.

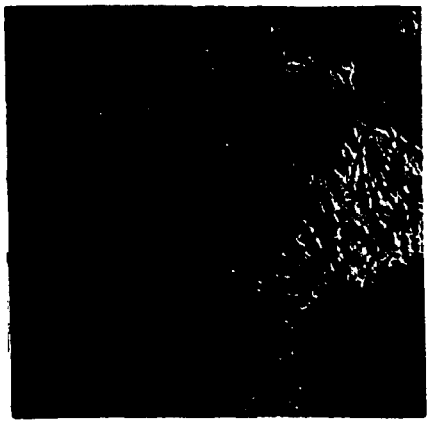
2a Phase contrast photomicrophotograph of radial outgrowth between chick cardiac explants cultured with Fraction 3, thymus protein. Incubation time: 20 hr. Culture medium: Simms. Additive: 45 µl of MMTCH purified on Sephadex G-100 (Fig. 11b). One explant beating. Culture date: 3/4/66.

2b Phase contrast photomicrograph of oriented outgrowth between cardiac explants cultured with Fraction 6, thymus protein. Incubation time: 20 hr. Culture medium: Simms. Additive: 45 µl of MMTCH purified on Sephadex G-100 (Fig. 11b). One explant beating. Culture date: 3/4/66.

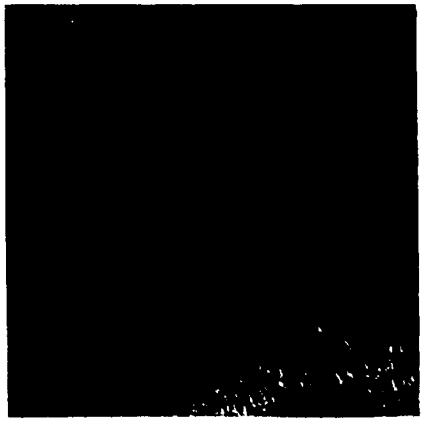
3a Phase contrast photomicrograph of radial outgrowth between cardiac explants cultured with Fraction 27, thymus protein. Incubation time: 20 hr. Culture medium: Simms. Additive: 60 µl of MMTACH purified on Sephadex G-100 (Fig. 11b). Explants beating. Culture date: 3/4/66.

3b Phase contrast photomicrograph of radial outgrowth between chick cardiac explants cultured with muscle extract. Incubation time: 15 hr. Culture medium: Simms. Additive: 15 µl of MMCH, Area A of the G-25 fractionation (Fig. 10a). Explants beating. Culture date: 7/17/64.

PLATE II



1a



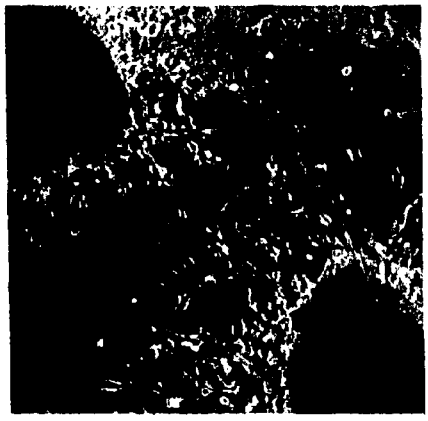
1b



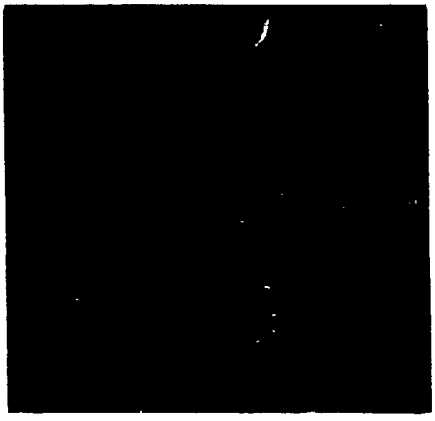
2a



2b



3a



3b

TABLE XXVIII

ASSAY OF MALE MOUSE THYMUS AND MUSCLE CRUDE HOMOGENATE FRACTIONATIONS

MATERIAL ADDED TO EXPERIMENTAL CARDIAC CULTURES			CULTURE DATE	NO. OF CULTURES OBSERVED	TOTAL NO.	OUTGROWTH PATTERN
Amount (µl)	Sephadex Type	Fraction				
	THYMUS EXTRACT					
15	G-25	A of Fig. 11a	7/16, 17, 23/64	2, 2, 2,	6	Oriented
30	G-100	3 of Fig. 11b	2/26/66	1	1	Radial
45	G-100	3 of Fig. 11b	2/26, 3/4/66	1, 1,	2	Radial
60	G-100	3 of Fig. 11b	3/4/66	1	1	Radial
45	G-100	6 of Fig. 11b	3/4/66	1	1	Oriented
60	G-100	6 of Fig. 11b	3/4/66	1	1	Oriented
30	G-100	27 of Fig. 11b	2/26/66	1	1	Radial
45	G-100	27 of Fig. 11b	2/26, 3/4/66	1, 1	2	Radial
60	G-100	27 of Fig.11b	3/4/66	1	1	Radial
	MUSCLE EXTRACT					
15	G-25	A_1 of Fig. 10a	7/16, 17, 23/64	2, 2, 2	6	Radial

TABLE XXIX

WEIGHT ESTIMATION OF PROTEIN IN 1 ML OF EXTRACT, AREA A OF G-25 FRACTIONATION, NOVEMBER, 1963

DATE	WEIGHT (grams)		DATE	WEIGHT (grams)	
	Membrane A	Membrane B		Membrane A + 1 ml of extract	Membrane B
9/ 8/65	0.59450	0.61172	11/29/64	0.58827	0.60324
9/10/65	0.59475	0.61240	11/30/64	0.58825	0.60340
9/20/65	0.59382	0.61190	11/30/64	0.58837	0.60370
9/27/65	0.59400	0.61180	12/ 1/64	0.58823	0.60338
9/30/65	0.59376	0.61172	12/ 2/74	0.58828	0.60341
AVERAGE	0.59417	0.61191	AVERAGE	0.58828	0.60343
			4/11/65	0.59000	0.60450
			4/12/65	0.59002	0.60449
			4/23/65	0.59009	0.60500
			4/24/65	0.59001	0.60515
			4/27/65	0.59007	0.60500
			AVERAGE	0.59004	0.60483

	CALCULATION A -- 11/29 to 12/2	CALCULATION B -- 4/11 to 4/27
Correction factor:	0.61191 - 0.60343 = 0.00848	0.61191 - 0.60483 = 0.00708
Empty membrane weight: (corrected)	0.59417 - 0.00848 = 0.58769	0.59417 - 0.00708 = 0.58709
Wt. of protein in 1 ml:	0.58828 - 0.58569 = 0.00259	0.59003 - 0.58709 = 0.00294
	approximately 3 mg/ml	approximately 3 mg/ml

the average angle of the axes of the emerging cells was observed with an increase from 11 µg to 20 µg or from 27 µg to 45 µg of the added protein. In the range between 20 µg and 27 µg, increase in the amount of added protein resulted in marked reduction of the average axis angles of the outgrowing cells from +17° to 0°. At the higher concentrations of the extract, therefore, the axes of the outgrowing fibroblast-shaped cells in the zone where the angles were measured were almost parallel with the midline between the two explants. At all concentrations of the extract the average slope of the cell axes was less for the experimental cultures than for the controls (Fig. 12; Tables XXX-XXXVIII). Plate III shows the cellular outgrowth pattern in the cultures which contained 20 µl, 27 µl, or 45 µl of water in the control cultures or the same amount of protein extract in the experimental cultures.

Molecular Weight Estimation of the Submaxillary Outgrowth Orienting Protein

The void volume of four of the Sephadex G-100 gel beds used, determined by the application of a 0.2% solution of Blue Dextran, was 26.8 ml (Fig. 13; Table XXXIX), 32.8 ml (Fig. 14; Table XL), 33.4 ml (Fig. 13; Table VII), and 34.4 ml (Fig. 14; Table XXI). The relation of the elution volume to the void volume (V_e / V_o) was 1.41 and 1.35 ($\frac{37.9}{26.8}$, $\frac{44.4}{32.8}$) for bovine serum albumin, 1.07 and 1.08 ($\frac{28.8}{26.8}$, $\frac{35.1}{32.8}$) for bovine serum albumin dimer, and 2.74 and 2.34 ($\frac{74.8}{26.8}$, $\frac{76.8}{35.8}$) for trypsin (Fig. 13 and 14; Tables XXXIX and XL). This same relationship for a sample of MMSCH was 1.97 ($\frac{65.8}{33.4}$) and for a sample of the ammonium sulfate purified MMSCH was 2.01 ($\frac{69.0}{34.4}$). The V_e/V_o values for the

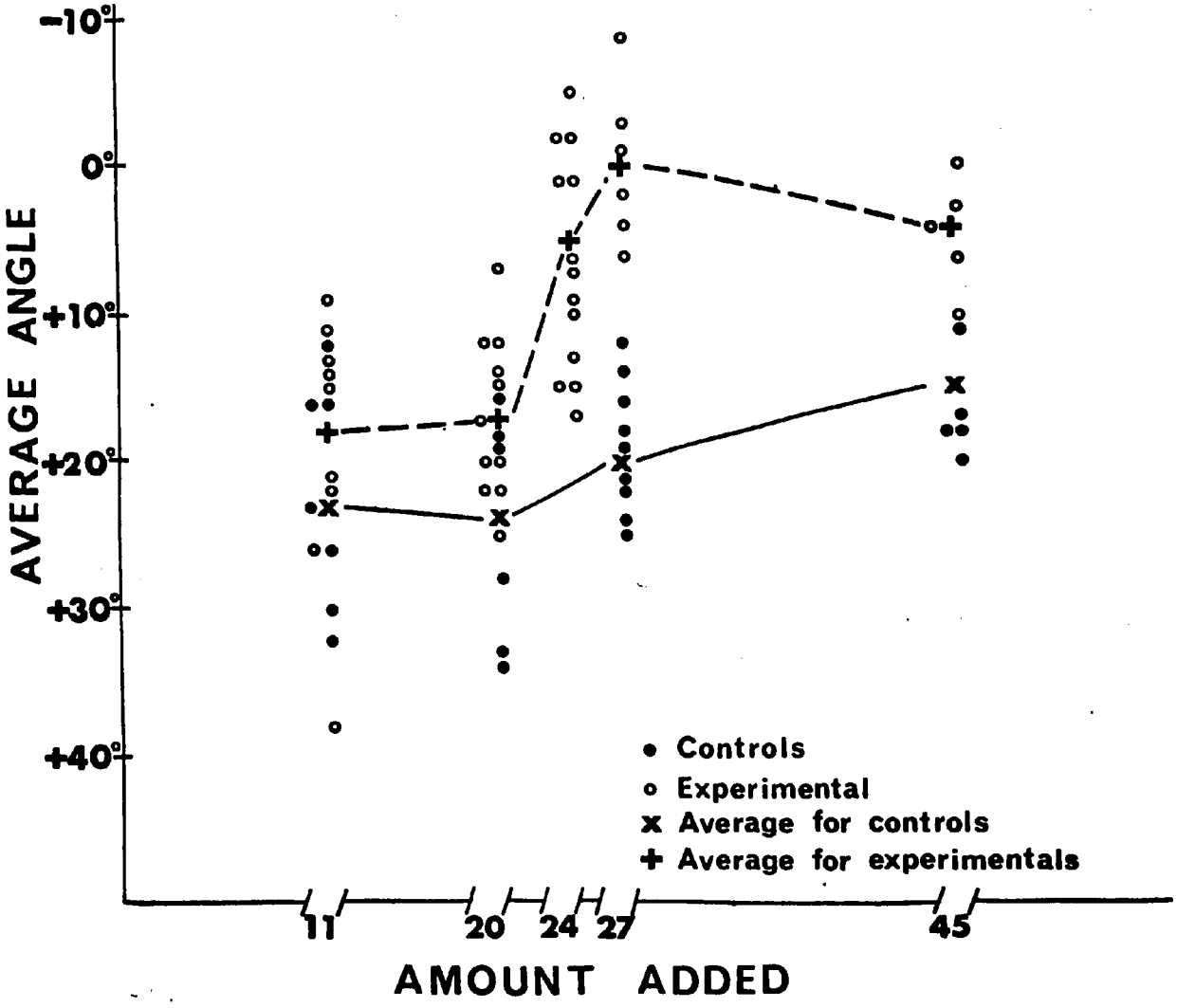


FIGURE 12: Change in the Average Angle of the Axes of Cells with the Midline as the Concentration of Submaxillary Protein Varied. In the control cultures the indicated amount of water, in µl, was added to the 2.5 ml of medium in the slide. In the experimental cultures the indicated amount of lyophilized protein represented by Area A of the G-25 gel fractionation, in µg, was added to the 2.5 ml of medium in the slide.

PLATE III

THE EFFECT OF VARIOUS CONCENTRATIONS OF SUBMAXILLARY EXTRACT UPON OUTGROWTH PATTERN

1a Phase contrast photomicrograph of radial outgrowth between chick cardiac explants in a control culture. Incubation time: 15 hr. Culture medium: Simms. Additive: 20 µl of water. One explant beating. Date: 2/18/66.

