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AN AMINO ACID BINDING PROTEIN FROM RAT NERVE

by

DONNA M. PEACH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled AN AMINO ACID BINDING PROTEIN FROM RAT NERVE submitted by DONNA MAY PEACH in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in EXPERIMENTAL SURGERY

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ABSTRACT

The transport of material along the nerve axon (axoplasmic transport) ensures a supply of the materials needed for repair and maintenance of the nerve structure. Pulse labelling experiments with amino acids have provided much of our present knowledge of axoplasmic transport. It has been well demonstrated that amino acids are transported along the nerve after incorporation into a protein. However, other modes of transport are possible including the transport of free material and the transport of bound material. This research project was designed to test these other possibilities. A study was made of the *in vitro* leucine binding properties of protein from homogenates of rat sciatic nerve. These samples were incubated with tritiated leucine, under conditions similar to those used by other investigators for the assembly of microtubules.

Leucine is competitively bound to a soluble protein of 97,000± 26,000 MW. The kinetic characteristics of this binding process were calculated, in order to describe the number of binding sites, the equilibrium constant, rate constants, and the changes in entropy and enthalpy. The leucine binding protein assembles into a very large protein polymer, which may contain up to five other types of

proteins. The rate at which leucine binding protein polymerizes is increased by the presence of free Mg^{++} ions in the incubating solution, and decreased by the presence of urea. The formation of the polymer does not appear to alter the conformation of the leucine binding sites. Trypsin and large amounts of colchicine inhibit the polymer formation.

There is Mg^{++} activated ATPase activity present in the smaller molecular weight fractions, and also in association with the large protein polymer. There is no measurable (Na^{+} + K^{+}) ATPase activity.

Proline, thymidine and succinic acid bind to the polymer fraction, particularily in the presence of Mg^{++}, while colchicine appears to bind to a subunit of the polymer. Proline and thymidine do not compete for the leucine binding sites, but appear to have other binding sites available.

The presence of specific binding sites on the protein from rat sciatic nerve suggests that leucine may be transported or stored in rat sciatic nerve in a protein bound form rather than incorporated into a protein structure.

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CHAPTER 1

INTRODUCTION

Nerve axons and terminals depend upon axonal transport to supply enzymes and other compounds to maintain their structural integrity and viability. Much of the knowledge of axonal transport comes from pulse labelling experiments with amino acids. These labelled amino acids are injected into or near the neuron cell body, and are subsequently transported along the nerve. Generally, the labelled material appears to be transported in association with protein (see review by Ochs, 1972). Several modes of transport might be possible. These are; transport of free labelled material, which may later become associated with protein in the neuron; transportation in a protein-bound form, or incorporation of the label into a protein structure. It is widely accepted that amino acids are transported along the axon following incorporation into protein (Ochs, 1972).

Several neuronal structures have been examined in an attempt to assign the axoplasmic transport of material to a specific functional unit. Microtubules, together with their side arms or bridges, microfilaments, and neurofilaments have been the main subjects of this search. Microtubules

have been implicated in cellular movement (Rudinska, 1965, 1967; Bikle et al, 1966). A general feature of the recent theories of the role of microtubules, is that the microtubules are not the force generating mechanism (Schmitt, 1969). In cilia, the microtubules do not appear to contract, but rather slide past one another (Satir, 1965, 1968). This is similar to the actomyosin system of skeletal muscle. Bridges have been found associated with almost all microtubule associated motile mechanisms (see review by Tilney, 1971). This structure is thought to be the force generating mechanism. Gibbons (1965) has found that the microtubules of cilia bind a large ATPase (dynein) which forms side arms or projections. Dynein may generate the forces that slide microtubules parallel to their neighbours (Summers and Gibbons, 1971). Sliding systems have been implicated in other motile systems which have microtubules as components, but there is less information available about the structure and function of the intertubule bridge.

The function of neurofilaments has not yet been established. A possible relationship between microtubules and neurofilaments has been suggested. During the development of the optic nerve, tubules were found to be replaced by filaments as the development proceeded (Peters and Vaughn, 1967). If the nervous tissue was treated with colchicine, there was a reversible replacement of tubules by

filaments (Wisniewski and Terry, 1968). Some investigators believe that neurofilaments may function as a part of the force generating mechanism in axoplasmic transport (Weiss and Mayr, 1971). Microfilaments are thought to be the contractile machinery of non-muscle cells (Wessells et al, 1971). The microfilaments in nerve appear to function in nerve elongation, and are found in the growth cone (Yamanda et al, 1970). Microfilaments and actin are of similar diameter, and are both inhibited by cytochalasin B (Wessells et al, 1971; Spudich and Lin, 1972). Spudich and Lin (1972) speculated that microfilaments may be actin-like proteins.

Microtubules, neurofilaments and microfilaments can be differentiated by the use of the drug colchicine. It disrupts microtubules (Tilney, 1968), but not microfilaments or neurofilaments (Wessels, 1971). However, colchicine induced proliferation of neurofilaments has been reported (Shelanski and Taylor, 1970).

Specific proteins have been isolated from neuronal structures, and characterized. Microtubule protein; tubulin and a tubulin dimer (colchicine binding protein) have been the subject of many investigations (Shelanski and Taylor, 1967, 1968; Renaud, 1968; Taylor, 1965; Borisy and Taylor, 1967). An actin-like protein (neurin) has been found in the membrane portion of the synaptosomal fraction from mammalian

brain (Puszkin et al, 1972; Berl et al, 1973). It has been suggested that this protein may be associated with certain microfilaments in close proximity to plasma membrane in neurons and glia (Wessells et al, 1971). A myosin-like protein (stenin) has been isolated from vesicles in the synaptosomal fraction of mammalian brain (Puszkin et al, 1972; Berl et al, 1973). Like muscle actomyosin, these proteins exhibit Ca, Mg ATPase activity (Puszkin et al, 1972). Thus, all the components of a system capable of functioning in a similar manner to the sliding filament system of muscle actomyosin, appear to be present in brain. The sequence of events and any relationship to axoplasmic transport has not been elucidated.

The research on the molecular mechanisms of axoplasmic transport reported in this thesis developed during investigations on the in vivo transport of ^{3}H-leucine in rat sciatic nerve. In order to examine the possibility of leucine binding, and eliminate the incorporation of leucine into protein, homogenates of rat nerve were used. The ^{3}H-leucine was added after homogenation, incubated and separated by gel filtration using Sephadex. Competitive binding techniques were employed, which were a slight modification of the techniques developed by Russell and Doty (1973). The conditions used in the incubation mixtures were similar to those shown to favor in vitro assembly of

microtubules (Weisenberg, 1972; Borisy and Olmsted, 1972) They were also appropriate for the measurement of ATPase activity (Charnock, Russell and Doty 1971). It became apparent that leucine was bound to a soluble protein fraction from rat sciatic nerve. This leucine binding protein assembles into a large protein polymer, at a rate which is influenced by the presence of Mg^{++} ions. Under the conditions used for binding and polymerization, a Mg ATPase is activated, which at times becomes associated with the protein polymer fraction.

The properties of the leucine binding protein were examined. The number of binding sites per molecule of protein, the amount of binding protein and the kinetic data for the binding process were evaluated using competitive binding techniques and measurement of protein concentration (Lowry, 1951). Arrhenius plots were made of the effect of temperature on binding. In order to evaluate a possible relatioship between the colchicine binding protein (microtubule protein) and leucine binding protein, the binding of ^{3}H-colchicine was also studied. Some kinetic data for colchicine binding has been published (Owellen et al, 1972). It was used in a comparison with the kinetic data for leucine binding.

In order to characterize the leucine binding protein, SDS acrylamide gel electrophoresis was employed. Using the method of Weber and Osborn (1969), the molecular weight of all the proteins was estimated. The combination of proteins, in each gel filtration fraction, was assesed as polymerization proceeded. The data was compared to the values for bound ^{3}H-leucine in duplicate fractions. This established a relationship between the presence of a specific protein and the bound ^{3}H-leucine. In order to characterize any association of the leucine binding protein with known neuronal structures, colchicine, urea, trypsin, ATP and RNAase were added to the incubation mixtures.

Any possible involvement of the leucine binding protein with the process of axoplasmic transport, would imply the presence of an energy source. Fast axoplasmic transport depends upon oxidative phosphorylation, with adenosine triphosphate (ATP) supplying the energy to the transport mechanism (Ochs, 1972). ATPase activity has been associated with microtubules (dynein-Gibbons, 1965) and neurostenin (Puszkin et al, 1972; Berl et al, 1973). For this reason, the ATPase activity ($Na^{++}K^{+}$, and Mg^{++}) was monitored during the binding of leucine and the polymerization of the leucine binding protein. The ATPase activity was measured using a rapid method established in this laboratory (Charnock, Russell and Doty, 1971).

The following chapter will survey significant work published in the field of microtubules, microfilaments and neurofilaments. The properties of actin-like and myosin-like proteins will also be reviewed, along with the significant features of ATPase activity present in the nervous system.

LITERATURE REVIEW

A. MICROTUBULES

1. Structure and distribution

Microtubules are not unique to neuronal tissue. Homologous structures are found in the cytoplasm of virtually all eukaryotic cells. The microtubule is a cylindrical structure of indefinite length, with a diameter generally reported as 240± 20 A°. On cross section, the wall of the microtubule consists of 12-13 subunits, 4-5 nm in diameter. In longitudinal view, the tubule has rows of parallel protofilaments arranged to form a hollow cylinder (Pease, 1963; Barnicot, 1966; Porter, 1966). These microtubules are frequently arranged parallel to the long axis of cellular extensions.

Cross bridging between neighbouring microtubules is found only when they are arranged in closely packed bundles, as in the mitotic spindle, in intial segments of the neurcnal axon (Palay et al, 1968), or in the axons of Purkinje cells (Kohno, 1964). It has been postulated that the bridges serve to slide tubules or other organelles with respect to one another. In dividing cells, tubules separated by distances greater than 40nm are found to have arms

morphologically identical to cross bridges, arranged at regular intervals on the surface of the microtubule (Gibbons, 1963). Cross bridging or attachment between microtubules and other cellular organelles also occurs. Cross bridging between microtubules and synaptic vesicles has been shown in the spinal cord of lamprey larve (Jalfors and Smith, 1969).

2. Function

Microtubules seem to be associated with diverse cellular functions including; chromosome movement in cell division (Nicklas, 1967; Luykx, 1969); sensory transduction (Moran and Varela, 1971); development and maintenance of cell form (Tilney and Gibbons, 1968); intracellular transport of material, ie. movement of cytoplasmic constituents especially in relationship to axoplasmic flow (Rudinska, 1965, 1967; Bilke et al, 1966; Freed and Lebowitz, 1970; Schmitt, 1969) and cellular motility (Goldman, 1970). The distribution of cytoplasmic constituents from the site of synthesis to the site of utilization is a vital cell function. In the long processes of neurons, product utilization may occur at great distances from the perikaryon. Various microtubule disruptive treatments have been used to interrupt axoplasmic flow in

neurons (Dahlstrom, 1968; Kreutzberg, 1969) and the saltatory movement of particles in cultured cells (Freed and Lebowitz, 1970). Disruption of cells with colchicine causes them to lose the ability to secrete histamine (Gillespie et al, 1968) and insulin (Malaisse-Lagae et al, 1971). The data thus far suggests microtubules are involved in the ordered movement of cytoplasmic constituents, particularily those which are membrane enclosed.

The exact role of microtubules in generating motive forces is unclear. Microtubules in cilia probably slide past each other, rather than contract (Satir, 1965). This process would presumably require ATP, which is hydrolyzed by the high molecular weight ATP'ase arms (dynein) found bound to the outer doublet microtubule of cilia (Summer and Gibbons, 1971).

C. TUBULIN

1. Structure and distribution

From a variety of sources of microtubules a class of closely related proteins (called tubulins) has been isolated. These comprise the subunits of microtubules in

cilia, flagella and sperm cells (Shelanski and Taylor, 1967, 1968; Renaud et al, 1968; Stephens, 1968a), and in all major cellular elements of mammalian brain (Raine and Wisniewski, 1970; Peters and Vaughn, 1967; Friede, 1970). The 'native' form of disassembled tubules is a dimeric protein with a sedimenation velocity of 6S and a molecular weight reported as 100,000 to 130,000 (Mohri, 1968). When tubulin is denatured in guanidine-HCL or sodium dodecyl sulphate (SDS), two subunits of approximately the same size are obtained, Tubulin A and Tubulin B. In flagella, Tubulin A is 53,000 MW while Tubulin B is 56,000 MW (Olmsted et al, 1971). Recently evidence has been obtained that these subunits differ not only in molecular weight but in cyanogen bromide peptide maps and amino acid composition (Cohen and Rubhen, 1970). Native microtubular protein is phosphorylated specifically on the B Tubulin (Eipper, 1972). The amino acid composition of the tubulins resembles that of the material isolated from the outer doublets of cilia and flagella (Weisenberg et al, 1968). In cilia, microtubules are associated with dynein (an ATPase) which attaches to one tubule (A tubule) of the outer doublet, forming arms or projections (Gibbons, 1963).