1b Phase contrast photomicrograph of radial outgrowth between chick cardiac explants cultured with submaxillary protein. Incubation time: 15 hr. Culture medium: Simms. Additive: 20 µl of water + 20 µg of lyophilized MMSCH, Area A of the G-25 fractionation (Fig. 1a). Explants beating. Culture date: 2/18/66.

2a Phase contrast photomicrograph of radial outgrowth betwee chick cardiac explants in a control culture. Incubation time: 15 hr. Culture medium: Simms. Additive: 27 µl of water. One explant beating. Culture date: 2/18/66.

2b Phase contrast photomicrograph of oriented outgrowth between chick c ardiac explants cultured with submaxillary protein. Incubation time: 15 hr. Culture medium: Simms. Additive: 27 µl of water + 27 µg of lyophilized MMSCH, Area A of the G-25 fractionation (Fig. 1a). Explants beating. Culture date: 2/18/66.

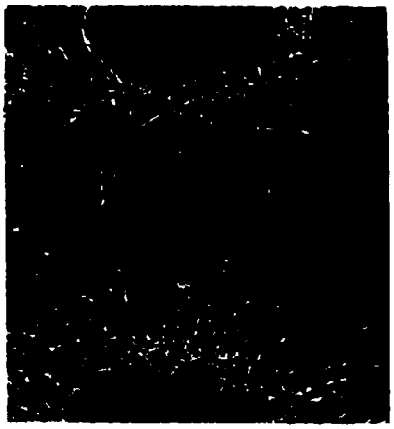
3a Phase contrast photomicrograph of radial outgrowth between chick cardiac explants in a control culture. Incubation time: 15 hr. Culture medium: Simms. Additive: 45 µl of water. Explants beating. Culture date: 2/18/66.

3b Phase contrast photomicrograph of oriented outgrowth between chick cardiac explants cultured with submaxillary protein. Incubation time: 15 hr. Culture medium: Simms. Additive: 45 µl of water+ 45 µg of lyophilized MMSCH, Area A of the G-25 fractionation (Fig. 1a). Explants beating. Culture date: 2/18/66.

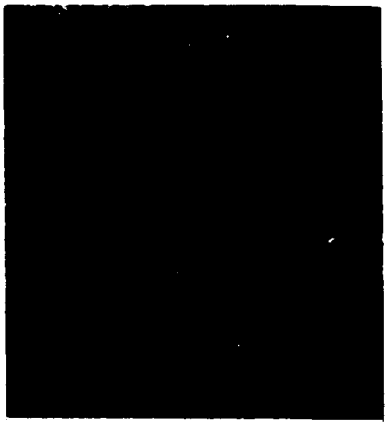
PLATE III



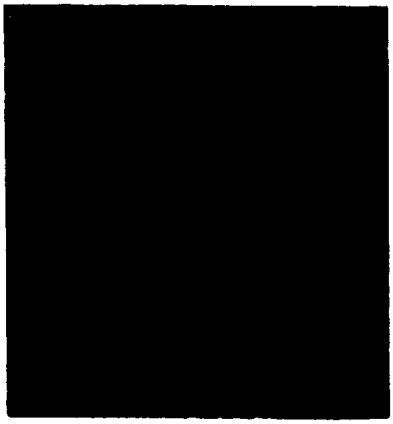
1a



1b



2a



2b



3a



3b

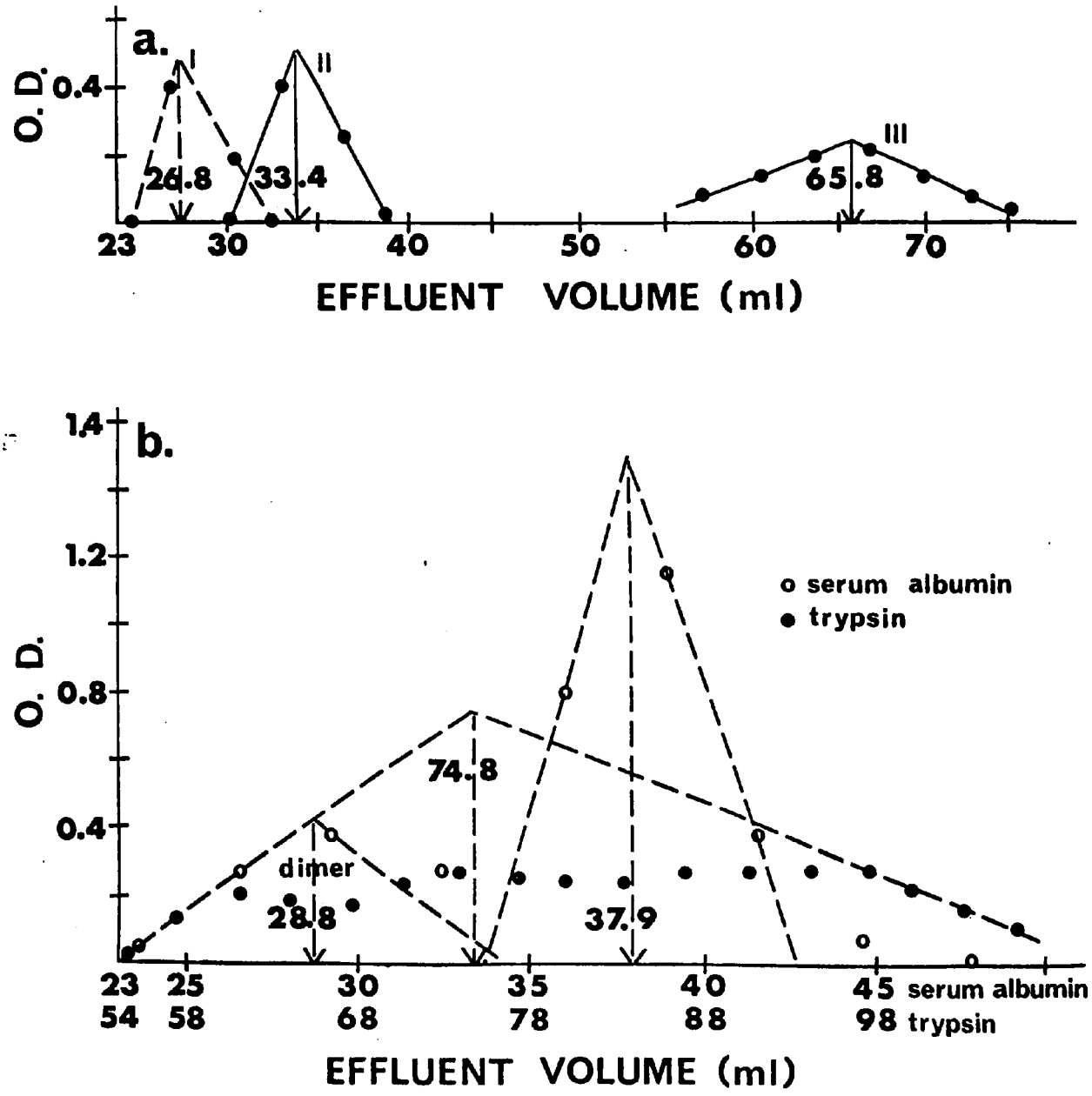


FIGURE 13: Elution Volumes of Blue Dextran, Peak IV from Male Mouse Submaxillary Crude Homogenate, Trypsin, and Bovine Serum Albumin. G-100 gel, Column I, 0.5 ml sample volumes, and 0.02 M sodium phosphate buffer, pH 6.5, were used.

a. Elution volumes of Blue Dextran and Peak IV from MMSCH. a-I = void volume for the gel bed used for trypsin and bovine serum albumin. a-II = void volume for the gel bed used for MMSCH. a-III = Peak IV from MMSCH; flow rate, 0.4 ml/min; gel height, 40.5 cm.

b. Elution volume of 10 mg trypsin and 12.5 mg bovine serum albumin. Flow rate: 0.2 ml/min.

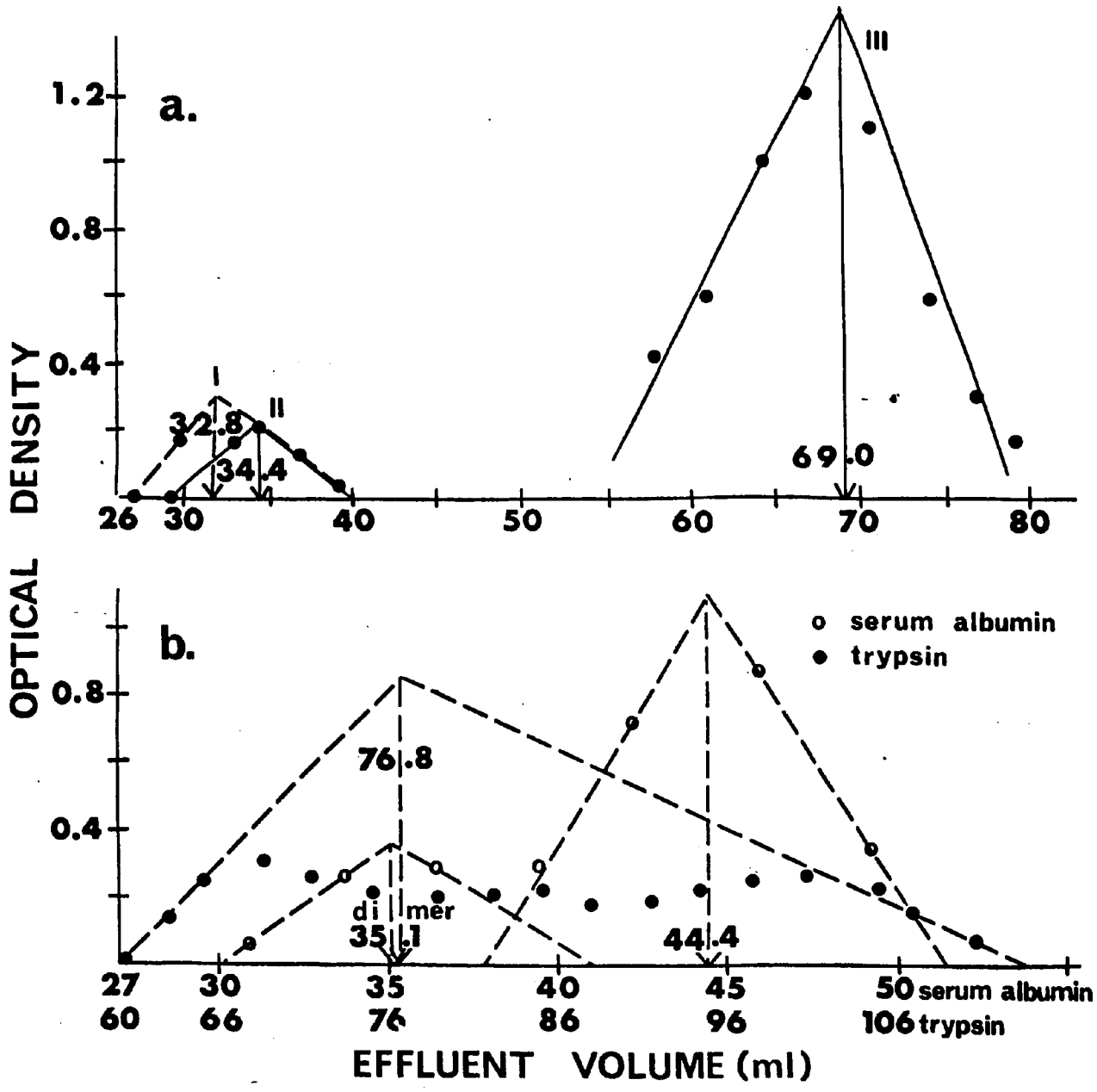


FIGURE 14: Elution Volumes of Blue Dextran, Peak IV from Ammonium Sulfate Purified Male Mouse Submaxillary Crude Homogenate, Trypsin, and Bovine Serum Albumin. G-100 gel, Column I, 0.5 ml sample volumes, and 0.02 M sodium phosphate buffer, pH 6.5, were used.

a. Elution volumes of Blue Dextran and Peak IV from ammonium sulfate purified MMSCH. a-I = void volume for the gel bed used for trypsin and bovine serum albumin. a-II = void volume for the gel bed used for ammonium sulfate purified MMSCH. a-III = Peak IV from ammonium sulfate purified MMSCH; flow rate, 0.3 ml/min; gel height, 45.6 cm.

b. Elution volume of 10 mg trypsin and 12.5 mg bovine serum albumin. Flow rate: 0.5 ml/min. Gel height: 45.1 cm.

proteins of known molecular weight are plotted against the common logarithm of their molecular weight in Fig. 15. From the straight line drawn between these points, the log of the molecular weight of the outgrowth orienting protein is 4.61. Therefore, the molecular weight is approximately 41,000.

Lability of the Submaxillary Outgrowth Orienting Protein

Activity of the outgrowth orienting protein was destroyed by heating the protein in phosphate buffered solution to its boiling point. The orienting factor was also inactivated by an exposure to 0.1 N NaoH or to 0.1 N HCl for 1 hr (Table XLI).

Outgrowth Response between Explants from Tissues other than Chick Cardiac to the Outgrowth Orienting Protein

Explants of embryonic mouse ventricle or embryonic chick kidney, liver, gut, limb, or pancreas tissue were grown in plasma clots. Fifteen µl of the submaxillary extract represented by Area A of the G-25 fractionation were added to the experimental cultures. Outgrowth between the explants was observed and photomicrographed. Radial outgrowth was observed in the control cultures of these various issues. Oriented outgrowth was observed in the experimental cultures (Table XLII). The processes from nerve tissue explants were also observed and photographed in control and experimental cultures. This study is summarized in Table XLIII. Radial outgrowth was observed in both the control and in the experimental cultures. Plate IV shows representative microphotographs of the cellular outgrowth from pancreas, gut, and nerve explants.

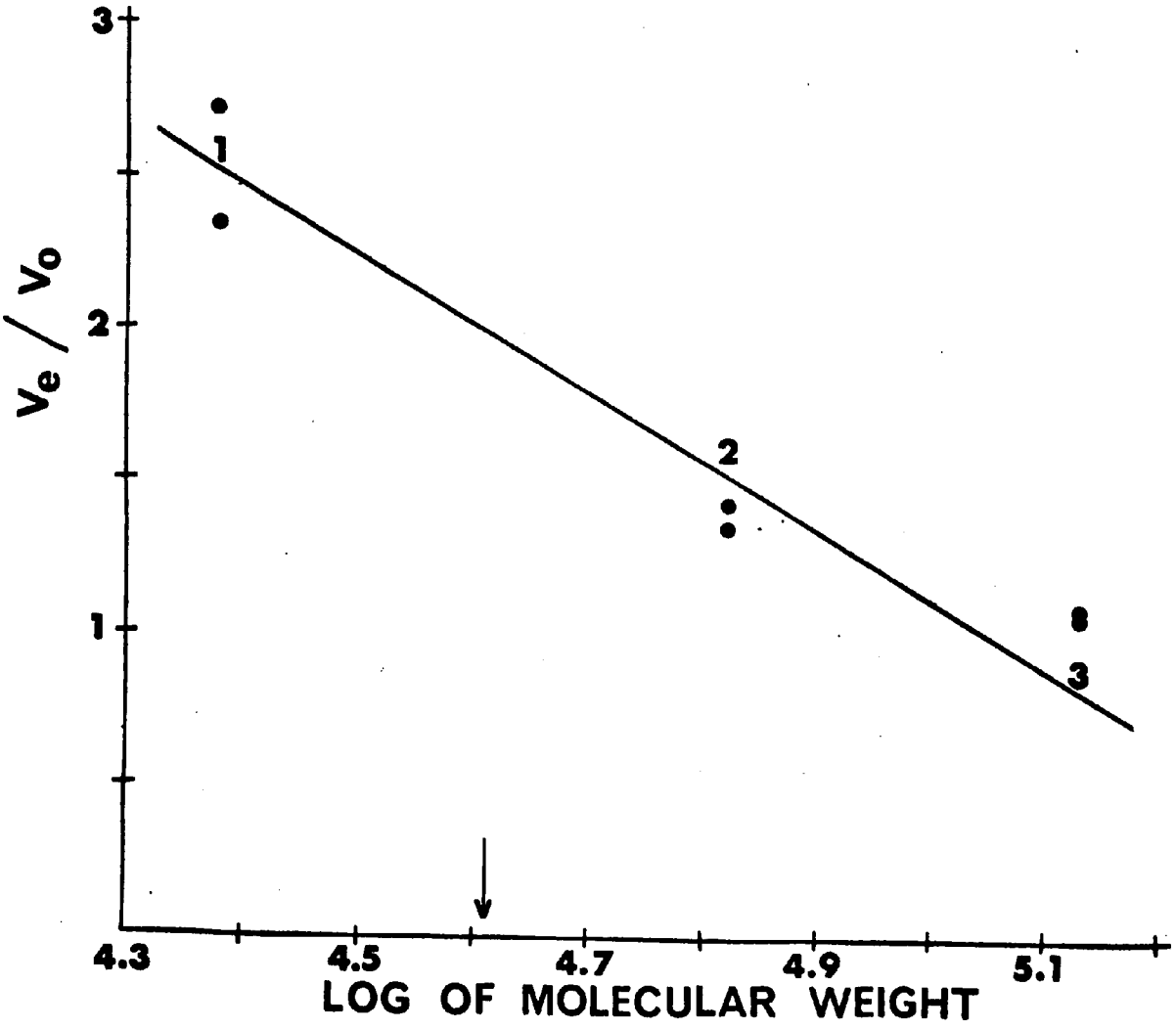


FIGURE 15: The Relation between V_e / V_o of Proteins and their Molecular Weight. 1. Log of mol. wt. of trypsin, 23,800 (Cunningham, 1954) = 4.38; V_e / V_o = 2.74 and 2.34. 2. Log of mol. wt. of bovine serum albumin, 67,000 (Loeb and Scheraga, 1956) = 4.83; V_e / V_o = 1.42 and 1.35. 3. Log of mol. wt. of bovine serum albumin dimer, 134,000 (Sogami and Foster, 1962) = 5.13; V_e / V_o = 1.08 and 1.07. V_e / V_o for Peak IV of MMSCH = 1.97 and for Peak IV of ammonium sulfate purified MMSCH = 2.01. 4.61 = log of 40,800.

TABLE XLI

LABILITY OF THE OUTGROWTH ORIENTING PROTEIN

CULTURE DATE	EXTRACT	CONTROL CULTURES		CONDITION OF THE MMSCH IN EXPERIMENTAL CULT.			
		Normal	15 µl 0.1 N NaOH	Normal	Heated to 100°C	Exposed to 0.1 N NaOH	Exposed to 0.1 N HCl
5/12/64	A of G-25[1]	2[2] - R[3]		1 - 0	2 - R		
6/ 3/64	A of G-25	2 - R		2 - 0	3 - R		
6/ 8/64	A of G-25	1 - R		2 - 0	2 - R		
6/10/64	A of G-25	2 - R	2 - R	2 - 0	2 - R	2 - R	
7/23/64	A of G-25	2 - R					2 - R
2/19/66	D_2 of G-200[4]	2 - 0				2 - R	

[1] MMSCH, Area A of the G-25 fractionation of 11/63 (Fig. 1a); 15 µl added to culture

[2] Number of slides observed

[3] Pattern of the outgrowth: R = Radial; 0 = Oriented.

[4] MMSCH, D_2 (Fig. 5d); 45 µg of protein in 11 µl of water added to culture.

TABLE XLII

SUMMARY OF THE NUMBER OF SLIDES CULTURED CONTAINING EXPLANTS OTHER THAN CHICK CARDIAC OR NERVE

CULTURE DATE	NUMBER OF SLIDES OBSERVED													
	Chick Tissue												Mouse Tissue	
	Ventricle		Kidney		Liver		Gut		Limb		Pancreas			
	C*	E**	C*	E**	C*	E**	C*	E**	C*	E**	C*	E**	C*	E**
4/ 7/64	3	3							3	1				
6/ 8/64	1	2											2	2
6/10/64	2	2											3	2
6/15/64	2	2			2	1	1	2	2	2				
6/17/64	1	1	2	2	2	2	2	2	2	2				
6/24/64	2	2	3	3	2	2	2	2	2	2				
7/ 7/64											2	2		
5/11/65											2	2		
9/16/65											2	2***		
	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Totals	11	12	5	5	6	5	5	6	9	7	6	6	5	4

* Control cultures--outgrowth pattern radial.

** Experimental cultures contained 15 µl MMSCH, Area A of G-25 fractionation of 11/63, Fig. 1a--outgrowth pattern oriented.

*** Experimental cultures contained 30 or 45 µl of purified MMSCH, Fraction 4 of G-100 fractionation of 8/65, Fig. 2a, concentrated once with Sephadex G-25--outgrowth pattern oriented.

TABLE XLIII

SUMMARY OF CHICK NERVE TISSUE CULTURES

Culture Date	Age of Embryo (days)	Type of Tissue	No. of Control Cultures	Experimental Cultures	
				Amt. of Extract* (µl)	No. of Cultures
6/22/64	7	Brain Stem	1	15	1
6/24/64	9	Brain Stem	3	15	3
7/ 1/64	7	Brain Stem	3	15	2
7/23/64	7	Brain Stem & Spinal Cord	2	5	1
				15	1
7/30/64	7	Spinal Cord	1	5	1
				10	1
				15	1
11/23/64	7	Brain Stem & Spinal Cord	2	1.5	2
				5	2
				15	2
				150	2
12/ 1/64	15	Spinal Cord	2	5	2
				15	2
4/ 1/64	7	Brain Stem	2	5	2
				15	2
4/ 1/64	14	Spinal Cord	2	15	2
5/ 4/65	8	Spinal Cord	1	5	1
				10	1
5/ 4/65	8	Brain Stem	2	5	1
				10	2
5/11/65	15	Spinal Cord	2	5	2
				10	2
				15	2

* MMSCH, Area A of G-25 fractionation of 11/63

PLATE IV

PATTERN OF OUTGROWTH BETWEEN PANCREAS, GUT, AND NERVE EXPLANTS

1a Phase contrast photomicrograph of radial outgrowth between chick pancreas explants in a control culture. Incubation time: 15 hr. Culture medium: Simms. Additive: None. Culture date: 7/7/64.

1b Phase contrast photomicrograph of oriented outgrowth between chick pancreas explants cultured with submaxillary protein. Incubation time: 15 hr. Culture medium: Simms. Additive: 15 µl of MMSCH, Area A of the G-25 fractionation (Fig. 1a). Culture date: 7/7/64.

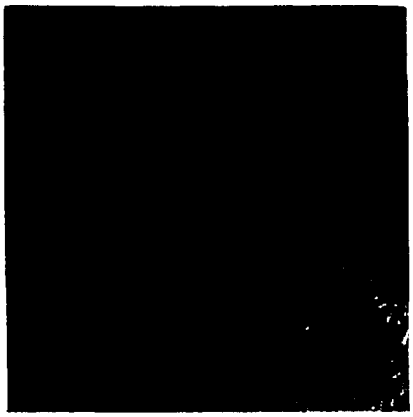
2a Phase contrast photomicrograph of radial outgrowth between chick gut explants in a control culture. Incubation time: 15 hr. Culture medium: Simms. Additive: None. Culture date: 6/15/64.

2b Phase contrast photomicrograph of oriented outgrowth between chick gut explants cultured with submaxillary protein. Incubation time: 15 hr. Culture medium: Simms. Additive: 15 µl of MMSCH, Area A of the G-25 fractionation (Fig. 1a). Culture date: 6/24/64.

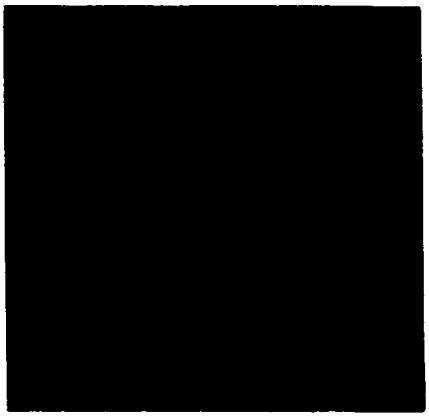
3a Phase contrast photomicrograph of oriented outgrowth between chick spinal cord explants in a control culture. Incubation time: 24 hr. Culture medium: Simms. Additive: None. Culture date: 7/1/64.

3b Phase contrast photomicrograph of radial outgrowth between chick spinal cord explants cultured with submaxillary protein. Incubation time: 24 hr. Culture medium: Simms. Additive: 15 µl of MMSCH, Area A of the G-25 fractionation (Fig. 1a). Culture date: 7/1/64.