2. Chemistry

a. Colchicine binding activity The binding of the drug to tubulin appears to be very specific since it binds only to the 120,000 MW dimer of microtubular protein and not to the intact microtubules (Wilson, 1970). It is not known whether the dimer is composed of A or B Tubulin or both. Under favorable conditions one mole of colchicine is bound per 120,000 daltons of tubulin. The optimal binding conditions are pH=6.7-6.8, low ionic strength, GTP (1mM), and Mg^{++} (1mM). Destruction of the binding activity by urea, trypsin or trichloroacetic acid suggests activity is associated with a protein (Wilson , 1970). Identical electrophoretic patterns have been obtained from colchicine binding material and intact microtubules isolated from brain (Kirkpatrick et al, 1970). Therefore it is commonly assumed that the colchicine binding fraction in neuronal tissue is in the microtubule subfraction.

The lack of response of assembled cilia and flagella as well as centrioles to various microtubule disruptive treatments has been well documented. However, these assemblies are susceptable to colchicine (Turner, 1970). It has been postulated that structures consisting of highly ordered arrangements of microtubules may be stabilized through intermicrotubular linkages, or differential

modification of the protein itself (Tilney, 1968). It has been generally accepted that the binding of colchicine to the microtubule subunits might prevent the formation of intermicrotubular linkages. In most cells, colchicine binding activity (CBA) is almost totally found in the 100,000g supernatant. However, approximately one-half of the CBA was found in the particulate fraction by Feit and Barondes, (1970). Subfractionation of this particulate fraction showed the greatest specific activity in nerve endings and in microsomes sedimenting to the 1.0-1.2M sucrose interface on discontious gradients. They therefore conclude that the action of colchicine cannot be directly on the neurotubules.

Colchicine inhibits the release of catecholamines from the adrenal medulla (Poiser and Bernstein, 1971). From the above results, Berl et al (1973) proposed that colchicine was acting on the actomyosin-like proteins at the nerve endings.

b. Nucleotide binding activity Isolated microtubule protein thus far has been found to contain bound guanine nucleotide, in a mixture of GDP and GTP. For each 6S dimer there are 2 moles of GTP noncovalently bound (Borisy, 1970). One mole is tightly bound and nonexchangable while the other has a turnover time of 15 minutes. The aggregation of tubulin was

initially thought to be dependent upon the presence of GTP. More recent evidence shows that the addition of ATP (Marx, 1973) or solutions containing 1M sucrose or 4 M glycerol (Olmsted and Borisy, 1973) also favors polymerization. In the presence of sucrose or glycerol, the time of polymerization is extended to 5 hours.

c. Phosphatase and kinase activity : Recently it has been reported that purified tubulin can be phosphorylated in the presence of an AMP stimulated protein kinase, as well as serving as a protein kinase itself (Soifer et al, 1972). Phosphate is covalently linked to a serine residue in the faster moving of the two electrophoretically separated tubulin bands. The ability of microtubular protein dimers to polymerize could be regulated by the state of phosphorylation of protein subunits (Eipper, 1972).

3. Axoplasmic flow of tubulin

There is some evidence to suggest not only a role for tubulin in axoplasmic transport but that tubulin is transported as well (Shelanski et al, 1968). The presence of tubulin in nerve endings and its transport by axoplasmic flow has been reported (Feit et al, 1971a).

4. Assembly of tubulin

A. Assembly in vivo: Although microtubules in different cell types appear to be similar morphologically and chemically, they differ in association and dissociation stability. Colchicine (10^{-7} M) inhibits polymerization of microtubules from porcine brain extracts (Olmstead and Borisy, 1973). Colchicine also blocks the neurite outgrowth of neuroblastoma cells in culture (Seeds et al, 1970), and of neurons (Yamada and Wessells, 1971). The more stable microtubules are insensitive to colchicine (Behnke and Forer, 1967), hydrostatic pressure (Tilney and Gibbins, 1968), and low temperature (Behnke and Forer, 1967). Polymerization of microtubules requires Mg^{++} (<10mM), EDTA or EGTA, and Na^{+}. The doublet arrangement is less stable than the nine peripheral pairs in cilia (Behnke and Forer, 1967). The control of microtubule distribution in cells is believed to be dependent upon the dynamic equilibrium which exists between the polymer and its monomer, maintaining an adequate pool of re-usable monomer (Inoue, 1964). It has been suggested that colchicine binds to the monomer and prevents reassembly. This has been accepted as a reasonable explanation for in vivo assembly. Changes in the length of the existing microtubule might be controlled by transient conditions within the cell affecting this equilibrium.

Colchicine is believed by some to prevent polymerization by shifting equilibrium to the monomeric state (Borisy and Taylor, 1967). The control and distribution of microtubules in cells must also involve loci which affect the nucleation of the microtubules. These are thought to include the basal body (Renaud and Swift, 1964), centriole (Gibbins et al, 1969) and associated satellites, the kinetochore (Brinkley and Nicklas, 1968) and other dense particles, many of which are membrane associated. Bridges connecting adjacent microtubules appear to control distribution of the microtubules as well as being active in motility (Tilney and Byers, 1969). The bridges may provide the active force in diverse motile processes, a motion which might be carried out by relative sliding of tubules past each other (McIntosch and Porter, 1967; Subriana, 1968).

B. Assembly in vitro The assembly of microtubules in extracts of porcine brain tissue has been characterized by viscometry. Conditions for maximum polymerization of microtubules are pH=6.7-6.8, 37° C, GTP >2.0mM or other nucleotides, (ATP CTP, or GDP and GTP). Polymerization occurs to a lesser extent in 1M sucrose or 4M glycerol. In vitro polymerization of microtubules also requires Mg^{++}, EDTA or EGTA and Na^{+} (Olmsted and Borisy, 1973). Colchicine (0.1 M) inhibits viscosity development, but only causes

partial depolymerization of formed tubules (Olmsted and Borisy, 1973). These authors report the morphology, kinetics, drug sensitivity and temperature dependence of the polymerization of porcine brain tubulin in vitro is similar to the in vivo assembly of microtubules in the mitotic spindle.

The in vitro assembly of microtubules, using subunits obtained from sea urchin sperm tails by treatment with mild detergent, does not appear to be analogous to that postulated for the mitotic spindle. The polymerization reaction was not inhibited by cold or colchicine (Stephens, 1968).

Microtubule assembly in the low speed extracts of porcine brain proceeded with the concomitant disappearance of disc like structures. The discs reappeared if the tubules were destroyed by cold, colchicine or Ca^{++}. These structures may be nucleating centres. In the high speed extracts, subunits form microtubules at 37° after some hours, as compared to minutes in the low-speed extracts. No disc like substances were present in the high speed extracts (Durham et al, 1971).

3. Homology to Actin

Microtubule protein from cilia has similar properties to those of actin (Gibbons, 1963). The extraction in low ionic strength solutions, the electrophoretic mobility on 7.5% SDS (sodium dodecyl sulfonate) acrylamide gels, the presence of bound nucleotides, associated ATP'ase activity, as well as association of microtubules with motile organelles are similar (Shelanski and Taylor, 1967; Gibbons, 1963). Colchicine binding protein from blood platlets and mammalian brain has the actin like characteristics of inducing the activation of Mg^{++} ATP'ase of myosin (Pushkin and Berl, 1970). In sucrose solutions, both actin and tubulin do not require the presence of nucleotides to polymerize although polymerization is slower. Addition of ATP to mixtures of myosin and colchicine binding protein resulted in a decrease in the relative viscosity, which increased again in 30-60 minutes. This is similar to the muscle myosin and actin preparations (Puszkin and Berl, 1970). Microtubular protein and actin differ in molecular weight, electrophoretic mobility in 5 % SDS gels and amino acid composition. The actin-like protein may be associated with the synaptcsomal membrane or with microfilaments. The 3-methyl histidine marker found in actin is not found in microtubules. The data suggests that as in actin polymerization, the binding and hydrolysis of guanine

nucleotides might be significant in the microtubule assembly. The association of guanine nucleotides with isolated microtubule protein is significant in stabilizing the native configuration of the protein.

C. NEUROFILAMENTS

The neurofilament is an 80-100 A° diameter filament, found normally in the cell body and axon of the neuron. It is composed of globular subunits and often has small arms protruding from it (Wuerker, 1970). A subunit of 60,000 MW has been isolated from mammalian brain (Albert et al, 1970). A larger subunit of 73,000 MW has been isolated from squid axon (Davison and Taylor, 1960; Huneeus and Davison, 1970).

The [redacted]tion of the neurofilament has not been established. Some researchers believe that it may function as a part of the force generating mechanism in axoplasmic transport (Weiss and Mayr, 1971). Some cases have been cited, where there seems to be a reversible replacement of tubules by neurofilaments (Peters and Vaughn, 1967; Wisniewski et al, 1968).

The neurofilament is dissimilar to microtubules, in amino acid composition. Unlike microtubules, it does not bind GTP or colchicine (Davison and Huneus, 1970). A number

of agents; aluminum, colchicine, vinblastine, podophyllotoxin, various nitriles and acrylamide have been reported to induce proliferation of neurofilaments (Shelanski and Taylor, 1970; Wisniewski et al, 1970).

D. MICROFILAMENTS

Microfilaments are fine, filamentous structures, 40-60 A° in diameter. They have been found in a variety of plant and animal cells, at points where contraction is believed to take place. They are found in the tips of growing axons and in the microspikes which project from this growth cone. Microfilaments are arranged in a polygonal pattern in the growth cone and in an extended linearly orientated pattern in the microspikes (Yamada et al, 1970).

The location of microfilaments in cells, as determined by electron microscopy, suggests that they may be involved in cytoplasmic streaming, cytokinesis, nerve axon production, changes in cell shape during embryo production, blood clot retraction, amoeboid movement and other forms of cell motility (see reviews by Jahn and Bovee, 1969; Wessells et al, 1971). The microspikes, which contain microfilaments, continually extend and retract providing direction for the elongating axon (Hughes, 1953; Nakai, 1956).

The structure of microfilaments is reversibly disrupted by cytochalasin B (Schroeder, 1970; Wessells et al, 1971). Cytochalasin B also interacts with muscle actin and appears to compete with muscle myosin for the binding of actin. It has been suggested that microfilaments may be actin-like proteins (Spudich and Lin, 1972).

E. ACTIN AND MYOSIN LIKE PROTEINS

The isolation of actomyosin-like, actin-like and myosin-like proteins from various sources, including mammalian brain have been described. The proteins from mammalian brain were named neurostenin, neurin, and stenin, respectively.

1. Actin like proteins

a. Distribution: Actin like protein has been isolated from sea urchin eggs (Miki-Noumura and Oosawa, 1969), sperm tails and the mitotic spindles of the crane fly (Behnke et al, 1971), mammalian brain and from cultures of chick sympathetic ganglia (Berl et al, 1973).

b. Function: The neurin, like muscle actin, stimulates the Mg^{++} ATP'ase activity of stenin (myosin-like neuroprotein). The relative viscosities of mixtures of neurin with stenin,

neurin with myosin, and actin with stenin were increased and became sensitive to added ATP, in a similar manner to muscle actin and myosin mixtures (Berl et al, 1973). The mixtures rapidly decreased in visocity, upon addition of ATP, and then increased again as the ATP was hydrolyzed. Similarly to actin, the neurin contains the 3-methylhistidine marker. SDS gel electrophoresis on polyacrylamide gels has indicated the MW of neurin to be approximately 47,000. This value is close to reported values for muscle actin of 43-47,000 MW. The sucrose gradient of membrane protein from a rat brain synaptosomal fraction was similar to neurin, in that it demonstrated little enzyme activity alone but enhanced the Mg^{++} ATPase activity of muscle myosin (Berl et al, 1973 1966; Freed and Lebowitz, 1970; Schmitt, 1969) and cellular motility (Goldman, 1970). Actin-like protein may be associated with the membranes or with microfilaments in close proximity to plasma membranes in neurons and in glia (Wessels et al, 1971).