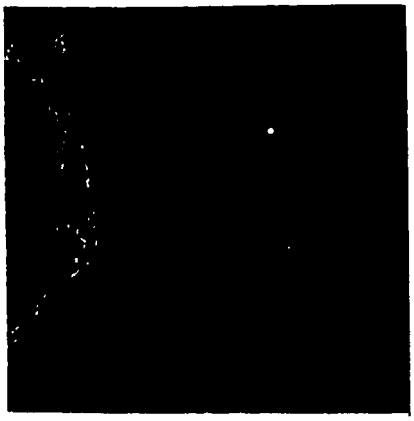
PLATE IV



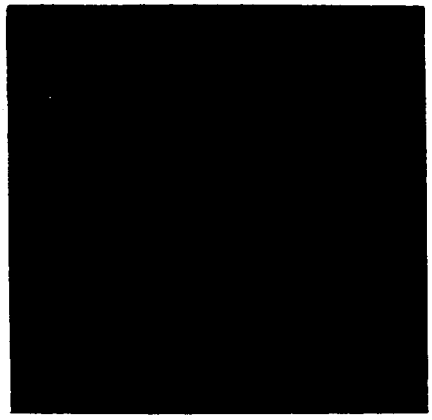
1a



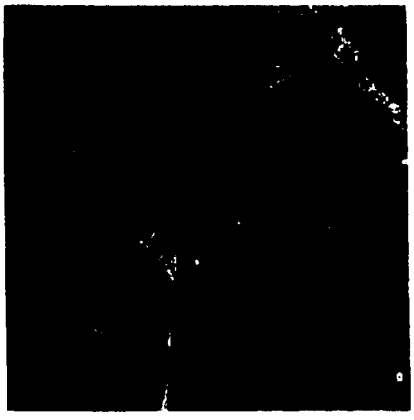
1b



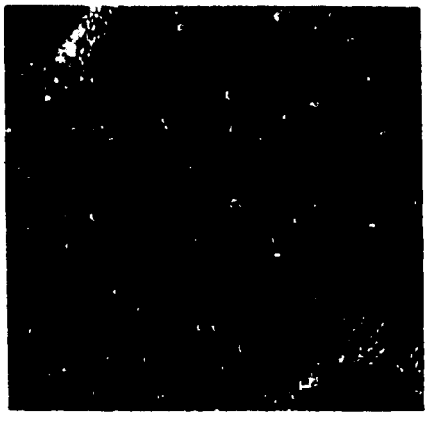
2a



2b



3a



3b

Effect of Different Kinds of Medium on the Action of the Outgrowth Orienting Protein

Oriented outgrowth was observed in the experimental cultures which contained MMSCH when various kinds and combination of culturing medium were used. Besides the usual medium composed of 80% Simms salt solution and 20% embryo extract, oriented outgrowth was also observed in the 4 experimental culture slides which contained 2 ml of Simms solution and 5 drops of horse serum, in the 7 which contained 2 ml of Simms solution and 5 drops of agamma horse serum, and in the 2 which contained 70% Hanks solution, 10% Tissue Culture Medium #199, and 20% agamma horse serum. Radial outgrowth was observed in the same number of control cultures which contained one of the various types of medium but no male mouse submaxillary gland extract (Table XLIV).

Monolayer Outgrowth Response to the Orienting Protein

Oriented outgrowth was not observed in the experimental cultures, which contained the usual amount (15 µl/2.5 ml of culture medium) of the submaxillary extract represented by Area A of the G-25 fractionation, when the outgrowth from the explants was a monolayer of cells adhering to a glass or plastic surface. Nine control and nine experimental culture slides, cultured on 6 different days, were observed and photographed. The culture medium consisted of 70% Hanks solution, 10% Tissue Culture Medium #199, and 20% agamma horse serum. Double the amount of medium and 30 µl of extract was used on two different occasions in a total of four 30 ml plastic flasks, but oriented outgrowth was never observed in these flasks although monolayer outgrowth flourished around the 40-60

TABLE XLIV

TYPES OF CULTURE MEDIA USED FOR CHICK CARDIAC EXPLANTS

CULTURE MEDIUM	CULTURE DATE	NO. OF SLIDES OBSERVED	EXTRACT* ADDED	PATTERN OF OUTGROWTH
Simms solution + horse serum	3/31/64	4 4	None 15 µl	Radial Oriented
Simms solution + agamma horse serum	3/3/; 4/7; 5/12/64	4, 2, 1 4, 2, 1	None 15 µl	Radial Oriented
Hanks solution + 199 + agamma horse serum	7/1; 7/25/64	1, 1 1, 1	None 15 µl	Radial Oriented
Simms solution + embryo extract	Routinely 1/22; 3/31/ 4/7;5/12;	2, 4, 3, 2, 2, 1, 3, 1	None 15 µl	Radial Oriented
	6/8; 10, 15, 17, 24;	1, 2, 2, 1, 2 2, 2, 2, 1, 2	None 15 µl	Radial Oriented
	7/16, 17, 23; 11/23/64;	2, 2, 2, 2 2, 2, 2, 2	None 15 µl	Radial Oriented
	4/1; 9/2, 23; 10/16/65	2, 2, 2, 2 2, 2, 2, 2	None 15 µl	Radial Oriented

* MMSCH, Area A of G-25 fractionation of 11/63 (Fig. 1a)

beating cardiac explants in each culture. The same number and type of control cultures (nothing added to the medium in these cultures) were also observed. The outgrowth pattern in the control cultures was similar to that in the experimental cultures.

CHAPTER IV

DISCUSSION

Heat lability, water solubility, absorption of light of 280 mμ, lability to strong acid and base, large molecular weight, and electrophoretic behavior support the statement that the *in vitro* outgrowth orienting factor studied in this work is a protein. The solubility of the outgrowth orienting protein in pure water indicated that it was an albumin. This solubility was predicted because the salts present within the tissue during the preparation of the crude homogenate material and the salts of the 0.02 M sodium phosphate buffer were removed on G-25 gel columns which were packed and washed with glass distilled water. The solubility of the outgrowth orienting protein in pure water was confirmed by dissolving the lyophilized material in pure water and the outgrowth orienting activity was retained.

The detection of the amount of proteins spectrophotometrically at the wave length of 280 mμ is a very sensitive method with the advantage that the sample is not destroyed. However, the absorption of light is due entirely to the tyrosine, tryptophan, and phenylalanine present. The amount of light absorbed at a wave length of 280 mμ may vary by a factor of 5 or more for equal concentrations of proteins since their amino acid composition may differ considerably (Sober, *et al.*,

1965). Therefore, the amount of absorption indicated by the various heights of the different peaks of the elution curve for a sample is not an indication of the absolute amount of the protein present. A concentration curve specific for each peak is necessary before absorbance at a wave length of 280 mμ can be related to the amount of protein present. The outgrowth orienting protein was not sufficiently purified to make a study of the amino acid composition profitable.

The molecular weight of the outgrowth orienting protein was determined by gel filtration. Whitaker (1963) obtained an excellent linear relation between the common logarithm of the molecular weight and the ratio of the elution volume (V_e) to the void volume (V_o). Calibration of Column I was done with samples of bovine serum albumin and trypsin. Two peaks were obtained from the sample of bovine serum albumin. The detection by elution of a dimer in bovine serum albumin was not mentioned by Whitaker (1963) nor by Leach and O'Shea (1965), but was reported by Andrews (1964). Whitaker (1963) quoted Wagner and Scherage as reporting that bovine serum albumin is known to contain at least two proteins. Although trypsin presented a broad elution curve the excessive trailing was not due to improper packing of the column because the elution curve for Dextran Blue presented a narrow sharply rising and falling curve. The wide peak is probably due to the impurity of the trypsin since Hakim, et al., (1962) reported that both commercial beef and pork trypsin are heterogeneous.

The heights of the G-100 gel in the column varied but the V_e / V_o has been found to be a constant for each protein regardless of column size. The void volume determinations can be considered to be

accurate because Blue Dextran has an average molecular weight of 2,000,000 and would be totally excluded from the gel particles. No significant difference was reported in the V_e / V_o of proteins when the temperature varied a few degrees although a temperature effect was observed between 3.3°C and 25-27°C. Since the elution volumes were interpolated to the nearest 0.1 ml, the molecular weight is significant to three figures even though the void volume of the column used was less than 100 ml. Sometimes a second material was placed on the column before the first emerged (i.e., MMSCH sample was added before the Blue Dextran sample emerged) as the elution volume is not influenced by this procedure (Whitaker, 1963).

Electrophoresis was used to determine the number of dissimilarly charged proteins in some of the gel separated fractions. Concentration of the protein, one of the many critical parameters in electrophoresis, must be considered in this work since G-100 gel filtration in Column I diluted the 0.5 ml applied samples from 6.5 to 10.5 times. The amount of dilution depends upon the sample size, the void volume of the column, the column size, and the irregularities in the flow of the sample through the column. Fractions were concentrated by lyophilization and with dry Sephadex G-25 as the spaces within the gel particles permit water and salts to enter and the result is a concentration of the protein under conditions in which the pH and ionic strength of the solution remain constant (Beling, 1963). Although the practical concentration factor is only 2-5 times in each step, this is a very mild method and it can be repeated (Gelotte and Krantz, 1959).

Molecular sieve chromatography was used for purification as well as an analytical technique for establishing the molecular weight of the outgrowth orienting protein. The separations on the G-25 columns removed streptomycin sulfate, amino acids and small peptides. This treatment also divided the larger molecular weight material into the two fractions which are diagramed on Fig. 2a. The protein material represented by Area A contained the outgrowth orienting factor; that represented by Area B did not. The fractionation on the column packed with G-75 gel indicated that the molecular weight of the active material was large enough to warrant the use of gel particles with less cross-linking for the separation of the outgrowth orienting protein from the other proteins in the tissue extract.

The fractionation on the G-100 and G-200 gels not only removed the excess streptomycin sulfate, amino acids and small peptides but also separated the larger proteins. The shape of the elution peaks indicated the purity of the fractions on the basis of the molecular weight of the material (Whitaker, 1963), i.e., the shape of peak D in Fig. 5b indicated partial separation of the outgrowth orienting protein from other proteins since there were irregularities in its slope and it was asymmetrical. Separation was achieved between peaks D_2 and D_3 in Fig. 5d. The slope of peak D_3 was constant from base to apex and symmetrical. By using only the material represented by the indicated area under D_2 the incomplete separation of D_1 from D_2 can be disregarded because the fractions which contained mixtures of these two peaks were not used.

The most efficient method of obtaining the outgrowth orienting protein of the purity used in this work seems to be the following. Large

aliquots of the crude homogenate, such as those used on the G-25 gel, could be purified and desalted on G-25 gel equilibrated with distilled water. Small samples of the lyophilized protein could be applied in small volumes to a column of G-100 gel and the desired fraction selected from the effluent. The information from the fractionation on the G-200 gel, especially the separation of the material in peaks D_1 and D_3 from D_2 may be useful if an electrophoretically pure preparation of the orienting factor, obtained from a G-100 gel separation, is not possible on an ion-exchange column. The separation of D_3 from the outgrowth orienting protein present in D_2 represents the removal of material of a smaller molecular weight. No indication of such a separation was observed when the extract was fractionated on G-100 gel.

The variations which were used in the tissue culturing in the presence of the outgrowth orienting protein indicate the following. First, the orientation response in the tissue cultures does not appear to be due to an interaction between the components of the tissue culture medium since three combinations of media were used in addition to the usual combination of 20% embryo extract and 80% Simms salt solution. Oriented outgrowth was observed in experimental cultures and radial outgrowth was observed in the control cultures. Second, a three-dimensional medium seems to be necessary as oriented outgrowth is not observed when cells are grown on a plane surface of glass or plastic. Third, the results with the various kinds of embryonic tissues used--chick and mouse ventricle; chick kidney, liver, gut, pancreas, and skeletal muscle--indicate that the outgrowth orienting protein is not specific for cells from a particular organ. The shape of the oriented cells allow

classification as fibroblastic according to Willmer (1965). Although he states that it is usually difficult, if not impossible, to designate the class to which a cell belongs, e.g., epitheliocyte, mechanocyte (fibroblast), amoebocyte, by the shape of the cell, cell classification by shape may be made with some degree of accuracy during the early growth. All the tissue explants contained a mixed population of cells so that the 15-25 hr oriented outgrowth studied in this work may be composed mainly of mesenchymal cells.

The eighth type of tissue used, embryonic chick explants from the central nervous system, did not show oriented outgrowth. The pattern of the nerve processes around the tissue explants appeared to be the same in the control and the experimental cultures. This indicates that the outgrowth orienting protein is not orienting the fibers in the plasma clot since Weiss (1934) has shown that if the fibers in the clot are oriented, i.e., by tension, the nerve fibers in the outgrowth around the nerve tissue explant will be oriented. The many variations in the amount of the submaxillary gland extract added to the nerve cultures suggest that the absence of patterned outgrowth in these cultures is not due to too much or too little submaxillary gland extract.

Another variation used in the tissue culturing in the presence of the outgrowth orienting factor indicated that the amount of orientation is dependent upon the amount of protein added to the medium. Although the average angle which the cells made with the midline connecting the explants was always less in the experimental cultures than in the control cultures, the differences at the 11 µg and 20 µg levels were slight. In the range between 20 µg and 27 µg the amount of added

protein resulted in marked reduction of the average axis angles of the fibroblast-shaped cells. The marked reduction did not continue to become greater when the added protein was increased to 45 µg. The data presented in this study does not eliminate the possibility of a threshold response.

Cultures which contained 27 or 45 µg of the purified submaxillary gland protein showed oriented outgrowth. The control cultures did not exhibit oriented outgrowth. Since the distances between the explants in the former cultures averaged 0.48 mm (range of 0.13 mm to 0.75 mm) and the distances between the explants in the latter cultures averaged 0.36 mm (range of 0.18 mm to 0.67 mm) the outgrowth orientation observed in this study seems not to be due to the "two-centre" effect described by Weiss (1952). If the outgrowth orientation were due to the orientation of fibrin between the explants by the tension produced as a result of their growth one would expect the outgrowth pattern to be similar in all the cultures as the average distance and the range of the distance between the explants were similar.

The data presented here support the idea that the outgrowth orienting protein differs from the other proteins in the mouse submaxillary gland which have been studied. Attardi, et al., (1965) reported that the nerve growth factor was present in the protein represented by the first peak of the separation of mouse submaxillary extract, purified by precipitation with 40-70% ammonium sulfate, on Sephadex G-100 gel. The protein represented by the first elution peak, using the same preparation of submaxillary extract and the same buffer

(0.005 M Tris HCl, pH 7.2, Attardi, 1966) did not show cellular outgrowth orienting activity in the cardiac cultures (Fig. 12).

The elution curve reported by Attardi, et al., (1965) was similar to that obtained from the mouse submaxillary extract when a 0.02 M sodium phosphate buffer, pH 6.5, was used (Fig. 3 and 9). Therefore, the lack of orientation of outgrowth in the cultures which contained the protein represented by peak 3, Fig. 2a, also support the idea that the nerve growth stimulating protein does not mediate the orientation effect. The molecular weight of the nerve growth factor was estimated from ultracentrifugation by Cohen (1960) to be approximately 44,000. The molecular weight of the outgrowth orienting factor is estimated from gel filtration to be approximately 41,000. Although the molecular weight difference was not large, the nerve growth protein has the larger molecular weight and would be expected to emerge from the G-100 gel column before the outgrowth orienting protein. The nerve growth factor on the elution curve reported by Attardi, et al., (1965) stated that the nerve growth factor does emerge first.

Analysis of the proteins present in a sample of MMSCH purified by ethanol precipitation, which is the first step in the purification of the nerve growth factor, indicated that the nerve growth protein and the outgrowth orienting protein are different. When the ethanol purified sample was fractionated on G-100 gel, the relative differences in the amount of light absorbed by the aliquots of effluent indicated that proteins other than the outgrowth orienting protein were being concentrated (Fig. 7). The outgrowth orienting protein was present in the material represented by Peak IV and the nerve growth factor was present in the

proteins emerging first. When the ethanol purified sample of submaxillary extract was analyzed by electrophoresis, the relative differences in the stained bands of protein indicated that protein other than the outgrowth orienting protein were being concentrated (Fig. 8). At the concentration of the protein solution used, electrophoretic analysis of the outgrowth orienting factor (Peak IV of the G-100 fractionations) showed, besides a band at the origin, proteins which migrated as cathodal Band 3 and cathodal Band 4.(Fig. 4). Figure 8 shows that the ethanol purification concentrates cathodal Band 2 especially cathodal Band 6.

The position of the macromolecular fraction in the elution curve is similar to that of the outgrowth orienting protein. Attardi, et al., (1965) reported that the growth effects produced by the macromolecular fraction from the submaxillary gland extract were the dedifferentiation of skeletal muscle and cartilage and the enhancement of mesenchymal growth and these effects were not observed when the submaxillary gland extract was replaced by extracts from thymus, liver, kidney, or pancreas tissue. The outgrowth orienting activity was produced by an extract of the thymus gland.

The outgrowth orienting protein differs from the other protein described from the mouse submaxillary gland, the epidermal outgrowth factor (Cohen, 1962). The epidermal outgrowth factor has an estimated molecular weight of approximately 14,600 and is heat stable. This molecule is considerably smaller than the outgrowth orienting protein and the outgrowth orienting protein is also heat labile.

CHAPTER V

SUMMARY

The outgrowth orienting factor has been shown to be a heat labile protein with a molecular weight of approximately 41,000. The patterned outgrowth response depends upon the concentration of the protein. Using the extract from the mouse submaxillary glands of mice, purified by filtration on Sephadex G-25 gel, there was little or no orientation response below 20 µg of protein per 2.5 ml of medium in the culture slides and a maximum effect above 27 µg of protein per 2.5 ml of medium. Although monolayer outgrowth from explants was not patterned, the orientation response does not seem to be due to the orientation of the ultrastructure of the plasma clot since nerve processes, which exhibited patterned outgrowth when the fibrin in the clot was oriented by tension, did not exhibit oriented outgrowth in the presence of the submaxillary extract. The outgrowth orienting factor has been shown to affect the fibroblastic outgrowth from embryonic chick pancreas, liver, gut, kidney, and skeletal muscle explants as well as fibroblastic outgrowth from embryonic ventricle tissue from mice and chickens.

The orientation of cellular outgrowth seems to be mediated by a protein which is different from the growth stimulating proteins which have been described from the submaxillary gland of mice. Analysis by

G-100 gel filtration indicated that the outgrowth orienting protein has a molecular weight smaller than the nerve growth factor since the nerve growth factor has been reported to emerge first from the gel column. The protein material represented by the first ultraviolet absorption peak was added to the culture slides, but it did not mediate the cellular orientation of the outgrowth from the cardiac explants. The protein material represented by the fourth absorption peak did mediate cellular orientation from cardiac explants. Molecular weight estimation of the outgrowth orienting protein from the ratio of elution volume to the void volume on a calibrated gel column, position differences in the effluent from gel filtration, and electrophoretic analysis of the first step in the purification of the nerve growth factor support the idea that the nerve growth factor and the outgrowth orienting factor are different proteins.

The outgrowth orienting factor is different from the epidermal growth factor which is heat stable and has a molecular weight of approximately 14,600. This molecule is considerably smaller than the outgrowth orienting protein and the outgrowth orienting protein is also heat labile.

The outgrowth orienting factor seems to differ from the protein or proteins in the macromolecular fraction from the mouse submaxillary gland which has been reported to affect mesenchymal tissue outgrowth <u>in vitro</u>. An extract of the thymus gland did not produce the reported effects upon mesenchymal tissue, but in this study an extract of the thymus gland did mediate oriented outgrowth.

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A P P E N D I X

TABLE III

FRACTIONATION OF MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE ON SEPHADEX G-75

EFFLUENT VOLUME (ml)	% TRANSMISSION	OPTICAL DENSITY
31.0	100	0.000
32.0	99	0.004
37.0	30	0.523
42.0	3	1.523
47.0	7	1.155
52.0	4	1.398
57.0	1	2.000
62.0	1	2.000
67.0	3	1.523
72.0	10	1.000
77.0	16	0.796
82.5	24	0.620
89.5	61	0.215
94.5	34	0.469
100.5	25	0.602
106.0	16	0.796
111.0	12	0.921
116.0	9	1.046
126.0	4	1.398
136.0	19	0.721
141.0	35	0.456

TABLE IV

FRACTIONATION OF MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE ON SEPHADEX G-100

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS MISSION	OPTICAL DENSITY
Fractionated on 8/26/65			Fractionated on 9/8/65		
6	84	0.076	3	92	0.036
12	95	0.22	6	92	0.036
15	86	0.066	9	92	0.036
18	84	0.076	13	89	0.051
21	85	0.071	19	90	0.046
24	87	0.061	22	90	0.046
27	89	0.051	25	92	0.036
30	89	0.051	28	92	0.036
33	89	0.051	31	92	0.036
36	64	0.194	34	57	0.244
39	62	0.208	37	67	0.174
42	66	0.181	40	67	0.174
45	72	0.143	43	72	0.143
48	75	0.125	46	72	0.143
51	71	0.149	49	67	0.174
54	68	0.168	52	63	0.201
57	69	0.161	55	63	0.201
60	69	0.161	58	65	0.187
63	69	0.161	62	56	0.252
66	49	0.310	64	44	0.357
69	41	0.387	67	35	0.456
72	43	0.367	70	33	0.482
75	56	0.252	73	40	0.398
78	68	0.168	76	51	0.292
81	77	0.114	79	65	0.187
84	80	0.097	82	73	0.137
87	84	0.076	85	76	0.119
90	84	0.076	88	79	0.102
93	85	0.071	91	90	0.046
96	86	0.066	94	80	0.097
99	82	0.086	97	77	0.114
102	75	0.125	100	72	0.143
105	69	0.161	103	65	0.187
108	58	0.237	106	58	0.237
111	51	0.292	109	50	0.301
114	42	0.377	112	42	0.377
117	40	0.398	115	38	0.420
120	47	0.328	118	42	0.377
			121	47	0.328
			124	55	0.260
			127	60	0.222
			130	61	0.215

TABLE V

ABSORPTION SPECTRA OF FRACTIONS 1, 2, AND 3

WAVE LENGTH	FRACTION 1 8/26/65		FRACTION 2 9/8/65		FRACTION 3 8/26/65		FRACTION 3 9/8/65	
(mμ)	% T	O. D.	% T	O. D.	% T	O. D.	% T	O.D.
245	84	0.076	87	0.061	66	0.181	53	0.276
250	85	0.071	90	0.046	69	0.161	60	0.222
255	85	0.071	92	0.036	69	0.161	61	0.215
260	86	0.066	92	0.036	69	0.161	63	0.201
265	86	0.066	93	0.032	67	0.174	61	0.215
270	86	0.066	93	0.032	65	0.187	60	0.222
271	86	0.066	93	0.032	65	0.187	60	0.222
272	86	0.066	93	0.032	65	0.187	60	0.222
273	86	0.066	93	0.032	64	0.194	59	0.230
274	86	0.066	92	0.036	64	0.194	59	0.230
275	86	0.066	92	0.036	64	0.194	59	0.230
276	87	0.061	93	0.032	64	0.194	58	0.237
277	87	0.061	93	0.032	64	0.194	59	0.230
278	87	0.061	93	0.032	64	0.194	58	0.237
279	87	0.061	94	0.027	64	0.194	58	0.237
280	87	0.061	94	0.027	64	0.194	58	0.237
281	88	0.056	94	0.027	64	0.194	60	0.222
282	88	0.056	94	0.027	65	0.187	60	0.222
283	88	0.056	94	0.027	65	0.187	60	0.222
284	89	0.051	94	0.027	65	0.187	61	0.215
285	88	0.056	92	0.036	66	0.181	61	0.215
290	90	0.046	93	0.032	71	0.149	65	0.187
295	91	0.041	94	0.027	77	0.114	71	0.149
300	91	0.041	95	0.022	82	0.086	75	0.125
305	92	0.036	95	0.022	85	0.071	77	0.114
310	93	0.032	96	0.018	87	0.061	81	0.092
320	94	0.027	97	0.013	87	0.061	81	0.092
330	94	0.027	97	0.013	90	0.046	83	0.081

% T = Percent Transmission
O. D.= Optical Density

TABLE VI

ABSORPTION SPECTRA OF FRACTIONS 4 AND 5

WAVE LENGTH (mμ)	FRACTION 4				FRACTION 5			
	8/26/65		9/8/65		8/26/65		9/8/65	
	% T	O. D.	% T	O. D.	% T	O. D.	% T	O. D.
245	61	0.215	54	0.268	54	0.268	47	0.328
250	67	0.174	61	0.215	54	0.268	47	0.328
255	65	0.187	60	0.222	51	0.292	45	0.347
260	59	0.229	53	0.276	46	0.337	42	0.377
265	51	0.292	46	0.337	42	0.377	40	0.398
270	45	0.347	39	0.409	41	0.387	37	0.432
271	44	0.356	38	0.420	40	0.398	37	0.432
272	42	0.377	37	0.432	40	0.398	37	0.432
273	42	0.377	36	0.444	39	0.409	36	0.444
274	41	0.387	36	0.444	39	0.409	36	0.444
275	40	0.398	35	0.456	40	0.398	36	0.444
276	40	0.398	34	0.469	41	0.387	37	0.432
277	40	0.398	34	0.469	41	0.387	37	0.432
278	39	0.409	34	0.469	41	0.387	38	0.420
279	39	0.409	33	0.482	42	0.377	38	0.420
280	39	0.409	33	0.482	43	0.367	38	0.420
281	39	0.409	33	0.482	43	0.367	39	0.409
282	39	0.409	34	0.469	44	0.356	40	0.398
283	39	0.409	34	0.469	45	0.347	40	0.398
284	40	0.398	35	0.456	46	0.337	42	0.377
285	42	0.377	36	0.444	47	0.328	43	0.367
290	50	0.301	44	0.357	52	0.284	50	0.301
295	66	0.181	60	0.222	57	0.244	55	0.260
300	80	0.097	75	0.125	60	0.222	58	0.237
305	89	0.051	85	0.071	61	0.215	59	0.229
310	92	0.036	87	0.061	62	0.208	60	0.222
320	95	0.022	92	0.036	65	0.187	64	0.194
330	97	0.013	93	0.032	72	0.143	66	0.181

% T = Percent Transmission
O. D.= Optical Density

TABLE VII

VOID VOLUME DETERMINATION WITH 0.5 ML BLUE DEXTRAN AND FRACTIONATION OF 0.5 ML OF MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE ON SEPHADEX G-100

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
	Blue Dextran		Continued from Column 1--MMSCH		
29.60	100	0.000	66.62	60	0.222
32.56	38	0.420	69.98	70	0.155
35.64	55	0.260	72.79	82	0.086
38.89	96	0.018	75.24	89	0.051
41.90	100	0.000	78.02	92	0.036
			80.82	94	0.027
	MMSCH		83.02	95	0.022
			85.87	96	0.018
21.54	100	0.000	88.43	97	0.013
24.70	99	0.004			
27.55	100	0.000	91.14	96	0.018
			94.05	95	0.022
30.69	98	0.009	96.86	92	0.036
33.60	78	0.108			
36.53	78	0.108	100.00	88	0.056
			103.34	81	0.092
39.42	81	0.092	106.52	74	0.131
42.41	85	0.071			
45.27	87	0.061	110.03	69	0.161
			113.50	76	0.119
48.13	85	0.071	116.42	83	0.081
51.25	80	0.097			
54.08	81	0.092	119.49	79	0.102
			123.09	72	0.143
57.24	89	0.051	127.52	84	0.076
60.50	74	0.131			
63.80	63	0.201	130.74	98	0.009
			134.00	100	0.000