C. Chemistry: Neurin isolated from brain tissue contains bound nucleotides, which exchange with free ^{14}C ATP. Neurin polymerizes in the presence of 0.1 M KCL and 0.1 mM Mg^{++} with the release of inorganic phosphate. The ability to isolate neurin from neurostenin is like that described for muscle actomyosin (Szent-Gyorgyi, 1951). The neurin from

bovine brain and also the colchicine binding material reacted immunologically with antiserum to neurostenin, to form a single band. This seems to indicate that common antigenic properties exist between the protein isolated from the microtubules and from neurin (Berl et al, 1972).

2. Myosin-like proteins

Myosin like protein has been obtained from non-muscle tissues such as acanthameoeba (Pollard and Korn, 1971), slime mold (Adelman and Taylor, 1969) and mammalian brain (Berl et al, 1972).

The relative viscosity of mixtures of actin with stenin, and neurin with stenin, were increased by and sensitive to, added ATP as described for actin like proteins. The vesicicle protein isolated from the rat synaptosomal fraction, was similar to stenin isolated from whole brain or synaptosomal neurostenin. Myosin-like proteins may be located in the vesicular membrane, or associated with its exterior or partially embeded in it (Berl et al, 1973). Myosin-like proteins and vesicle protein exhibit Ca^{++} stimulated ATP'ase activity and little Mg^{++} stimulated activity (Berl et al, 1973).

Similar to striated muscle myosin, stenin contains the same 3-methylhistidine and N-methyllysine markers (Berl et

al, 1973). SDS gel electrophoresis on polyacrylamide gels indicates that the MW of stenin is 240,000 (Berl et al, 1973), which is close to the accepted value for muscle myosin of 190,000-210,000.

3. Actomyosin like proteins

Contractile proteins similar to actomyosin have been isolated in ascites sarcoma cells (Hoffman-Berling, 1956), blccd platlets (Bettex, Galland and Luscher, 1960), adrenal medulla , slime mold plasmodia (Adelman and Taylor, 1969) and synaptosomal fractions from bovine and rat brain (Berl et al, 1972). These could not however be isolated from purified mitochondrial, microsomal, myelin or supernatant fractions. From 8-10% of the synaptosomal protein was isolated as neurostenin.

There are common antigenic properties between neurostenin and colchicine binding protein (ie. microtubular protein) and neurin (Puszkin and Berl, 1972). The isolation of neurostenin from nerve endings in the synaptosomal subfraction of brain tissue raises the speculation that this protein may function in movement to the nerve endings in association with plasma membranes and neurofilaments.

F. PROTEIN BINDING

Many proteins are able to interact with small molecules, and form complexes by secondary valence forces. This binding can either be specific or non specific. There may be more than one species of binding site for a ligand. Examples of binding are found in inhibitor or substrate binding to an enzyme; binding of drugs to plasma proteins and protein binding of steriods, vitamins or metal ions (see Murphy, 1970; Wood and Cooper, 1970 for reviews). The binding of the anti-mitotic drugs, colchicine, vinblastine and vincristine to microtubule protein has also been shown (Weisenberg, Borisy and Taylor, 1968).

The presence of specific binding sites on a protein make it possible to develop a sensitive, specific assay for the bound material using competitive binding techniques. This would indicate the level of the circulating bound material in addition to the level of the free material which is normally measured (Murphy, 1970).

Subsequent chapters describe an <u>in vitro</u> protein binding system from rat sciatic nerve. The properties of this system are used to develop a model for the axoplasmic transport in peripheral nerve.

CHAPTER 2

METHODS AND MATERIALS

A. PREPARATION OF BINDING PROTEIN

Wistar male rats (300-450g), were purchased from Woodlyn Farms, Guelph, Ontario and boarded in the Health Sciences Animal Centre. They were anesthesized with 45 mg/kg Sodium Pentobarbital (Diabutal), without proir starvation. The sciatic nerve was exposed dorsally by dissection from the distal end of the tibia to the spinal cord, in the region where the L5 dorsal root makes its entry. Each sciatic nerve (excluding the ganglion) was removed within a 15 minute time interval, and placed in 1 ml of 50 mM phosphate buffer containing 0.3M sucrose and 2.5mM EDTA, at pH=6.8. The nerves were then homogenized with a Potter-Elvejheim homogenizer. Charcoal (600 mg of Norit A-Fisher Chemical Co.,Montreal, Quebec), was then added. The preparation was then refrigerated overnight, in order to strip the endogenous material from the protein binding sites. The nerve preparation was then centrifuged at 1000g for 10 minutes at 2° C. The supernatant was recentrifuged twice more, under the same conditions.

The high-speed fraction (100,000g supernatant) was

Prepared following essentially the same procedure as above, with one further centrifugation. The low-speed supernatant was centrifuged at 35,000 rpm for 30 minutes in a Beckman L2-50 ultracentrifuge at 2° in a type 50.1 rotor. The supernatant was then removed and stored at 0° C. Urea (1.3M) was added to several of the nerve preparations (1:1), after centrifugation was complete.

B. ASSAY SYSTEM

L-leucine 4-5-^{3}H (30Ci/mM), L-proline-3-4-^{3}H (29.8 Ci/mM), thymidine methyl-^{3}H (50.3Ci/mM), or succinic-2-3-^{3}H acid (5.0Ci/mM), (New England Nuclear, Dorval, Quebec) were diluted with distilled water to 6×10^{-3} mC/ml. Colchicine (ring; C methoxyl-^{3}H; Amersham/Searle, Chicago, Illinois), was diluted with absolute ethanol. Aliquots (0.1 ml) were pipetted into 13x100 mm test tubes and dried in a vaccuum oven at 22° C.

L-leucine, L-proline, thymidine and succinic acid (Sigma Chemical Co., St. Louis) were dissolved in distilled water to give a final concentration of 100 mg/ml. Standards of 10×10^{-6} g/ml were prepared from this solution. Test tubes containing 1-10 x 10^{-6} g of the standards were prepared and dried in a vaccuum oven at 22° C.

C. GEL FILTRATION

G-200 Sephadex and G-25 Sephadex (Pharmacia, Uppsula, Sweden) were prepared in slurry form by soaking with 50mM phosphate buffer, pH=6.8. Glass columns fitted with 20 ml reservoirs were used. The columns were plugged with fine glass wool, and filled with 250 mg of the Sephadex slurry.

D. PROTEIN BINDING

Binding studies were conducted by adding 0.1 ml samples of the nerve preparation (1000g supernatant) to tubes containing the dried, tritiated leucine. Mg^{++} (2.5mM) was added to some of the incubation mixtures, as was colchicine (0.25 mM and 2.5 mM) or 3 mM ATP (Adenosine 5-triphosphate; Sigma Chemical Co., St. Louis). The samples were incubated for varying lengths of time, normally at 22° C, unless otherwise stated.

The incubated radioactive protein preparation was placed in the glass column just above the level of Sephadex, with a Pasteur pipet. When the sample had passed into the Sephadex column, it was followed by a 0.1 ml buffer rinse, then 10 mls of buffer was added to the column resevoir. The

eluate from the column was collected in 1 drop (0.053 ml) fractions, directly into liquid scintillation counting vials. Aquasol (7 mls; New England Nuclear) was added to each vial, the samples cooled and counted in a Nuclear-Chicago Mark 2 liquid scintillation counter.

Blanks, consisting of buffer incubated with radioactive leucine were run through the Sephadex columns. Control samples were incubated simultaneously with experimental samples to determine the total radioactivity present in the preparation.

E. COMPETITIVE PROTEIN BINDING

1. ^{3}H leucine

Aliquoits of 0.1 ml of nerve preparation were added to 13x100 mm test tubes containing the dried, radioactive leucine, and incubated at 22° C for 6 hours. At this time these protein-^{3}H-leucine samples were removed from the incubation tubes and placed in test tubes, in which 1-10 x 10^{-6} g of non-radioactive leucine had previously been dried. Tubes containing the neuroprotein, radioactive leucine and non-radioactive leucine (as required) were left for a further 18 hour period, at 22° C. Samples were then

fractionated on G-200 Sephadex and the percent radioactive leucine bound was determined. A blank sample (labelled leucine, 0.1ml buffer) and a control sample (labelled leucine, 0.1ml protein) were run simultaneously.

Standard binding curves were constructed by plotting percent bound ^{3}H leucine as a function of the total ^{3}H leucine present.

2. Specificity of binding sites

Aliquots of 0.1 ml of the nerve preparation were incubated with ^{3}H-leucine and 2.5 mM Mg^{++} for 6 hours at 22° C, as previously described. After this incubation, samples were removed and added to test tubes containing $1\text{-}10 \times 10^{-6}$ g of radioactive proline, thymidine, or succinic acid and incubated for a further 18 hours. The samples were then fractionated on G-200 Sephadex columns and counted. Blanks and control samples were run with each set of samples.

The amount of bound ^{3}H leucine was then estimated in samples with increasing amounts of these other compounds added in order to determine the specificity of the leucine binding sites.

F. ATPASE MEASUREMENT

The ATPase activity was measured by a H^+ measuring procedure previously reported (Charnock, Doty, Russell, 1971). In this method, the hydrolysis of ATP by the preparation is continously monitored by measurement of the change in pH associated with the formation of inorganic phosphate from substrate ATP. Each sample was back titrated with 0.1N NaOH, in order to calibrate the system. The slope of the pH recording is converted to equivalents of H^+ /min./gm of protein.

Samples of nerve preparation (0.1 ml, 2.5 mM Mg^{++} - added), were mixed with 1 ml of buffer (80mM Na^+, 20mM K^+, 0 mM Mg^{++}, 2.5mM PO^{4-}), pH=6.8. This solution is stabilized at 37° C, and 1mM Adenosine 5-triphosphate (disodium; Sigma Chemical Co.) added. The ensuing reaction was recorded. Ouabain (0.2mM; Sigma Chemical Co.) was selectively added prior to the ATP addition, in order to inhibit the $Na^+ + K^+$ ATPase activity but not the Mg^{++} ATPase activity.

G. PROTEIN CONCENTRATION

Protein concentration was determined by the Lowry method (Lowry et al,1951). A 0.1 ml sample of the nerve preparation was generally fractionated on G-200 Sephadex columns before the protein concentration was measured. The standard used for the determination was prepared from bovine serum albumin. Samples were read, at 750 mu, in a Unicam SP 500 Spectrophotometer.

H. GEL ELECTROPHORESIS

Sodium dodecyl sulphate (SDS) gel electrophoresis on 7.5% acrylamide gels (1% SDS, 1% mercaptoethanol,8M urea) were prepared by the method outlined by Weber and Osborn,(1969). These gels were 5 mm in diameter and 6 cm im length. The voltage used was 8 mA/tube. Separation of native proteins on polyacrylamide gels in the presence of the anionic detergent sodium dodecyl sulphate, has been shown to be dependent on the molecular weight of the polypeptide chains. Samples of 0.1 ml of the nerve preparation with 2.5mM Mg++ present were incubated with ^{3}H-leucine for 1 and 6 hours at 22° C. These samples were then fractionated on G-

200 Sephadex columns prior to gel electrophoresis. Samples of mouse muscle myosin, C protein and actin were prepared by the above procedure and run on 7.5% gels. These were used as controls.

Collagenase (0.05 mls, Form 3), (Advance Biofactures Corp. Lynbrook, N.Y.), was incubated with the high molecular weight protein fractions for 1 hour at 37° prior to gel electrophoresis, in order to test for the presence of collagen in the samples. A blank containing collagenase and buffer was also incubated for 1 hour at 37° C and run in the gel electrophoresis experiments.

I. CHARACTERIZATION OF BINDING MATERIAL

1. Trypsin

A 0.1 ml sample of the nerve preparation was incubated with ^{3}H-leucine and 2.5mM Mg^{++} for 6 hours at 22° C. This preparation was then fractionated on G-200 Sephadex. The high molecular weight fraction (fraction# 5,6) containing the bound leucine was separated out and 0.05% trypsin (w/v) (Baltimore Biological Laboratory, Baltimore Maryland), was added to the incubation mixture, for 1 hour at 37° C. This sample was again run through a G-200 Sephadex column and

protein determinations run on each sample.

2. RNAase

RNAase was added to nerve preparation, (1×10^{-4} g/0.1ml sample). This sample was incubated with ^{3}H-leucine and 2.5mM Mg^{++}, for 4 hours at 37°C. Subsequent fractionation on G-200 Sephadex was used to determine the position of radioactive leucine and protein. Unfractionated samples were used as controls to determine total radioactivity.

J. ION MEASUREMENT

Na^{+}, and K^{+} ions were measured in samples of rat nerve preparation, rat gastrocnemius muscle, rat blood samples, in the phosphate buffer solution used for Sephadex filtrations, and salt solutions used in the ATP'ase measurements. These measurements were made using an IL Flame Photometer, Model 143.

Total concentration of Mg^{++} and Ca^{++} in rat nerve preparations (with and without 2.5mM Mg^{++} added) and in the rat muscle preparations were determined by atomic absorption techniques.

H. TISSUE SPECIFICITY OF BINDING PROTEIN

Rat gastrocnemius muscle (0.2 g/ml) was added, to the buffer (0.05M PO^{4}; 0.3M sucrose; 2.5mM EDTA, pH=6.8). Rat blood (1.0 mls) was also added to a 10 ml aliquct of buffer. Both of these samples were prepared in the same manner as the lcw-speed rat sciatic nerve preparations. Samples (0.1 ml) of each of the preparations were incubated with radioactive leucine (2.5mM Mg^{++}) for 6 hours at 22° C. These samples were then separated on G-200 Sephadex cclumns and the fractions ccunted to determine the amount of radioactivity which might be protein bound in either of these samples.

I. ION CONCENTRATIONS

Varicus ion concentrations (Mg^{++}, Ca^{++}, Na^{+},K^{+}), were measured in the 1000g supernatant of rat nerve and muscle homogenates, and in the various sclutions used in the experiments. The results are as follows:

A. Rat sciatic nerve homogenate

Na^{+} =70 mM

K^{+} =7.5 mM

Mg^{++} =1.0 mM

Ca^{++} =2.01 mM

B. Rat sciatic nerve homogenate with 2.5 mM Mg^{++} added.

Mg^{++} =3.56 mM

Ca^{++} =1.84 mM

The actual addition of Mg^{++}, as measured by atomic absorption techniques =2.56 mM.

C. Rat muscle homogenates.

Na^{+} =65 mM

K^{+} =7.5 mM

Mg^{++} =1.36 mM

Ca^{++} =1.96 mM

D. Phosphate buffer used in column preparation and for eluting buffer.

Na^{+} =94 mM

K^{+} =152 mM

E. Salt solutions used for ATPase measurements.

Na^{+} =85 mM

K^{+} = 29.5 mM

J. EFFECT OF EDTA

EDTA binds Mg^{++} and Ca^{++} ions in a ratio of 1:1. It has a greater affinity for Ca^{++} ions (log K Ca^{++}=10.6), than for Mg^{++} ions (log K Mg^{++} =8.7). On this basis, it may be shown that free, unbound Ca^{++} in the 1000g nerve homogenate, after addition of 2.5 mM EDTA , is equal to 0.025 mM. The free, unbound Mg^{++} is equal to 0.5 mM. In samples with 2.5 mM Mg^{++}

added, the total free Mg^{++} is equal to 3.0 mM Mg^{++}.

CHAPTER 3

RESULTS

A. LEUCINE BINDING

^{3}H-leucine (1.1×10^{-8} M) binds *in vitro* at 22° C, to a high molecular weight fraction in the 1000g supernatant from rat sciatic nerve. Fractionation of this labelled material on G-200 indicates the labelled peak to be in the vicinity of fraction #5,6 (Figs. 1 and 2). This region will hereafter be referred to as peak 1. The area after fraction 17 contains free (unbound) leucine, and small proteins or polypeptides. Reference markers indicate the retention volume of 302,000 MW collagen (fraction #8) and 68,000 MW bovine serum albumin (fraction # 16).

Fig. 1 shows ^{3}H-leucine binding in the presence of 2.5 mM Mg^{++}. The ^{3}H-leucine is associated with the high molecluar weight fraction (peak 1) within the initial one hour incubation period, and increases with time. In the absence of Mg^{++}, with other factors held constant (Fig. 2), the ^{3}H-leucine appears bound to a lower molecular weight

fraction, #10,11. This bound fraction appears in progressively larger molecular weight fractions, until at 17 hours, it becomes associated with the peak 1 area (Fig.2).

The time course of *in vitro* binding of ^{3}H-leucine, with and without Mg^{++}, is illustrated in Fig. 3. Each data point represents the total bound leucine in fractions 1-16 (inclusive). The initial rate of binding with no Mg^{++} present, is much slower than the rate with Mg^{++} present, and appears to be a two step reaction. This is also evident in Figs. 1 and 2.

Samples of the ~~100,000~~g supernatant from rat sciatic nerve, were incubated *in vitro* with ^{3}H-leucine, and Mg^{++} and fractionated on G-25 Sephadex (Fig. 4). The amount of ^{3}H-leucine present in fractions #9,10 increased with increasing time.

Thymidine-methyl-^{3}H (1.0×10^{-3}M), Fig.5; L-proline-3-4 ^{3}H (2.2×10^{-8} M), Fig.6 and succinic acid-2,3 ^{3}H (1.7×10^{-8} M), Fig.7 were incubated *in vitro* for 24 hours with the 1000g supernatant, under the same conditions used with ^{3}H-leucine (22°, 2.5 mM Mg^{++}, G-200 Sephadex). All of these compounds bind in the peak 1, high molecular weight fractions. When Mg^{++} is not added, the binding is minimized, and present in the lower molecular weight fractions. Proline (Fig. 6) may be an exception to this, binding slightly to

the peak 1 fraction without Mg^{++} present. A small degree of binding occurs in the peak 1 area without Mg^{++} present.

Colchicine (C-methoxyl-^3H) binds *in vitro* to the low speed, 1000g supernatant, in the presence of 2.5 mM Mg^{++} during a 24 hour incubation period (Fig.8). After fractionation on G-200 Sephadex, the radioactive colchicine is found on the smaller molecular weight fractions, eluted at fraction #9, as compared to the binding of the other compounds to fractions #5,6 (Figs. 1,5,6,7). Free colchicine appears after fraction #17.

B. COMPETITIVE BINDING

A typical leucine binding curve over the range of 1-10 x 10^{-6} g/ 0.1 ml of preparation is shown in Fig. 9. Each data point in the upper curve represents the total amount of leucine bound in fractions #1-16, from a 1000g supernatant. Nontritiated leucine was added to the preparation after ^3H-leucine had been allowed to bind for 6 hours. The incubation was continued for an additional 18 hours. The conditions were the same as in previous experiments, where the *in vitro* binding took place with 2.5 mM Mg^{++} present, and at 22° C.

Each data point in the lower curve represents the amount of leucine bound only to the peak 1 area, under the same conditions as above. A significant fall in percent bound occured between 0-4 x 10^{-6} g, in both curves. This is equivalent to a concentration of 4 mg leucine/l of nerve preparation. The average percent bound ^{3}H-leucine, after incubation and fractionation through G-200 Sephadex columns was 53.6%± 0.5 (SD), n=6.

Non radioactive proline and thymidine were added to the ^{3}H-leucine bound preparation in the same manner as the nonradioactive leucine. The competitive efficiency of 10 x 10^{-6} g/0.1 ml of proline for the leucine sites was 3± 1.9% (SD), n=4. where competitive efficiency is defined as the concentration of leucine causing an equivalent change in binding / the concentration of prcline present. The competitive efficiency of 10 x 10^{-6} g/ 0.1 ml of thymidine was 2± 1.5% (SD), n=4.

Competitive binding of leucine was also demcnstrated in the high speed supernatant after fractionation on G-25 Sephadex(Fig. 10). Within a 10 minute incubation period, significant ^{3}H leucine binding occured. Binding at these low time intervals could not be demcnstrated on G-200 The molecular weight ranges for these two types of Sephadex differ widely. Since leucine binds to the lower molecular

weight ranges initially, ^{3}H-leucine binding would be more evident on G-25 Sephadex at the shorter time intervals.

C. CHEMICAL INTEREFERENCE WITH BINDING

Fig. 11 demonstrates that the presence of 0.65 M urea in the low speed supernatant, inhibits the ability of ^{3}H-leucine (2.5 mM Mg^{++}) to bind to the high molecular weight fractions in peak 1 (Fig. 1).

The inclusion of colchicine, Fig. 12, (0.25 mM, 24 hours, 22° C) or RNAase (RNAase:protein, 4 hours, 37°C), Fig. 13, did not effectively reduce the amount of ^{3}H-leucine bound, when compared with the data from Fig. 1. The majority of leucine bound remained in the peak 1 area.

D. PROTEIN CONCENTRATION

Figs. 15 and 16 show the appearance of a protein polymer in peak 1, the same area where the ^{3}H-leucine appears bound in Fig. 1. When the 1000g supernatant was incubated at 22° C, with 2.5 mM Mg^{++} present and fractionated on G-200 Sephadex, this protein polymer was evident in the peak 1 position at early time intervals (Fig. 14). The amount of protein polymer in this area

progressively increased with time, with a corresponding reduction in the amount of smaller molecular weight proteins present. The time interval for appearance of protein polymer in the peak 1 position corresponds to the time interval of ^{3}H-leucine binding in the same area (Fig. 1).

When these measurements were repeated without the addition of Mg^{++}, the appearance of protein at peak 1 was much slower (Fig. 15). This time interval corresponds to the data shown in Fig. 2, which demonstrates a similar time lag before bound ^{3}H-leucine appears in the peak 1 region.

E. CHEMICAL INHIBITION OF PROTEIN FORMATION

Fig. 16 illustrates the fractionation of proteins on G-200 Sephadex from the 1000g supernatant, when the preparation was incubated in the presence of 0.65 M urea and 2.5 mM Mg^{++}, and fractionated on G-200 Sephadex. Urea (0.65M) did not inhibit the ability of the large molecular weight protein to form, although the polymer formed does not appear to be as stable as the protein formed in the absence of urea (Fig. 14). Although protein polymer was found in peak 1 region, with urea present, binding of ^{3}H-leucine did not occur (Fig. 11).

Colchicine (0.25 mM) at 22° C (Fig. 17) or at 37° C

(Fig. 18) did not significantly inhibit the formation of this protein polymer.

The pretreatment of this preparation with RNAase, (Fig. 20) or 3 mM ATP, (Fig. 21), both with 2.5 mM Mg^{++} present, does not appear to reduce the protein in peak 1 position.

Trypsin was added to peak 1 protein, already formed by a 6 hour incubation at 22° C, with 2.5 mM Mg^{++} present. The protein present in peak 1 area was reduced to 6 % of that originally present.

Peak 1 protein formed during a 24 hour, 22° C incubation with 2.5 mM Mg^{++} present was separated from the other proteins, by fractionation on G-200 Sephadex. Urea (8M), was added, and the protein originally in peak 1 fraction (#5,6), was now found in the #9 fraction. Repeating the above experiment, with 1.3M urea and mercaptoethanol present, peak 1 protein was broken down as follows: 11% in fractions #3,4; 9.2% in fractions #5,6 (peak 1); 42.6% in fractions #8,9; 27.8% in fractions #16-20. The fraction #9 region corresponds to the fraction which binds ^{3}H colchicine (Fig. 8).

F. TISSUE SPECIFICITY

Fig. 22 illustrates the *in vitro* binding of rat muscle protein (0.2g/ml) and rat plasma proteins (0.06g/ml), under the same experimental conditions as the rat nerve preparations. Neither rat muscle protein nor plasma protein bind leucine to any degree.

G. EFFECT OF TEMPERATURE

Fig. 23 is an Arrhenius plot of the temperature dependance of the binding of leucine (1.0×10^{-8}) at equilibrium (24 hours incubation). The lines were drawn using a least square best fit method. Each data point on the upper graph represents the total binding in all fractions from #1-16. The enthalpy change on binding, (ΔH), is equal to 5000± 800 cal/mole. Each data point on the lower graph represents only the binding of leucine in the peak 1 (#5,6) area. H is equal to 4900 ± 1400 cal/mole. These are not significantly different ($p>0.05$), and appear to represent the same process.

H. ATPase ACTIVITY

Samples of 1000g supernatant, incubated at 22° C, with or without Mg^{++} present, and then fractionated on G-200 Sephadex show differences in measured ATPase activities. There was no measurable inhibition of the activity when ouabain was added. The nerve preparations incubated with Mg^{++} showed a higher ATPase activity in fraction #12,13 and #14,15 than did the preparation with no Mg^{++} included (Fig. 24). There is an association of the ATPase activity with the peak 1 region, both with and without Mg^{++}, although the time dependance of the association differs.