TABLE VIII

VOID VOLUME DETERMINATION WITH 0.5 ML BLUE DEXTRAN AND FRACTIONATION OF 0.5 ML LYOPHILIZED PROTEIN FROM AREA A, FIG. 1a ON SEPHADEX G-100 IN 0.02 M SODIUM PHOSPHATE BUFFER, pH 6.5

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
Blue Dextran		
28.15	100	0.000
30.95	63	0.201
33.85	46	0.337
36.81	82	0.086
Submaxillary Protein		
25.66	100	0.000
29.15	83	0.081
32.08	63	0.201
35.09	70	0.155
38.05	66	0.181
41.14	66	0.181
44.01	73	0.137
46.91	73	0.137
49.64	66	0.181
52.64	60	0.222
55.80	64	0.194
58.74	68	0.168
61.45	60	0.222
64.20	44	0.357
67.12	31	0.509
69.78	29	0.538
72.53	40	0.398
75.18	60	0.222
78.00	75	0.125
81.25	86	0.066
84.55	90	0.046
87.32	91	0.041
90.07	92	0.036
93.15	92	0.036
95.88	94	0.027
98.64	95	0.022
101.47	96	0.018
104.90	95	0.022
107.99	95	0.022
111.17	96	0.018
114.14	96	0.018
117.13	98	0.009
120.34	99	0.004
123.73	99	0.004
126.51	99	0.004
129.79	100	0.000
133.29	99	0.004

TABLE IX

VOID VOLUME DETERMINATION WITH 0.5 ML BLUE DEXTRAN AND FRACTIONATION OF 0.5 ML LYOPHILIZED PROTEIN FROM AREA A, FIG. 1a, ON SEPHADEX G-100 IN 0.005 M TRIS HCl BUFFER, pH 7.2

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
	Blue Dextran		Submaxillary Protein, Continued		
27.62	100	0.000	61.28	63	0.201
30.52	87	0.061	64.16	71	0.149
33.38	31	0.509	67.06	79	0.102
36.38	85	0.071	70.08	88	0.056
39.94	100	0.000	72.82	94	0.027
			75.80	97	0.013
	Submaxillary Protein		78.64	100	0.000
			81.69	100	0.000
27.84	100	0.000	84.46	100	0.000
30.70	98	0.009			
33.51	69	0.161	87.32	100	0.000
			91.32	100	0.000
35.54	78	0.108	94.29	100	0.000
37.97	84	0.076			
40.96	80	0.097	97.00	100	0.000
			100.00	100	0.000
43.66	75	0.125	102.84	101	--
46.51	78	0.108			
49.51	81	0.092	105.58	102	--
			109.32	101	--
52.66	80	0.097	111.70	102	--
55.69	70	0.155			
58.40	62	0.208	114.45	102	--

TABLE X

DENSITOMETER ANALYSIS OF ELECTROPHORETIC SEPARATIONS OF PROTEIN REPRESENTED BY AREA A, FIG 1a AND PEAK IV, FIG 3a

DISTANCE (mm)	Area A		Peak IV		DISTANCE (mm)	Area A		Peak IV	
	% T	O. D.	% T	O. D.		% T	O. D.	% T	O. D.
-16	100	0.00	95	0.02	Continued from Column 1				
-15	100	0.00	98	0.01					
-14	95	0.02	98	0.01	20	63	0.20	91	0.04
-13	95	0.02	98	0.01	21	74	0.13	95	0.02
-12	95	0.02	100	0.00	22	80	0.10	91	0.04
-11	95	0.02	100	0.00	23	76	0.12	87	0.06
					24	72	0.14	91	0.04
-10	95	0.02	100	0.00					
- 9	95	0.02	100	0.00	25	72	0.14	95	0.02
- 8	95	0.02	100	0.00	26	69	0.16	98	0.01
- 7	95	0.02	100	0.00	27	66	0.18	100	0.00
- 6	95	0.02	100	0.00	28	69	0.16	100	0.00
					29	87	0.06	100	0.00
- 5	95	0.02	100	0.00					
- 4	95	0.02	100	0.00	30	91	0.04	100	0.00
- 3	91	0.04	100	0.00	31	95	0.02	100	0.00
- 2	87	0.06	100	0.00	32-44	100	0.00	100	0.00
- 1	87	0.06	100	0.00					
					45	100	0.00	100	0.00
0	87	0.06	95	0.02	46	91	0.04	100	0.00
1	87	0.06	98	0.01	47	87	0.06	100	0.00
2	98	0.06	100	0.00	48	83	0.08	100	0.00
3	87	0.06	100	0.00	49	87	0.06	100	0.00
4	87	0.06	100	0.00	50	91	0.04	100	0.00
5	87	0.06	100	0.00	51	91	0.04	100	0.00
6	83	0.08	100	0.00	52	95	0.02	100	0.00
7	80	0.10	100	0.00	53	100	0.00	100	0.00
8	76	0.12	100	0.00	54	100	0.00	100	0.00
9	74	0.13	100	0.00	55	100	0.00	100	0.00
10	76	0.12	100	0.00					
11	76	0.16	100	0.00					
12	69	0.16	100	0.00					
13	66	0.18	100	0.00					
14	57	0.24	100	0.00					
15	50	0.30	100	0.00					
16	55	0.26	100	0.00					
17	66	0.18	95	0.02					
18	66	0.18	91	0.04					
19	60	0.22	87	0.06					

% T = Per cent Transmission O. D. = Optical Density

TABLE XI

FRACTIONATION OF MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE ON SEPHADEX G-200, CHROMATOGRAPHIC COLUMN I

EFFLUENT VOLUME (ml)	% TRANSMISSION	OPTICAL DENSITY
3	98	0.009
6	98	0.009
9	98	0.009
12	98	0.009
15	98	0.009
18	98	0.009
21	98	0.009
24	98	0.009
27	99	0.004
30	95	0.022
33	89	0.051
37	84	0.076
39	84	0.076
42	87	0.061
45	85	0.071
48	82	0.086
51	79	0.102
54	76	0.119
57	76	0.119
60	77	0.114
64	74	0.131
67	64	0.194
70	58	0.237
72	56	0.252
75	57	0.244
78	51	0.292
80	39	0.409
85	24	0.620
88	24	0.620
90	33	0.482
93	47	0.328
96	60	0.222
99	66	0.181
102	63	0.201
105	58	0.237
108	52	0.284

TABLE XII

FRACTIONATION OF 2 ML OF MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE ON SEPHADEX G-200, CHROMATOGRAPHIC COLUMN II

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
	10/13/65		Continued from Column I		
42	100	0.000	193	84	0.076
57	100	0.000	196	89	0.051
60	100	0.000	199	90	0.046
63	100	0.000	201	89	0.051
66	98	0.009	204	83	0.081
69	93	0.032	207	53	0.276
72	91	0.041	210	51	0.292
75	94	0.027	213	42	0.377
78	96	0.018	216	40	0.398
81	97	0.013	219	37	0.432
84	96	0.018	222	37	0.432
87	94	0.027	225	39	0.409
			228	40	0.398
	11/3/65		231	59	0.229
132	Pooled		234	71	0.149
135	99	0.004			
138	100	0.000	248	89	0.051
141	100	0.000	261	95	0.022
144	96	0.018	264	85	0.071
			267	80	0.097
147	96	0.018	270	73	0.137
150	94	0.027			
153	93	0.032	273	66	0.181
156	94	0.027	276	59	0.229
159	100	0.000	279	53	0.276
			282	52	0.284
162	100	0.000	285	54	0.268
165	100	0.000			
168	98	0.009	288	60	0.222
171	98	0.009	290	66	0.181
174	97	0.013	294	76	0.119
			296	83	0.081
177	96	0.018	299	91	0.041
180	93	0.032			
183	94	0.027	302	97	0.013
186	90	0.046	306	100	0.000
189	88	0.056	309	100	0.000
			311	100	0.000

TABLE XIII

RECYCLING OF AREA D

EFFLUENT VOLUME (ml)	% TRANSMISSION	OPTICAL DENSITY
198	100	0.000
201	100	0.000
203	98	0.009
206	96	0.018
209	91	0.041
212	87	0.061
215	81	0.092
218	74	0.131
220	70	0.155
222	67	0.174
225	66	0.181
228	63	0.201
231	67	0.174
234	70	0.155
237	79	0.102
240	86	0.066
243	94	0.027
252	99	0.004
261	100	0.000

TABLE XIV

FRACTIONATION OF 1 ML OF MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE ON SEPHADEX G-200, CHROMATOGRAPHIC COLUMN II

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
130	Pooled		Continued from Colume 1		
133	90	0.036	195	72	0.143
136	93	0.032	198	66	0.181
139	98	0.009	201	60	0.222
142	97	0.013	204	55	0.260
145	97	0.013	207	52	0.284
148	100	0.000	210	51	0.292
152	100	0.000	214	54	0.268
155	100	0.000	217	61	0.215
159	99	0.004	220	72	0.143
161	91	0.041	223	78	0.108
165	98	0.009	226	90	0.046
168	97	0.013	229	95	0.022
171	94	0.027	231	92	0.036
174	87	0.061	234	89	0.051
177	86	0.066	237	91	0.041
180	85	0.071	240	97	0.013
183	82	0.086	243	97	0.013
186	83	0.081	245	96	0.018
189	81	0.092	248	93	0.032
192	77	0.114			

TABLE XV

DENSITOMETER ANALYSIS OF ELECTROPHORETIC SEPARATIONS OF PROETIN

REPRESENTED BY AREA A, FIG 1a, AND PEAK D_2 FIG. 5d

DISTANCE (mm)	Area A % T	Area A O. D.	Peak IV % T	Peak IV O. D	DISTANCE (mm)	Area A % T	Area A L. D.	Peak IV % T	Peak IV O. D.
-16	100	0.00	100	0.00	Continued from Column 1				
-15	95	0.02	95	0.02					
					7	76	0.12	87	0.06
-14	95	0.02	93	0.03	8	80	0.10	87	0.06
-13	95	0.02	93	0.03	9	83	0.08	87	0.06
-12	91	0.04	93	0.03					
					10	83	0.08	91	0.04
-11	91	0.04	95	0.02	11	80	0.10	91	0.04
-10	91	0.04	95	0.02	12	69	0.16	91	0.04
- 9	93	0.03	95	0.02					
					13	66	0.18	87	0.06
- 8	95	0.02	95	0.02	14	72	0.14	83	0.08
- 7	95	0.02	95	0.02	15	83	0.08	87	0.06
- 6	95	0.02	98	0.01					
					16	87	0.06	91	0.04
- 5	95	0.02	98	0.01	17	91	0.04	95	0.02
- 4	93	0.03	98	0.01	18	87	0.06	95	0.02
- 3	91	0.04	95	0.02					
					19	83	0.08	91	0.04
- 2	87	0.06	95	0.02	20	80	0.10	95	0.02
- 1	83	0.08	95	0.02	21	76	0.12	95	0.02
0	83	0.08	95	0.02					
					22	66	0.18	95	0.02
1	83	0.08	95	0.02	23	66	0.18	93	0.03
2	80	0.10	91	0.04	24	83	0.08	91	0.04
3	69	0.16	91	0.04					
					25	100	0.00	95	0.02
4	60	0.22	91	0.04	26-55	100	0.00	100	0.00
5	57	0.24	91	0.04					
6	66	0.18	87	0.06					

% T = Percent Transmission
O. D. = Optical Density

TABLE XVI

VOID VOLUME DETERMINATION WITH 0.5 ML BLUE DEXTRAN AND FRACTIONATION OF 0.5 ML MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE PURIFIED BY ETHANOL PRECIPITATION

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
Blue Dextran			Continued from Column 1		
23.43	100	0.000	64.55	96	0.018
26.33	100	0.000	67.78	95	0.022
28.66	90	0.046	73.23	98	0.137
31.33	29	0.538	76.12	100	0.000
34.32	76	0.119	79.80	100	0.000
36.42	96	0.179	83.18	100	0.000
39.41	100	0.000	86.43	98	0.009
			89.46	100	0.000
Submaxillary Extract			92.70	100	0.000
27.29	100	0.000	95.93	97	0.013
31.13	91	0.041	99.51	95	0.022
34.15	91	0.041	102.70	93	0.032
37.20	90	0.046	105.85	88	0.056
40.34	91	0.041	108.93	83	0.081
43.58	87	0.061	112.06	81	0.092
46.50	88	0.056	115.48	85	0.071
49.51	88	0.056	118.77	89	0.051
52.53	91	0.041	121.77	95	0.022
56.19	93	0.032	125.39	97	0.013
58.28	93	0.032	128.11	93	0.032
61.51	95	0.022	130.93	95	0.022
			135.29	97	0.013
			139.41	100	0.000