I. MOLECULAR WEIGHTS

Samples of the 1000g supernatant from rat nerve were incubated at 22° C, with 2.5 mM Mg^{++} for 1 and 6 hours. The samples were then fractionated on G-200 Sephadex and applied to 7.5% SDS acrylamide gels for electrophoresis. The peak positions and peak areas were recorded and protein mobilities and molecular weights calculated by the method of Weber and Osborn (1969). Standards prepared from muscle actin (MW=47,000; Sasasibara and Yagi, 1970), myosin (MW=200,000; Woods et al, 1963), C protein (MW=135,000; Offer et al,

1973), and collagen (MW=302,000; Atman and Dittmer, 1972) were run on 7.5% SDS gels in a similar manner. The position of collagen on the gels was indicated by pretreatment of some of the samples with collagenase. The best fit line was calculated using a least squares method. The resulting equation was: LOG MW=5.6-(0.71 ± 0.13) xM, where MW=molecular weight, and Mobility (M)=(distance of protein migration x length before staining) / (length after destaining x distance of dye migration) By using the method of linear interpolation, on the graph produced by this equation, the molecular weights were determined from their calculated mobilities. The molecular weights of the neuroprotein bands found in the rat nerve samples (identified by arbitrary letters) are as follows :

PA=160,000 ± 63,000 (SD)

PB=130,000 ± 44,000 (SD)

PC=97,000± 26,000 (SD)

PD=74,000 ± 16,000 (SD)

PE=56,000 ± 9,000 (SD)

PF= 42,000 ± 5,000 (SD)

PG=13,500 ± 700 (SD)

J. POLYMERIZATION

The proteins in all G-200 Sephadex fractions previously

incubated with 2.5 mM Mg^{++} for 1 and 6 hours were characterized using the SDS gel electrophoresis techniques discussed above. The increase in the proteins, contained in each Sephadex fraction was calculated as the polymerization time increased from 1 to 6 hours. This data is presented in Table 1. For purposes of comparison, the change in ^3H-leucine bound from 1 to 4.5 hours, and 1 to 6 hours is presented in Table 2. The leucine accumulates in fractions (#3,4) and (#5,6). By examining the data in Table 1, it may be seen that the protein previously identified as C (97,000 ± 26,000 MW), is the only protein which increases in the same fractions, over the same time interval.

K. RATE CONSTANTS AND THERMODYNAMIC VARIABLES FOR LEUCINE BINDING

The rate constants and thermodynamic variables were calculated for leucine binding at 22° C, with 2.5 mM Mg^{++} present. The calculations are based on the total leucine bound in fractions 1-16. The amount of protein used in the calculations was the amount of protein present in fractions 1-16 x 19.2%, which represents the amount of PC protein found in the 1000g supernatant samples (Table 1). The average value of 97,000 MW was used for the molecular weight of the PC protein. The K(equil.) is 1.33×10^4, the rate

constants are k_1=0.424 l/m/sec, k_{-1}=3.18 x 10^{-5} sec^{-1}. The entropy change upon binding, ΔS=35.33 eu. The enthalpy change on binding ΔH =4876 cal/mole. The neuroprotein (PC) apparently binds 10 moles leucine/mole protein. The kinetic data for leucine binding without Mg^{++} present was not calculated, as the data in Fig. 3 indicates the time course of binding is complex.

CHAPTER 4

DISCUSSION

The evidence presented documents the existence of specific leucine binding sites located on protein obtained from rat sciatic nerve. Under certain conditions, detailed in Fig. 10, it may be shown that this binding process occurs at incubation times under ten minutes.

Other tritiated compounds also bind to the nerve homogenates, under similar conditions to those used in the binding of leucine. Tritiated proline, thymidine and succinic acid bind to the high molecular weight fractions in the presence of Mg^{++}, as illustrated in Figs. 5,6 and 7. Thymidine and proline do not compete for the leucine binding sites, as shown by competitive binding results. There appear to be other binding sites, probably specific to these compounds. The competition of succinic acid for the leucine binding sites w[illegible]

The le[illegible]ne binding proces[illegible]ay be characterized with informat[illegible] derived from binding [illegible]ata and kinetic analysis. Since t[illegible] present results indicate the presence of specific bindin[illegible]sites on [illegible] rat neuroprote[illegible]n (PC), it was assumed that th[illegible]inding of leucine is [illegible] equilibrium process

represented by:

$$PC + L \underset{k_{-1}}{\overset{k_1}{\leftrightharpoons}} PCL \qquad (1)$$

Then equation 2 may be ed;

$$PCL = K \times [PC] \times [L] / (1 + K \times L) \qquad (2)$$

where $K = k_1 / k_{-1}$, L and PCL are the concentration of free leucine and the concentration of bound leucine respectively and PC represents the concentration of binding sites.

Following the theory of absolute reaction rates, the rate constants can be represented by equation 3 (Frost and Pearson, 1961).

$$k = (kT/h) \times \exp(\Delta S^{\dagger}/R) \times \exp(-\Delta H^{\dagger}/RT) \qquad (3)$$

The activation energy for the reaction is represented by $\Delta H^{\dagger}$, $\Delta S^{\dagger}$ is the entropy of activation, k is the Boltzmann constant, h is Planck's constant, R is the gas constant and T is the

absolute temperature.

Since $K = k_1 / k_{-1}$;

$$K = \exp(\Delta S_1 - \Delta S_{-1} / R) \times \exp(\Delta H^{\dagger}_{-1} - \Delta H^{\dagger} / RT) \quad (4)$$

$$\text{then } K = \exp(\Delta S/R) \times \exp(-\Delta H/RT) \quad (5)$$

where ΔS is the entropy change upon binding and ΔH is the enthalpy of reaction.

Differentiating equation 5 gives equation 6 for the slope of the classical Arrhenius plot.

$$d \ln K_i / d\ 1/T = \Delta H_i / R \quad (6)$$

In addition to characterization of the binding process, some properties of the binding molecule have been established. The molecule to which leucine binds has been identified as a soluble protein, arbitrarily called PC. It has a molecular weight of 97,000, and makes up 19% of the total protein found in the 1000g supernatant. This protein binds 10 moles of leucine per mole of protein. The leucine appears to bind to this protein initially. The protein (PC) polymerizes to a polymer which has a molecular weight greater than 302,000. This polymer has been identified as

peak 1, in Fig. 1.

The appearance of ^{3}H-leucine in the polymer is greatly influenced by the presence of free Mg^{++} in the incubation mixture. The addition of 2.5 mM Mg^{++} to the incubation mixture (ie. low speed homogenate and leucine), increases the rate at which ^{3}H-leucine appears in the polymer (peak 1) position (Fig. 1 and 3). This appears to be due to an increase in the rate at which the polymer is formed, as indicated in Fig. 14. When Mg^{++} is not added, the time course of binding appears to be complex and proceeds in a stepwise fashion as in Fig. 3. Without the addition of Mg^{++}, the leucine is initially bound to the smaller molecular weight protein fractions. These higher molecular weight fractions progressively increase in size as the polymer is formed. This process is graphically illustrated in Fig. 2. The ^{3}H label appears in the polymer position at approximately 17 hours incubation time without Mg^{++} and less than 1 hour with Mg^{++} present (Fig.1). The time interval for the appearance of the protein polymer in this region correlates with the data for the appearance of the radioactivity in the same region; 16.5 hours for incubations with no Mg^{++} present and less than 2.5 hours with Mg^{++} present.

The polymer may be assembled by the polymerization of

the PC protein itself, or in combination with some of the other proteins that are present. In the fraction where polymerized leucine binding protein is found, five other proteins are also found. From Table 1, it may be seen that during polymerization the amount of leucine binding protein (PC) in the polymer fraction increases by 20.9%, while the amount of a smaller molecular weight protein (PF; MW=42,000± 5,000) decreases by 27.9% (Table 1). The time of isolation of this polymer, would then, greatly influence the predominate protein species found. This has not been taken into consideration by other investigators. It is possible that PF protein (MW= 42,000± 5,000) may be the tubulin monomer, while PC (MW= 97,000± 26,000) or PB (MW= 130,000± 44,000) might be the tubulin dimer or colchicine binding protein.

The formation of the polymer does not appear to significantly alter the conformation of the leucine binding sites. This infers that the binding sites are accessable on the polymer. The effect of the Sephadex on the assay measurement is thought to be minimal as the sample passage time (10 minutes) is very small compared to the large unbinding time indicated by the rate constants. The leucine bound to the polymer is exchangeable, as shown by the competitive binding experiments. As indicated in Fig. 9, the leucine found bound to the smaller protein units is similar

in kinetic characteristics. In addition, Fig. 23 shows that the leucine bound to the polymer has the same enthalpy of binding (ΔH) as that found for the total leucine bound to all polymer fractions .

Addition of colchicine to the rat sciatic nerve homogenates inhibits the polymerization of leucine binding protein. Colchicine (1×10^{-6} M), has previously been reported to inhibit microtubule assembly (Olmsted and Borisy, 1973). The amount of colchicine required for inhibition is higher in the leucine binding system, than that reported in the literature for inhibition of microtubule protein. Colchicine (2.5 mM) inhibits the formation of peak 1 protein (Fig, 18), but 0.25 mM colchicine does not (Fig. 17). This may be due to experimental procedures which require the colchicine to be dried, to evaporate the ethanol carrier, prior to use. Colchicine is less soluble in water and may not be totally dissolved by the incubating solution. The binding of leucine to the large polymer can be inhibited by the addition of 0.65 M urea to the preparation, as shown in Fig. 11. However, some binding still occurs on the small molecular weight fractions. Unlabelled protein polymer still forms in the peak 1 area, as shown in Fig. 16, but the formation does not appear to be as stable as the protein formed without urea present. Urea tends to break secondary bonds, but not

covalent bonds (Watson, 1970). Thus leucine, or leucine binding protein may be held in the polymer by this type of bond. RNAase does not inhibit the binding of leucine (Fig. 13) nor the protein polymerization (Fig. 20). Trypsin does destroy the ^{3}H-leucine bound polymer, indicating the polymer to be a protein structure.

^{3}H-colchicine appears to bind to a subunit of the large protein polymer. ^{3}H-colchicine binds to a smaller molecular weight fraction, eluted in fraction #9,10 instead of polymer fraction #5,6. Urea or urea and mercaptoethanol break down the polymer protein into smaller molecular weight units, the major portion appearing in the colchicine binding fraction. When the colchicine binding fraction was separated out and reincubated in the original homogenization solution (with Mg^{++} present), 30% of this protein appeared in the higher molecular weight polymer fraction. Colchicine may function in inhibiting peak 1 polymer formation, by binding to the polymer subunit, much in the same manner as it has been suggested to function in the inhibition of microtubule assembly.

To examine the possibility of an energy coupled mechanism, ATPase activity was monitored throughout the binding and polymerization reactions of the 1000g supernatant. Mg^{++} activated ATPase is present in the low

speed homogenates of rat sciatic nerve. This activity is largely associated with the smaller molecular weight fractions (#12,13) and (#14,15) in Fig. 24. There is some ATPase activity evident, both with and without the addition of Mg^{++}, that appears to be associated with the protein polymer . It has not been determined whether the ATPase activity without Mg^{++} added is due to the effect of the small amount of free Mg^{++} present or to the small amount of Ca^{++} already present in the homogenizing mixture. No ouabain inhibited (ie. $(Na^{+}+/K^{+})$ ATPase) activity was found in the preparations.

The kinetic data for the binding of leucine to neuroprotein (PC), can be compared to kinetic data from other sources. Similar binding has been described for other types of compounds (see for instance Westphall, 1972). The results presented by Russell and Doty (1973), for plasma protein binding of epinepherine, show the existence of two distinct kinds of epinepherine binding sites. One type (site 1), has a large equilibrium constant for binding, $K = 1.82 \times 10^{6}$ 1/m, and is present in small numbers. This is similar to the K reported by Owellen et al, (1972) for the binding of colchicine to tubulin, $K = 1.8 \times 10^{6}$ 1/m and the binding of vinblastine, $K = 6.0 \times 10^{6}$ 1/m and vincristine, $K = 8.0 \times 10^{6}$ 1/m to tubulin. The other epinepherine binding site (site 2), has a smaller equilibrium constant, and the binding is

weaker in comparison. The kinetic data for the binding of leucine to neuroprotein resembles the characteristics of site 2 more than site 1. The rate constants (k_1 , k_{-1}) represent slower binding rates than the epinepherine-plasma binding system.

There are many similarities between the conditions for the assembly of microtubules, the conditions for the binding of colchicine, and the the conditions for the assembly of the leucine binding protein. Successful repolymerization of tubulin subunits into microtubule polymers has been accomplished in vitro. This was accomplished by removing calcium ions from the solution with a strong chelating agent (EGTA or EDTA). Polymerization also required the presence of Mg^{++} ions, Na^+, K^+ <150 mM, and ATP or GTP or a high concentration of sucrose (1M) or glycerol (4M). Maximum polymerization was obtained at pH=6.7-6.8 and at a temperature maximum of 27° C (Olmsted and Borisy, 1973). Colchicine, vinblastine, vincristine and low temperature disrupt microtubules and prevent reassembly. The stability and amount of colchicine binding activity decreases outside the pH range 6.7-6.8, and at high ionic strength. The presence of Mg^{++} (<10 mM), and 100 mM Na^+ stabilize the colchicine binding activity. The presence of GTP is not necessarily required for colchicine binding activity. Addition of vinblastine or vincristine stabilize the

colchicine binding activity. Polymerization of leucine binding protein (PC) was accomplished under very similar conditions to those used by other investigators for microtubule assembly. The incubating solution contained 0.15 M sucrose, phosphate buffer pH=6.8, 2.5 mM EDTA, 2.5 mM Mg^{++}, and the incubation temperature was 22° C.

Multiple binding sites are associated with both microtubule subunits and leucine binding protein. The evidence suggests that vincristine, vinblastine, colchicine and GTP all bind to the microtubule protein at different sites (Wilson, 1970; Owellen et al, 1972). Interaction with these sites modifies the ability of the subunit to function in the microtubule structure. Multiple binding sites are also associated with the polymer formed from leucine binding protein. Leucine, proline, succinc acid, and thymidine all bind to this fraction.

The molecular weights of microtubule subunits, and leucine binding protein are different. The leucine binding protein from rat sciatic nerve appears to be a soluble protein of molecular weight 97,000± 26,000. This molecular weight was measured after disruption by 8M urea, mercaptoethanol, SDS and heating to 100° C. With this treatment, all polymerized units should be broken down into the basic subunits. The protein polymer (peak 1) and the

colchicine binding fraction both contain leucine binding protein, as shown in Table 1. Although the molecular weight ranges for colchicine binding protein (MW= 100,000-130,000) and leucine binding protein (MW= 97,000± 26,000) appear to be very similar, colchicine binding protein is reported to be a dimer, while leucine binding protein does not appear to be.

ATPase activity has been attributed to three systems, in the nervous system. These are: the actomyosin like protein, neurostenin; dynein, a large ATPase bound to microtubules and mitochondrial ATPase. Dynein has been found in cilia and flagella. The doublet tubules (A tubule) bind this ATPase (dynein), which is essential for ciliary motility. It apparently, generates the force that slides microtubules parallel to their neighbour (Summer and Gibbons, 1971). Sliding systems have been implicated in other motile systems built from microtubules, but there is less available information concerning these. A sliding system could be modelled on the actomyosin muscle system, since similar contractile proteins, neurin, stenin, and neurostenin have been identified from brain homogenates. These proteins are capable of chemomechanical transduction (Berl et al, 1973).

Stenin (a myosin-like protein) alone exhibits Ca^{++}

ATPase activity and little Mg^{++} ATPase activity. Neurin (like muscle actin), demonstrates little enzyme activity alone, but enhances the Mg^{++} ATPase activity of stenin, and of myosin (Berl et al, 1973). Actin has been compared to colchicine binding protein, which also has the actin-like characteristics of increasing the Mg^{++} ATPase activity of myosin (Puszkin and Berl, 1970). However, colchicine binding protein and actin differ in many other properties such as amino acid composition, peptide maps and electrophoretic patterns on 50% SDS gels. (Stephens, 1970). It has been suggested that actin and colchicine binding protein are phylogenetically related (Schmitt and Sampson, 1968). This has now been suggested for neurin as well (Puszkin and Berl, 1972). Neurin and actin are similar, in that they both contain a 3-methyl histidine marker which colchicine binding protein (tubulin dimer) does not have (Stephens and Linck, 1969). There are however, common antigenic properties between neurin and colchicine binding protein (Puszkin and Berl, 1972).

Mitochondrial ATPase is found in two forms. The ATPase activity is normally part of the mitochondrial inner membrane and is a large complex (MW= 468,000). Soluble forms of ATPase can be released from, and rebind to this membrane. This soluble form (F1) has a molecular weight of 384,000, which is composed of five different subunit structures

ranging from 7,500 to 62,000 MW (Senior, 1973). The Mg^{++} activity associated with mitochondrial enzymes is not characteristic of actomyosin (Puszkin and Berl, 1972). The molecular weights of the subunits also differ. In actomyosin the molecular weight is equal to 200,000 + n(47,000), where n= the number of actin molecules. In mitochondrial ATPase, the subunits are less than 60,000 MW. Mitochondrial ATPase (F1) has a specific inhibitor which does not inhibit the other system.

The protein(s) responsible for the Mg^{++} ATPase activity in the leucine binding system have not been identified. Proteins are present in all fractions with ATPase activity, which fall in the range of stenin (ie. PA= 160,000± 63,000 MW) and neurin (ie. PF= 42,000± 5,000). There are also protein components which would be in the range of mitochondrial ATPase subunits (PE, PF, PG). Further investigation is needed in this area. The association of ATPase activity with the large polymer (peak 1) protein (Fig. 24), indicates that the ATPase enzyme must bind to the polymer fraction, as each of the proteins present in this fraction is too small to be found in this fraction in an unbound state. This ATPase-polymer may represent dynein or a dynein-like enzyme and/or the activation of stenin by neurin. It could also represent an ATPase enzyme from a mitochondrial system, where the subunits form large polymers

(F1= 360,000 MW) and an ATPase complex (468,000 MW) (Senior, 1973).

There are noteworthy similarities between the following three systems: microtubules, neurostenin and mitochondrial ATPase. All of these systems have associated ATPase characteristics. In each, there is one element present, whose ATPase activity is enhanced by the binding of another element. These are, the increase in the the Mg^{++} ATPase activity of stenin by the binding of neurin (Berl et al, 1973); mitochondrial membrane ATPase activity is enhanced by the binding of F1 (Senior, 1973) and microtubules by the binding of dynein (Summer and Gibbons, 1971). Similarily, all three of these systems have nucleotide binding sites. Neurin contains 0.77 moles of nucleotide binding sites per 50,000 g of neurin (Puszkin and Berl, 1972). Two mole sites are found per mole of F1 (Senior, 1973), and 2 moles of nucleotide binding sites per mole of tubulin dimer have also been found. In addition, these nucleotide binding sites in F1 and in the microtubule dimer, have other similar characteristics. One site binds tightly and relatively specifically, while the other site binds compounds more loosely (Senior, 1973; Borisy, 1970). In the case of F1, it is suggested that the tight site may be the site of binding F1 to the mitochondrial membrane (Senior, 1973).

The molecular weights of the subunits involved in the ATPase associated systems above are remarkably similar. The molecular weight of neurin (actin-like) is 47,000 (Berl et al, 19). The molecular weights of the two largest F1 subunits are (1) 49-57,000 MW, (2) 53-62,500 MW (Senior, 1973; Boirsy, 1971). The molecular weights of the tubulin subunits are (1) 53,000 and (2) 56,000 MW (Feit et al, 1971.

Two types of proteins (actin and tubulin), have already been examined to see if they are the same compounds. Other systems have not yet been compared. Actin and tubulin proteins have been examined by many investigators and found to differ in their electrophoretic mobility in 5% SDS acrylamide gels, amino acid sequence (Stephens and Linck, 1969) and peptide maps (Stephens, 1970). However, it has been suggested that actin and colchicine binding protein (tubulin dimer) may be phylogenetically related (Schmitt and Sampson, 1968). Puszkin and Berl (1972), suggest that this may also be the case for neurin. Some of these subunits (ie. tubulin, F1 subunits, neurin) may be the same compounds, or as suggested above, may be phylogenetically related. Since all three systems appear to have binding sites with similar characteristics, the binding of the compounds may be interchangeable.

The identification of the leucine binding protein or

polymer with a known neuronal structure, such as microtubules, has not yet been accomplished. The conditions for assembly and inhibition of the polymer by colchicine and the binding of ^{3}H-colchicine to a polymer subunit, all suggest the polymer may be a microtubule formation. However the leucine binding polymer, assembled from the 1000g supernatant, may also represent one of the following states; (1) the assembly of leucine labelled microtubules, neurofilaments or microfilaments, (2) the binding of labelled subunits onto mitochondria; (3) binding of mitochondria onto microtubules or some other fibrous structure, either of which might be labelled ; (4) the assembly of an ATPase complex or (5) a Schwann cell component. All of these possibilities would increase the molecular weight of the associated structure to the point where it could be present in the polymer fraction. The subunits of F1, tubulin and neurin are all too small to be the leucine binding protein (PC= 97,000± 26,000 MW). However, they may be involved in the binding or the polymer assembly.

The association of Mg^{++} ATPase with the peak 1 protein polymer, indicates that a mechanism for chemomechanical transduction is possibly available and could provide energy for movement. It is not known at this time which system(s) is responsible for the ATPase enzyme activity, nor if this

ATPase system is involved in the same structure as the leucine bound protein. It has been shown that both the polymerization of leucine binding protein and the activation of ATPase are accelerated by the addition of Mg^{++}. Both of these structures are found in the peak 1 polymer fraction .

Although the present work has been done in vitro sciatic neuroproteins labelled in vivo show some similar properties. Research reported by Ochs, (1972) shows that the injection of ^{3}H- leucine into the cell bodies of the L7 dorsal root ganglia, in cat sciatic nerve, is followed by the appearance of a crest of activity. This crest is considered to be due to labelled proteins passing down the axon, both in vivo and in vitro, and may be transported or stored in this form. This radioactive wavefront, evident at post-injection times of over 2 hours, moves at a rate of 400± 35 mm/day. Ochs fractionated samples of high speed supernatant (100,000g) from homogenates of nerves previously labelled in vivo, for 6 and 21 hours. This fractionation showed a discernable increase in both the amount of high molecular weight protein (480,000 MW), and the degree of ^{3}H-leucine labelling on this protein (Ochs, 1972). These are similar to the in vitro work documented in this thesis. The interpretation has been that the ^{3}H-leucine, once injected into the dorsal root ganglia, is incorporated into the protein components, which move down

the axon at the rate mentioned above. In view of the evidence presented in this thesis, indicating that leucine is competitively bound to a neuroprotein (PC), consideration should be given to the idea that leucine is bound rather than completely incorporated *in vivo* as well as *in vitro*. The appearance of the wave front movement (Ochs, 1972), may actually represent the movement of the large polymer unit, formed with the smaller ^{3}H-leucine binding protein. The presence of proline, thymidine, and succinic acid binding sites suggests this may be a generalized phenomenon, perhaps utilized for transport or storage of material. The source of this binding protein has not been established.

The data documented in this thesis could be applied to a more detailed study of the binding properties of neuroproteins. Variations in the number and type of sites, Mg^{++} concentration, rate of polymerization, and ATPase activity can now be examined and compared. The competitive binding techniques could be used to quantitatively measure amounts of leucine, proline, thymidine or succinic acid, already bound *in vivo*. This would be done by addition of ^{3}H-leucine or the appropriate tritiated compound, to the preparation after homogenation. Then the amount of previously bound compound could be interpolated from a standard graph. Investigation of the molecular arrangements and the type of bonds formed in polymerization could now

follow.

This type of investigation could be applied to several different lines of research. The types of proteins produced at different stages of neural development, in disease, in the presence of drugs or anesthesia, or with a change in environment are likely to vary. A comparison between the various systems, may give us further understanding of the molecular biology of the nerve.

Further research is needed to clarify the following questions: Which structure is associated with the polymerization of the leucine binding protein (PC)? Are all the proteins present in the polymerized fraction bound into the polymer, or is there more than one polymer present and with the same molecular weight? What are the kinetics of polymerization with very low concentrations of Mg^{++} present? Is the colchicine binding protein the same protein that binds leucine? Is the ATPase activated without the addition of Mg^{++} due to the low free Mg^{++} present (0.5 mM) or to the low free Ca^{++} present (0.025 mM)? Which protein(s) is responsible for the ATPase activity? Is the ATPase activity due to the presence of neurin, stenin, or dynein, or mitochondrial ATPase, or due to some other effect?

FIGURE 1

G-200 Sephadex gel filtration of the low-speed (1000 g) supernatant from rat nerve homogenates. Samples of 0.1 ml were incubated with 12,000 cpm of L-leucine-4,5 ^{3}H and 2.5 mM Mg^{++}, for varying time intervals, at 22° C. Markers are placed to indicate the position of the protein polymer (1), the retention volume of 302,000 MW collagen (A), and the retention volume of 68,000 MW bovine serum albumin (B). Free leucine is eluted after fraction 17.