TABLE XVII

DENSITOMETER ANALYSIS OF 35 MIN ELECTROPHORETIC SEPARATION OF PROTEIN FROM AREA A, FIG 1a

DISTANCE (mm)	% T	O. D.	DISTANCE (mm)	% T	O. D.	DISTANCE (mm)	% T	O. D.
			Continued			Continued		
			7	91	0.04	34	100	0.00
-16	100	0.00	8	91	0.04	35	95	0.02
-15	100	0.00	9	93	0.03	36	91	0.04
-14	100	0.00	10	95	0.02	37	91	0.04
-13	100	0.00	11	95	0.02	38	95	0.02
-12	100	0.00	12	93	0.03	39	95	0.02
-11	100	0.00	13	91	0.04	40	95	0.02
-10	95	0.02	14	85	0.07	41	98	0.01
-9	93	0.03	15	83	0.08	42	95	0.02
-8	91	0.04	16	87	0.06	43	95	0.02
-7	87	0.06	17	95	0.02	44	95	0.02
-6	87	0.06	18	100	0.00	45	91	0.04
-5	83	0.08	19	100	0.00	46	91	0.04
-4	80	0.10	20	100	0.00	47	91	0.04
-3	76	0.12	21	100	0.00	48	91	0.04
-2	74	0.13	22	100	0.00	49	91	0.04
-1	72	0.14	23	100	0.00	50	91	0.04
0	74	0.13	24	100	0.00	51	91	0.04
1	76	0.12	25	100	0.00	52	95	0.02
2	80	0.10	26	100	0.00	53	95	0.02
3	83	0.08	27	100	0.00	54	95	0.02
4	91	0.04	28	100	0.00	55	95	0.02
5	91	0.04	29	100	0.00			
6	91	0.04	30	100	0.00			
			31	100	0.00			
			32	100	0.00			
			33	100	0.00			

% T = Percent Transmission; O. D.=Optical Density

TABLE XVIII

DENSITOMETER ANALYSIS OF 35 MIN ELECTROPHORETIC SEPARATION OF PROTEIN FROM MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE ETHANOL PRECIPITATION

DISTANCE (mm)	% T	O. D	DISTANCE (mm)	% T	O. D.	DISTANCE (mm)	% T	O. D.
-16	100	0.00	Continued			Continued		
-15	91	0.04						
			7	95	0.02	34	91	0.04
-14	91	0.04	8	95	0.02	45	87	0.06
-13	83	0.08	9	100	0.00	36	76	0.12
-12	84	0.08						
			10	100	0100	37	66	0.18
-11	80	0.10	11	100	0.00	38	63	0.20
-10	95	0.02	12	100	0.00	39	60	0.22
-9	87	0.06						
			13	100	0.00	40	52	0.28
-8	83	0.08	14	100	0.00	41	44	0.35
-7	80	0.10	15	100	0.00	42	40	0.39
-6	69	0.16						
			16	100	0.00	43	45	0.34
-5	63	0.20	17	100	0.00	44	41	0.38
-4	63	0.20	18	100	0.00	45	41	0.38
-3	55	0.26						
			19	100	0.00	46	38	0.42
-2	52	0.28	20	100	0.00	47	38	0.42
-1	51	0.29	21	100	0.00	48	39	0.41
0	60	0.22						
			22	100	0.00	49	39	0.41
1	66	0.18	23	100	0.00	50	36	0.44
2	66	0.18	24	100	0.00	51	38	0.42
3	66	0.18						
			25	100	0.00	52	39	0.40
4	72	0.14	26	100	0.00	53	52	0.28
5	76	0.12	27	100	0.00	54	83	0.08
6	80	0.10						
			28	100	0.00	55	100	0.00
			29	100	0.00			
			30	100	0.00			
			31	100	0.00			
			32	100	0.00			
			33	95	0.02			

% T = Percent Transmission; O. D. Optical Density

TABLE XIX

DENSITOMETER ANALYSIS OF 90 MIN ELECTROPHORETIC SEPARATION OF PROTEIN FROM AREA A, FIG. 1a

DISTANCE (mm)	% T	O. D.	DISTANCE (mm)	% T	O. D	DISTANCE (mm)	% T	O. D.
			Continued			Continued		
-16	100	0.00	7	95	0.02	34	87	0.06
-15	95	0.02	8	95	0.02	35	83	0.08
			9	93	0.03	36	80	0.10
-14	95	0.02	10	91	0.04	37	91	0.04
-13	95	0.02	11	91	0.04	38	100	0.00
-12	95	0.02	12	93	0.03	29	100	0.00
-11	95	0.02	13	95	0.02	40	100	0.00
-10	95	0.02	14	91	0.04	41	100	0.00
-9	95	0.02	15	87	0.06	42	100	0.00
-8	95	0.02	16	83	0.08	43	100	0.00
-7	98	0.01	17	80	0.10	44	100	0.00
-6	98	0.01	18	76	0.12	45	100	0.00
-5	98	0.01	19	80	0.10	46	100	0.00
-4	95	0.02	20	83	0.08	47	100	0.00
-3	95	0.02	21	87	0.06	48	100	0.00
-2	95	0.02	22	91	0.04	49	100	0.00
-1	91	0.04	23	93	0.03	50	100	0.00
0	91	0.04	24	91	0.04	51	100	0.00
1	91	0.04	25	85	0.07	52	100	0.00
2	91	0.04	26	83	0.08	53	100	0.00
3	91	0.04	27	83	0.08	54	100	0.00
4	95	0.02	28	87	0.06	55	100	0.00
5	95	0.02	29	91	0.04			
6	95	0.02	30	93	0.03			
			31	91	0.04			
			32	91	0.04			
			33	89	0.05			

% T = Percent Transmission O. D. = Optical Density

TABLE XX

DENSITOMETER ANALYSIS OF 90 MIN ELECTROPHORETIC SEPARATION OF PROTEIN FROM MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE PURIFIED BY ETHANOL PRECIPITATION

DISTANCE (mm)	% T	O. D.	DISTANCE (mm)	% T	O. D.	DISTANCE (mm)	% T	O. D.
			Continued			Continued		
-16	100	0.00	7	91	0.04	34	95	0.02
-15	100	0.00	8	91	0.04	36	95	0.02
			9	91	0.04	36	95	0.02
-14	95	0.02	10	91	0.04	37	100	0.00
-13	93	0.03	11	91	0.04	38	100	0.00
-12	91	0.04	12	91	0.04	39	100	0.00
-11	91	0.04	13	89	0.05	40	100	0.00
-10	91	0.04	14	87	0.06	41	100	0.00
-9	91	0.04	15	76	0.12	42	100	0.00
-8	95	0.02	16	69	0.16	43	100	0.00
-7	95	0.02	17	76	0.12	44	100	0.00
-6	95	0.02	18	91	0.04	45	100	0.00
-5	95	0.02	19	98	0.01	46	100	0.00
-4	91	0.04	20	100	0.00	47	100	0.00
-3	91	0.04	21	95	0.02	48	100	0.00
-2	87	0.06	22	95	0.02	49	100	0.00
-1	87	0.06	23	100	0.00	50	100	0.00
0	87	0.06	24	100	0.00	51	100	0.00
1	87	0.06	25	100	0.00	52	100	0.00
2	87	0.06	26	100	0.00	53	100	0.00
3	91	0.04	27	100	0.00	54	100	0.00
4	91	0.04	28	100	0.00	55	100	0.00
5	91	0.04	29	100	0.00			
6	91	0.04	30	100	0.00			
			31	100	0.00			
			32	100	0.00			
			33	100	0.00			

% T = Percent Transmission; O. D. = Optical Density.

TABLE XXI

VOID VOLUME DETERMINATION WITH 0.5 ML BLUE DEXTRAN AND FRACTIONATION OF 0.5 ML OF $(NH_4)_2SO_4$ PURIFIED MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE ON SEPHADEX G-100 IN 0.2 M

Sodium Phosphate Buffer, pH 6.5

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
Blue Dextran			Column 1, Continued		
26.34	Pooled		58,08	37	0.432
29.49	100	0.000	61.12	25	0.602
32.90	68	0.168	64.21	10	1.000
36.39	71	0.149	67.25	6	1.222
39.40	96	0.018	70.57	8	1.097
			73.92	26	0.585
Submaxillary Extract					
			76.70	51	0.292
18.63	Pooled		79.34	67	0.174
23.47	100	0.000	82.68	75	0.125
26.42	97	0.013			
			85.87	78	0.108
29.27	93	0.032	88.49	80	0.097
32.26	35	0.456	91.28	81	0.092
35.06	48	0.319			
			94.06	78	0.108
37.86	41	0.387	97.08	80	0.097
41.20	42	0.377	100.44	74	0.131
44.66	52	0.284			
			103.15	70	0.155
47.69	43	0.367	106.16	77	0.114
51.01	26	0.585	109.11	87	0.061
54.18	28	0.553			
			114.00	96	0.018
			118.72	100	0.000
			121.64	100	0.000

TABLE XXII

VOID VOLUME DETERMINATION WITH 0.5 ML BLUE DEXTRAN AND FRACTIONATION OF 0.5 ML OF $(NH_4)_2SO_4$ PURIFIED MALE MOUSE SUBMAXILLARY CRUDE HOMOGENATE ON SEPHADEX G-100

in 0.005 M Tris HCl Buffer, pH 7.2

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
	Blue Dextran	
16.83	Pooled	
29.10	100	0.000
32.02	58	0.237
37.88	97	0.013
41.38	100	0.000
	Submaxillary Extract	
24.55	Pooled	
27.44	100	0.000
31.00	86	0.066
33.87	24	0.620
36.77	19	0.721
40.04	35	0.456
43.19	45	0.347
45.86	27	0.569
48.77	21	0.678
51.52	29	0.538
54.48	37	0.523
57.50	30	0.523
	Continued from Column I	
60.30	16	0.796
63.36	10	1.000
66.14	10	1.000
68.82	22	0.658
71.74	40	0.398
75.24	57	0.244
75.24	57	0.244
78.83	64	0.194
81.78	64	0.194
84.87	63	0.201
88.46	60	0.222
91.45	60	0.222
95.50	56	0.252
98.77	58	0.237
101.70	70	0.155
105.23	78	0.108
108.37	82	0.086
111.39	86	0.066
114.32	89	0.051
117.34	89	0.051
120.19	92	0.036

TABLE XXIII

PURIFICATION OF MALE MOUSE MUSCLE CRUDE HOMOGENATE

AND MALE MOUSE THYMUS CRUDE HOMOGENATE ON SEPHADEX G-25

MUSCLE HOMOGENATE				THYMUS HOMOGENATE		
EFFLUENT VOLUME (ml)	WAVE LENGTH (mu)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
41.0	280	100	0.000	39.0	100	0.000
42.0	280	98	0.004	40.0	91	0.041
43.0	280	91	0.041	43.0	13	0.886
44.0	280	68	0.168	46.0	7	1.155
44.5	280	36	0.444	53.0	21	0.678
44.5	290	80	0.097	60.0	54	0.268
47.0	290	58	0.237	61.5	77	0.114
48.5	290	51	0.292	63.5	48	0.319
49.0	290	51	0.292	65.0	37	0.432
50.0	290	52	0.284	68.5	50	0.301
57.5	290	73	0.137	71.5	62	0.208
60.5	290	80	0.097	74.0	64	0.194
61.7	280	48	0.319	81.0	66	0.181
62.0	280	63	0.201	88.0	51	0.292
64.0	280	78	0.108	96.5	46	0.337
67.5	280	64	0.194			
68.3	280	70	0.155			
71.0	280	72	0.143			
78.0	280	74	0.131			
85.0	280	75	0.125			
92.0	280	69	0.161			
100.5	280	70	0.222			
110.0	280	46	0.337			
121.0	280	70	0.155			

TABLE XXIV

ABSORPTION SPECTRUM OF MALE MOUSE MUSCLE CRUDE HOMOGENATE

WAVE LENGTH (mμ)	% TRANSMISSION	OPTICAL DENSITY
245	65	0.187
250	68	0.168
255	67	0.174
260	64	0.194
265	62	0.208
270	58	0.237
271	58	0.237
272	58	0.237
273	57	0.244
274	57	0.244
275	57	0.244
276	57	0.244
277	57	0.244
278	57	0.277
279	57	0.244
280	57	0.244
281	58	0.237
282	58	0.237
283	59	0.237
284	58	0.229
285	60	0.222
290	72	0.143
295	77	0.114
300	79	0.102
305	81	0.092
310	83	0.081
320	84	0.076
330	85	0.071

TABLE XXV

VOID VOLUME DETERMINATION WITH 1.0 ML BLUE DEXTRAN AND FRACTIONATION OF 1.0 ML OF MALE MOUSE MUSCLE CRUDE HOMOGENATE ON SEPHADEX G-100