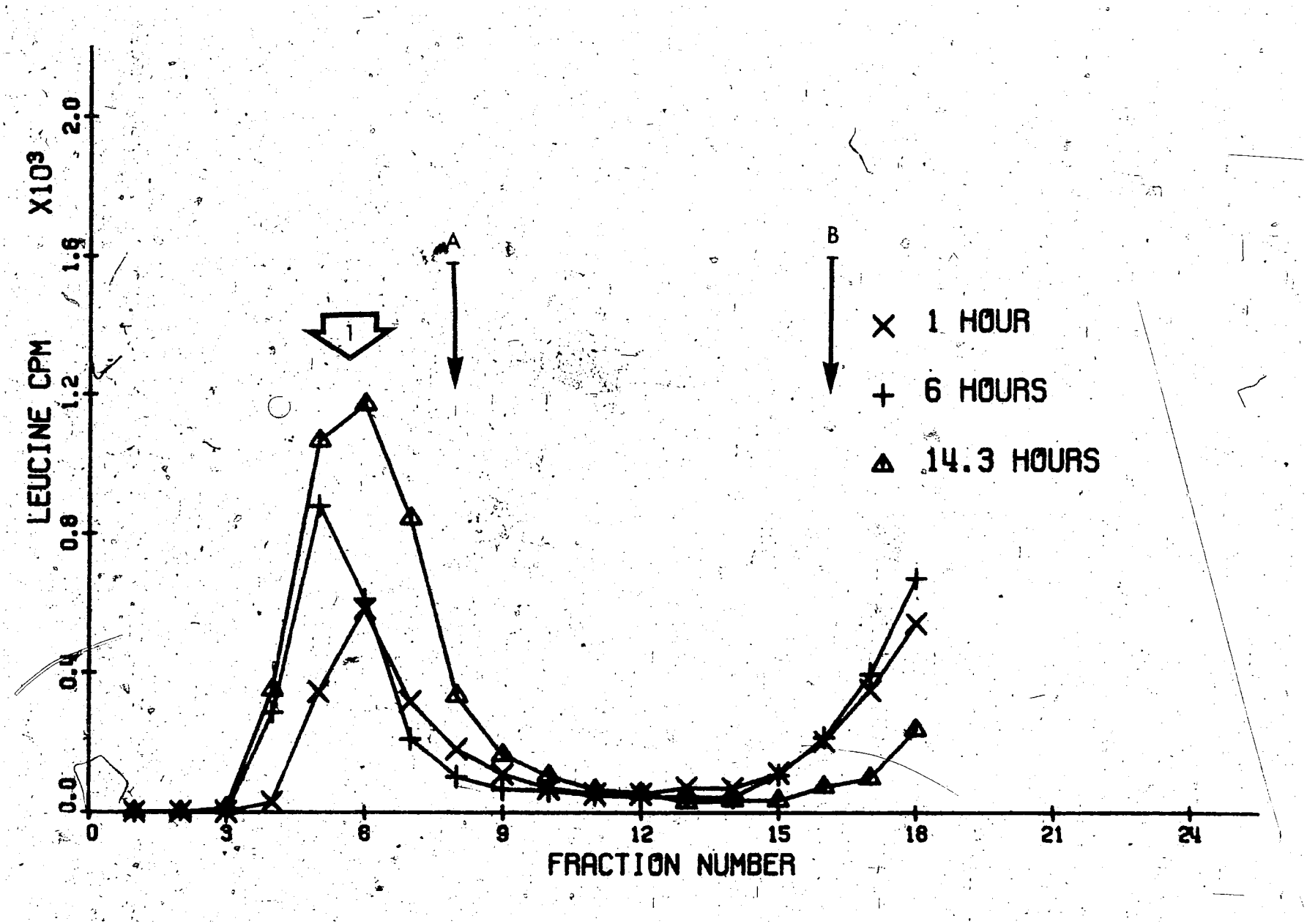
LEUCINE CPM X10³
2.0
1.6
1.2
0.8
0.4
0.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER
A
B
1
× 1 HOUR
+ 6 HOURS
△ 14.3 HOURS

FIGURE 2

Sephadex gel filtration of a 0.1 ml sample of the nerve preparation, incubated without the presence of Mg^{++}. Other conditions as in Fig. 1.

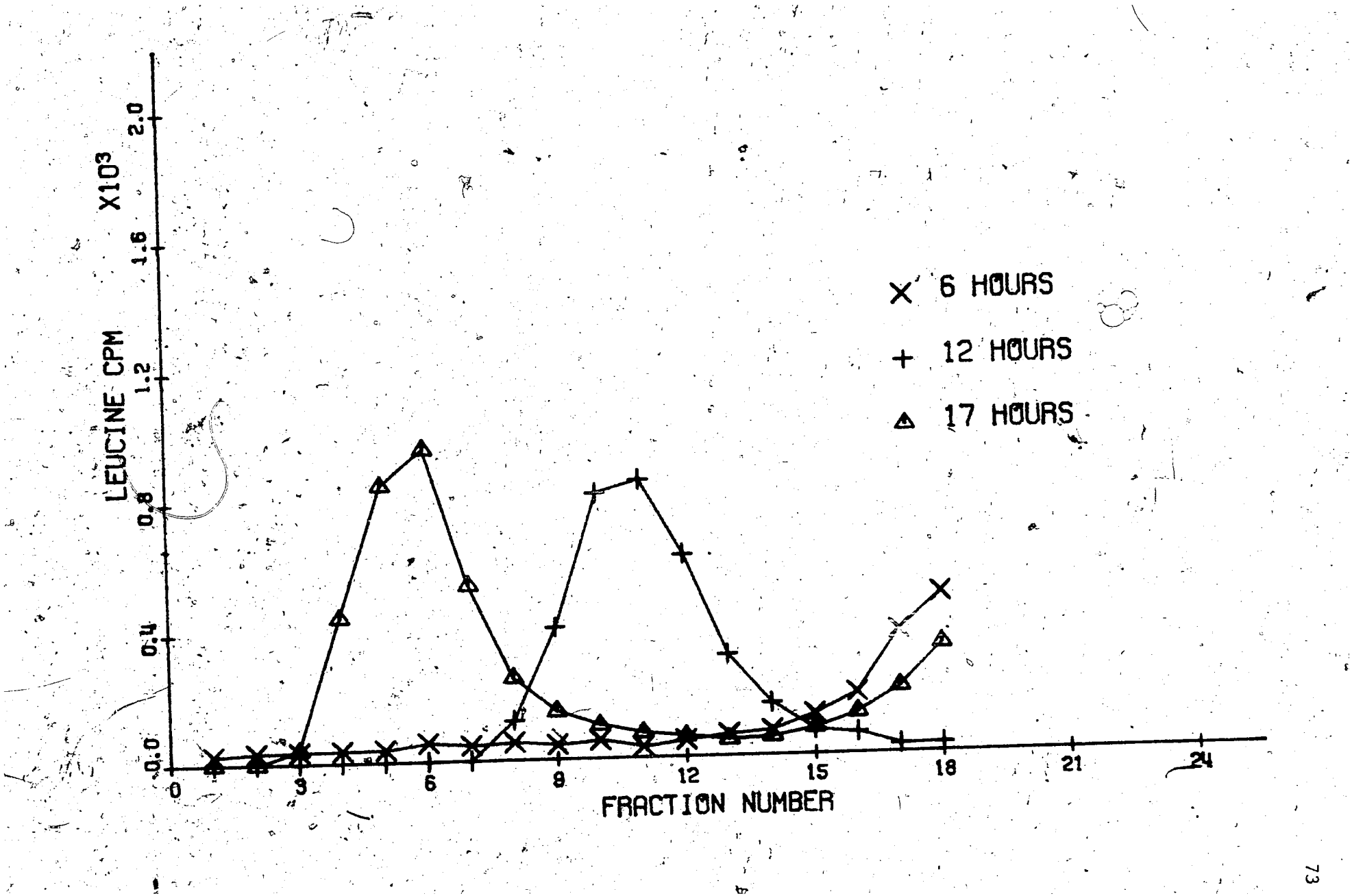
LEUCINE CPM X10³
2.0
1.6
1.2
0.8
0.4
0.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER
6 HOURS
12 HOURS
17 HOURS

FIGURE 3

The time course of the binding of leucine, with and without the presence of Mg^{++}. Each data point represents the total amount of bound leucine in fractions 1-16.

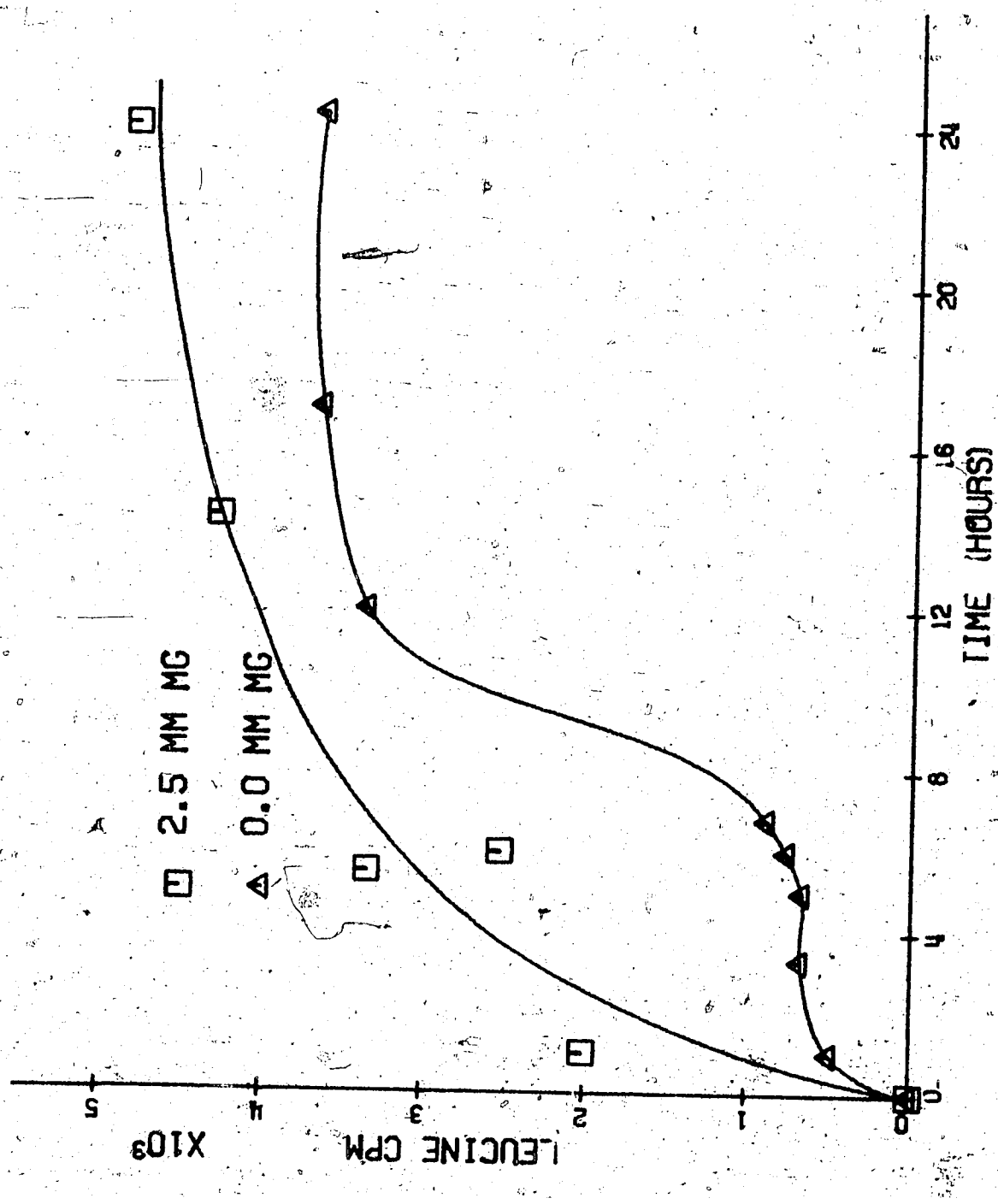
2.5 MM MG
0.0 MM MG
LEUCINE CPM
X10³
0
1
2
3
4
5
0
4
8
12
16
20
24
TIME (HOURS)

FIGURE 4

G-25 Sephadex gel filtration of a 0.1 ml sample of the high speed (100,000 g) supernatant from nerve homogenates. Samples were incubated with 40,000 cpm L-leucine. Other details as in Fig. 1. Free leucine is eluted after fraction 14.