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
Blue Dextran			Continued from Column 1		
30.96	100	0.000	72183	97	0.013
33.95	33	0.482	75.66	96	0.018
36.91	17	0.770	78.47	96	0.018
39.98	84	0.076	81.40	98	0.009
47.27	97	0.013	84.03	99	0.004
			86.81	98	0.009
MMMCH			89.64	99	0.004
			91.87	99	0.004
19.51	Pooled		94.63	97	0.013
22.49	100	0.000			
25.24	100	0.000	97.13	85	0.071
			99.95	75	0.125
29.25	100	0.000	102.93	69	0.161
32.14	100	0.000			
35.02	99	0.004	106.24	64	0.194
			109.44	62	0.208
37.86	93	0.032	111.85	63	0.201
40.70	88	0.056			
43.53	78	0.108	117.35	77	0.114
			119.89	94	0.027
46.35	72	0.143	122.81	96	0.018
49.26	81	0.092			
52.32	90	0.046	125.94	98	0.009
			128.61	100	0.000
55.47	95	0.022	131.58	100	0.000
58.47	98	0.009			
61.40	99	0.004			
64.59	97	0.013			
67.36	95	0.022			
70.16	95	0.022			

TABLE XXVI

VOID VOLUME DETERMINATION WITH 0.5 ML BLUE DEXTRAN AND FRACTIONATION OF 0.5 ML OF MALE MOUSE THYMUS CRUDE HOMOGENATE ON SEPHADEX G-100

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
	Blue Dextran		Continued from Column 1		
23.39	100	0.000	65.50	100	0.000
26.87	89	0.051	69.45	100	0.000
29.88	39	0.409	72.69	100	0.000
32.37	100	0.000	75.39	100	0.000
			78.70	100	0.000
	MMTCH		82.62	100	0.000
21.35	Pooled		85.56	100	0.000
24.34	100	0.000	88.60	100	0.000
27.28	97	0.013	91.39	98	0.009
30.08	63	0.201	94.81	93	0.032
32.92	87	0.061	97.63	80	0.097
35.78	87	0.061	100.56	65	0.187
38.48	85	0.071	103.90	54	0.268
41.60	92	0.036	107.01	57	0.244
44.38	96	0.018	110.28	77	0.114
47.35	97	0.013	112.68	82	0.086
50.65	98	0.009	115.15	85	0.071
53.50	99	0.004	118.41	94	0.027
56.56	98	0.009	121.87	100	0.000
59.45	98	0.009	125.14	100	0.000
62.44	100	0.000	128.32	100	0.000

TABLE XXX

MEASUREMENTS OF CELL AXIS ANGLES

Culture Slide contained 2.5 ml of Medium and 11 µl of Water

DATE	SLIDE NUMBER	NUMBER OF ANGLES MEASURED	ACCUMULATED TOTAL(°)	AVERAGE (°)	DISTANCE BETWEEN EXPLANTS (mm)
1/29/66	2	39	+1246	+31.9	0.53
2/ 5/66	2	30	+ 783	+26.1	0.35
2/19/66	2	65	+1486	+22.9	0.45
2/27/66	1	19	+ 555	+29.2	0.21
2/27/66	1	19	+ 311	+16.4	0.44
2/27/66	1	10	+ 157	+15.7	0.35
2/27/66	1	32	+ 384	+12.0	0.42
TOTALS		214	+4922		

AVERAGE OF TOTALS: +23.0

TABLE XXXI

MEASUREMENTS OF CELL AXIS ANGLES

Slide contained 2.5 ml of Medium and 11 µg of Lyophilized G-25 Fraction in 11 µl of Water

DATE	SLIDE NUMBER	NUMBER OF ANGLES MEASURED	ACCUMULATED TOTAL (°)	AVERAGE (°)	DISTANCE BETWEEN EXPLANTS (mm)
1/29/66	1	58	+1207	+20.8	0.30
2/ 5/66	2	47	+ 536	+11.4	0.67
2/19/66	1	36	+ 917	+25.5	0.44
2/19/66	1	59	+13-3	+22.1	0.40
2/27/66	1	12	+ 451	+37.6	0.32
2/27/66	1	15	+ 215	+14.3	0.22
2/27/66	1	22	+ 206	+ 9.4	0.57
2/27/66	1	45	+ 658	+14.6	0.28
2/27/66	1	26	+ 340	+13.1	0.18
TOTALS		320	+5833		

AVERAGE OF TOTALS: +18.2

TABLE XXXII

MEASUREMENTS OF CELL AXIS ANGLES

Culture Slide contained 2.5 ml of Medium and 20 µl of Water

DATE	SLIDE NUMBER	NUMBER OF ANGLES MEASURED	ACCUMULATED TOTAL (°)	AVERAGE (°)	DISTANCE BETWEEN EXPLANTS (mm)
2/19/66	1	67	+1089	+16.3	0.34
2/19/66	1	56	+1910	+34.1	0.38
2/19/66	1	64	+1206	+18.8	0.35
2/19/66	2	40	+1099	+27.5	0.26
2/27/66	1	35	+ 638	+18.2	0.28
2/27/66	1	22	+ 724	+32.9	0.18
TOTALS		284	+6666		

AVERAGE OF TOTALS: +23.5

TABLE XXXIII

MEASUREMENTS OF CELL AXIS ANGLES

Slide contained 2.5 ml of Medium and 20 µg of Lyophilized G-25 Fraction in 20 µl of Water

DATE	SLIDE NUMBER	NUMBER OF ANGLES MEASURED	ACCUMULATED TOTAL (°)	AVERAGE (°)	DISTANCE BETWEEN EXPLANTS (mm)
2/19/66	1	90	+1806	+20.1	0.24
2/19/66	1	71	+1573	+22.1	0.42
2/19/66	2	90	+1077	+12.0	0.50
2/27/66	1	39	+ 272	+ 6.9	0.22
2/27/66	1	31	+ 385	+12.4	0.45
2/27/66	1	62	+ 852	+13.7	0.35
2/27/66	1	38	+ 747	+19.6	0.10
2/27/66	1	40	+ 611	+15.3	0.68
2/ 5/66	1	34	+ 590	+17.4	0.24
2/ 5/66	1	27	+ 606	+22.4	0.25
3/ 5/66	2	13	+ 323	+24.8	0.38
TOTALS		535	+8842		

AVERAGE OF TOTALS: +16.5

TABLE XXXIV

MEASUREMENTS OF CELL AXIS ANGLES

Slide contained 2.5 ml of Medium and 24 µg of Lyophilized G-25 Fraction in 48 µl of Water

DATE	SLIDE NUMBER	NUMBER OF ANGLES MEASURED	ACCUMULATED TOTAL (°)	AVERAGE (°)	DISTANCE BETWEEN EXPLANTS (mm)
3/5/66	1	41	- 68	- 1.7	0.30
3/5/66	1	30	+ 40	+ 1.3	0.22
3/5/66	1	27	+397	+14.7	0.41
3/5/66	1	22	-113	- 5.1	0.25
3/5/66	2	15	+192	+12.8	0.36
3/5/66	2	34	+593	+17.4	0.51
3/5/66	2	55	+491	+ 8.9	0.31
3/5/66	2	42	+287	+ 6.8	0.28
3/5/66	3	25	+269	+10.7	0.29
3/5/66	3	17	+ 19	+ 1.1	0.63
3/5/66	3	89	-212	- 2.4	0.23
3/5/66	3	22	+329	+15.0	0.47
3/5/66	3	23	+142	+ 6.2	0.22
TOTALS		442	+2366		

AVERAGE OF TOTALS: +5.4

TABLE XXXV

MEASUREMENTS OF CELL AXIS ANGLES

Culture Slide contained 2.5 ml of Medium and 27 µl of Water

DATE	SLIDE NUMBER	NUMBER OF ANGLES MEASURED	ACCUMULATED TOTAL (°)	AVERAGE (°)	DISTANCE BETWEEN EXPLANTS (mm)
1/29/66	1	53	+ 627	+11.8	0.37
1/29/66	2	60	+1138	+19.0	0.29
2/19/66	1	40	+ 710	+17.8	0.41
2/19/66	1	78	+1917	+24.6	0.36
2/19/66	2	48	+1139	+23.7	0.39
2/19/66	2	41	+ 882	+21.5	0.52
3/ 5/66	1	37	+ 522	+14.1	0.57
3/ 5/66	1	38	+ 598	+15.7	0.31
3/ 5/66	1	24	+ 504	+21.0	0.30
TOTALS		419	+8037		
AVERAGE OF TOTALS	+19.2				

TABLE XXXVI

MEASUREMENTS OF CELL AXIS ANGLES

Slide contained 2.5 ml of Medium and 27 µg of Lyophilized G-25 Fraction in 27 µl of Water

DATE	SLIDE NUMBER	NUMBER OF ANGLES MEASURED	ACCUMULATED TOTAL (°)	AVERAGE (°)	DISTANCE BETWEEN EXPLANTS (mm)
1/29/66	1	95	+195	+2.1	0.75
1/29/66	2	105	+453	+4.3	0.66
2/19/66	1	106	-931	-8.8	0.53
2/19/66	1	74	- 34	-0.5	0.53
2/27/66	1	17	-42	-2.5	0.13
3/ 5/66	1	46	+258	+5.6	0.34
TOTALS		443	-101		

AVERAGE OF TOTALS: -0.2

TABLE XXXVII

MEASUREMENTS OF CELL AXIS ANGLES

Culture Slide contained 2.5 ml of Medium and 45 µl of Water

DATE	SLIDE NUMBER	NUMBER OF ANGLES MEASURED	ACCUMULATED TOTAL (°)	AVERAGE (°)	DISTANCE BETWEEN EXPLANTS (mm)
1/29/66	1	29	+ 529	+18.2	0.33
1/29/66	2	64	+1301	+20.3	0.34
2/10/66	1	62	+1072	+17.3	0.29
2/19/66	2	142	+1539	+10.8	0.31
3/ 5/66	1	25	+ 441	+17.6	0.51
TOTALS		322	+4882		

AVERAGE OF TOTALS: +15.3

TABLE XXXVIII

MEASUREMENTS OF CELL AXIS ANGLES

Slide contained 2.5 ml of Medium and 45 µg of Lyophilized G-25 Fraction in 45 µl of Water

DATE	SLIDE NUMBER	NUMBER OF ANGLES MEASURED	ACCUMULATED TOTAL (°)	AVERAGE (°)	DISTANCE BETWEEN EXPLANTS (mm)
1/29/66	2	31	+ 189	+6.1	0.51
1/29/66	2	36	+ 147	+4.1	0.71
2/19/66	1	177	+ 307	+1.7	0.34
2/19/66	2	62	+ 644	+10.4	0.52
3/ 5/66	1	6	+ 2	+0.3	0.22
TOTALS		312	+1289		

AVERAGE OF TOTALS: +4.1

TABLE XXXIX

VOID VOLUME DETERMINATION WITH 0.5 ML BLUE DEXTRAN AND THE ELUTION OF TRYPSIN AND BOVINE SERUM ALBUMIN ON SEPHADEX G-100 (12/6/65)

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
	Blue Dextran			Trypsin	
23.66	100	0.000	41.75	Pooled	
26.58	38	0.420	44.53	100	0.000
29.71	65	0.187	47.57	100	0.000
32.46	95	0.022	50.99	100	0.000
			54.32	92	0.036
			57.63	73	0.137
	Bovine Serum Albumin		60.91	64	0.194
			64.66	65	0.187
21.33	Pooled		67.59	65	0.187
23.48	92	0.036			
26.54	55	0.260	70.70	59	0.229
			74.05	54	0.268
29.24	42	0.377	77.52	55	0.260
32.48	54	0.268			
36.04	17	0.770	80.32	57	0.244
			83.57	58	01237
38.94	7	1.155	87.19	54	0.268
41.61	42	0.377			
44.69	85	0.071	90.41	52	0.284
			94.11	52	0.284
47.83	96	0.018	97.55	54	0.268
50.71	98	0.009			
53.62	99	0.004	100.37	61	0.215
			103.22	70	0.155
56.51	100	0.000	106.22	78	0.108
59.26	100	0.000			
			109.88	81	0.092
			112.94	89	0.051
			116.27	89	0.051
			119.37	94	0.027
			122.80	95	0.022

TABLE XL

VOID VOLUME DETERMINATION WITH 0.5 ML OF BLUE DEXTRAN AND THE ELUTION OF TRYPSIN AND BOVINE SERUM ALBUMIN ON SEPHADEX G-100 (2/17/66)

EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY	EFFLUENT VOLUME (ml)	% TRANS-MISSION	OPTICAL DENSITY
	Blue Dextran			Trypsin	
26.97	100	0.000	56.92	100	0.000
29.96	66	0.181	60.00	95	0.022
32.98	38	0.420	62.96	72	0.143
36.02	92	0.036	65.68	56	0.252
38.89	99	0.004	68.57	49	0.310
			71.68	55	0.260
	Bovine Serum Albumin				
			75.22	60	0.222
24.69	Pooled		79.31	62	0.208
27.61	97	0.013	82.12	61	0.215
30.81	86	0.066	85.05	62	0.208
			88.25	65	0.187
33.65	54	0.268	91.49	64	0.194
36.52	53	0.276	94.50	60	0.222
39.46	54	0.268			
			97.65	56	0.252
42.29	19	0.721	100.59	55	0.260
46.17	45	0.347	104.78	59	0.229
52.31	83	0.076	107.49	67	0.174
55.14	84	0.076	111.12	77	0.114
58.12	99	0.004	113.97	84	0.076
61.09	100	0.000			