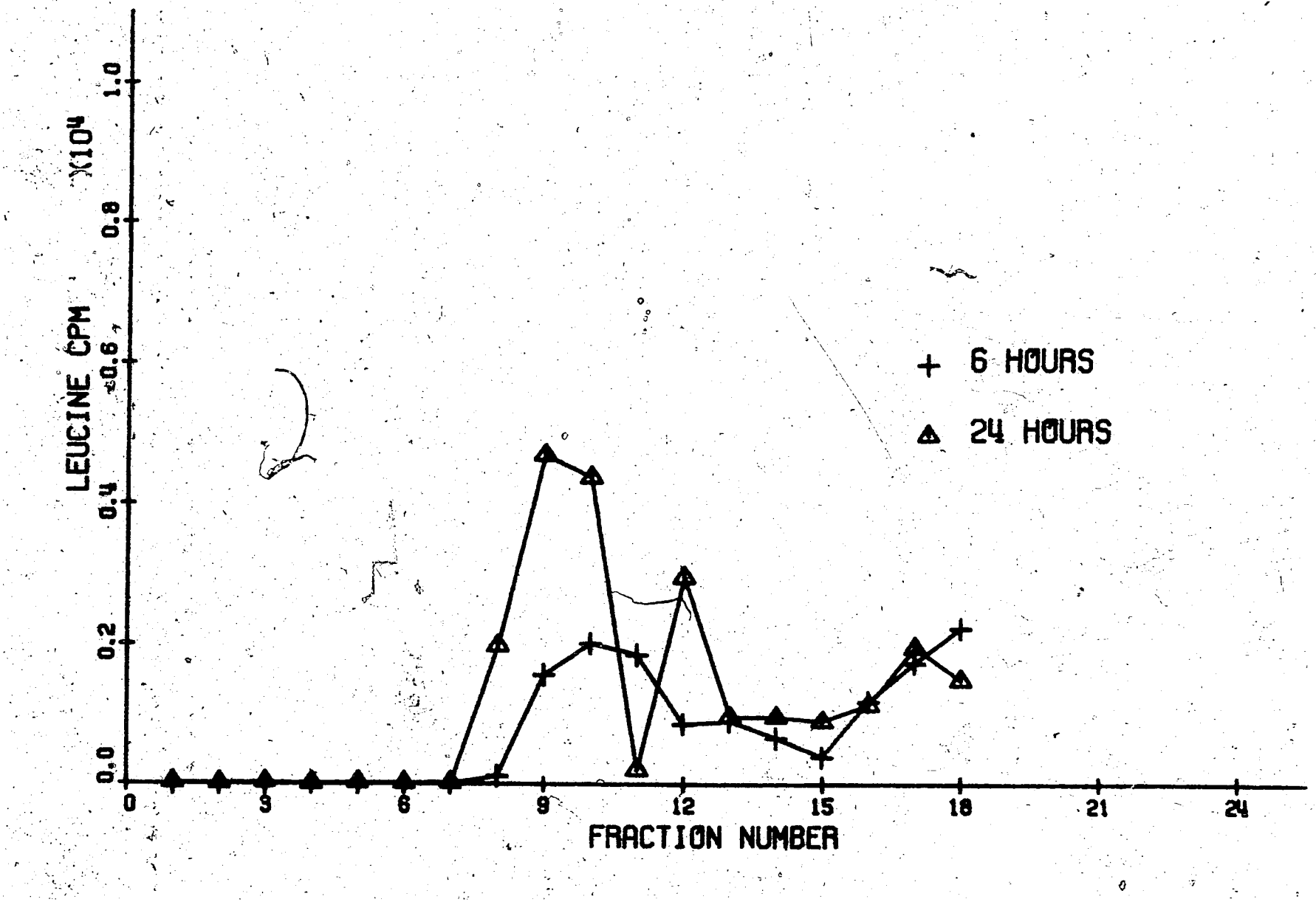
LEUCINE CPM X10⁴
1.0
0.8
0.6
0.4
0.2
0.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER
+ 6 HOURS
Δ 24 HOURS

FIGURE 5

Gel filtration of a 0.1 ml sample of the nerve preparation previously incubated with 25,000 cpm thymidime-methyl ^{3}H, with and without the addition of 2.5 mM Mg^{++}, for 24 hours at 22° C.

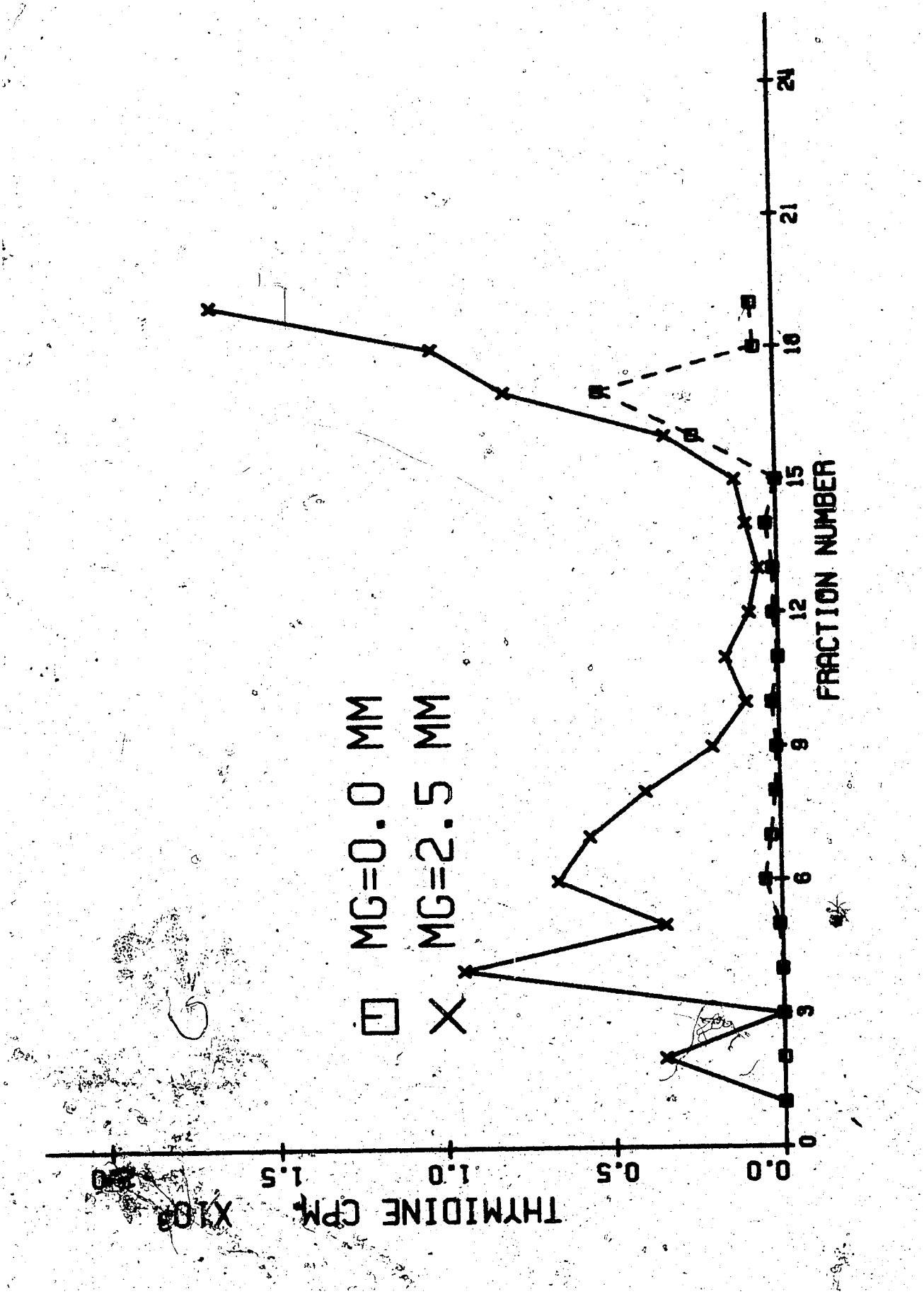
THYMIDINE CPM X10³
0.0
0.5
1.0
1.5
0
3
6
9
12
15
18
21
24
FRACTION NUMBER
□ MG=0.0 MM
X MG=2.5 MM

FIGURE 6

Gel filtration of a 0.1 ml sample of the preparation, when incubated with 15,000 cpm of L-proline-3,4-^{3}H, with and without the presence of Mg^{++}. The conditions of incubation were as stated in Fig.5.

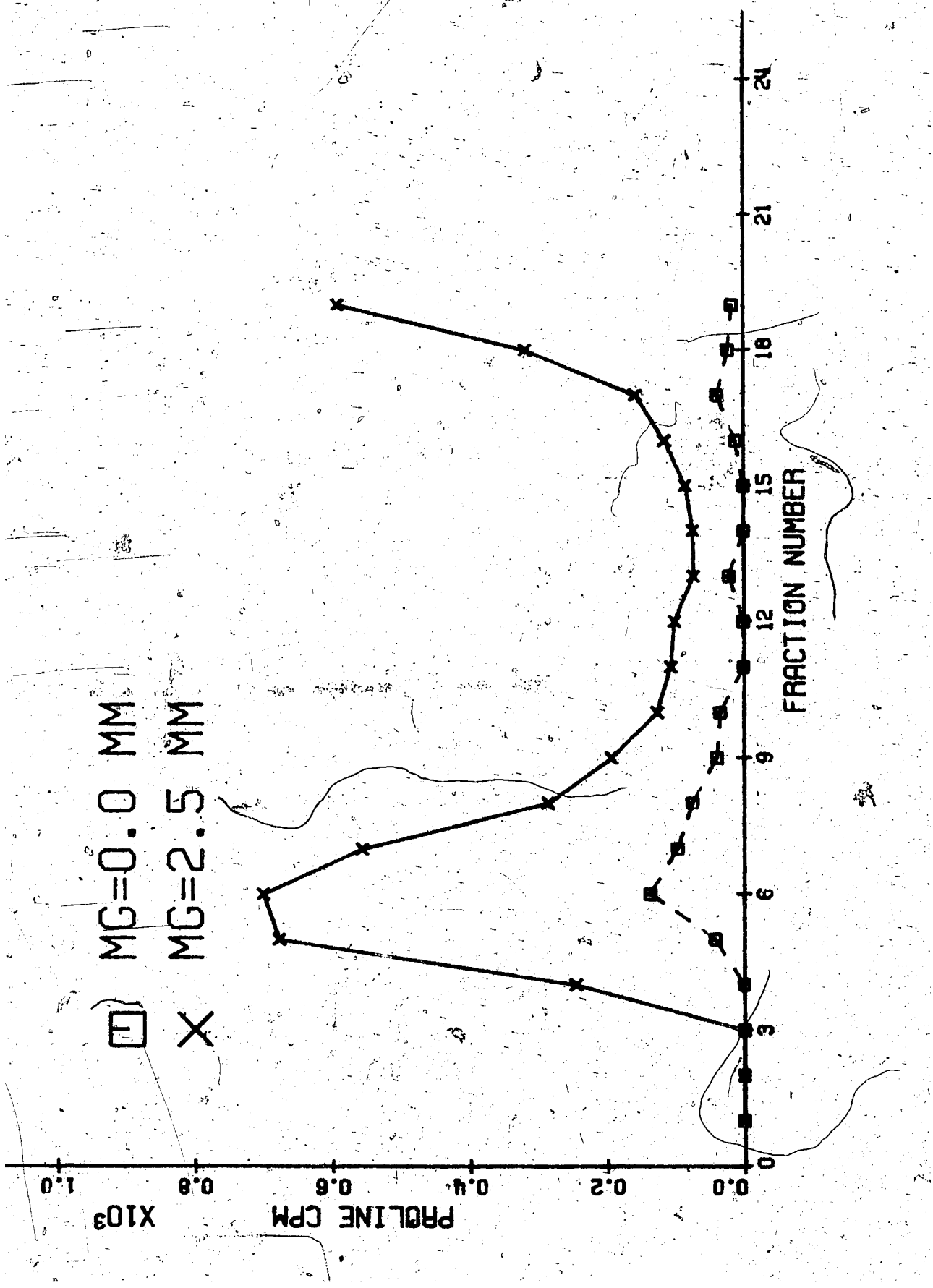
□ MG=0.0 MM
X MG=2.5 MM
PROLINE CPM
X10³
0.0
0.2
0.4
0.6
0.8
1.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER

FIGURE 7

Sephadex fractionation of a 0.1 ml sample of the nerve preparation after incubation with 2,000 cpm succinic-2,3-^{3}H acid, [illegible] and without the presence of Mg^{++}. Other details are as in Fig. 5

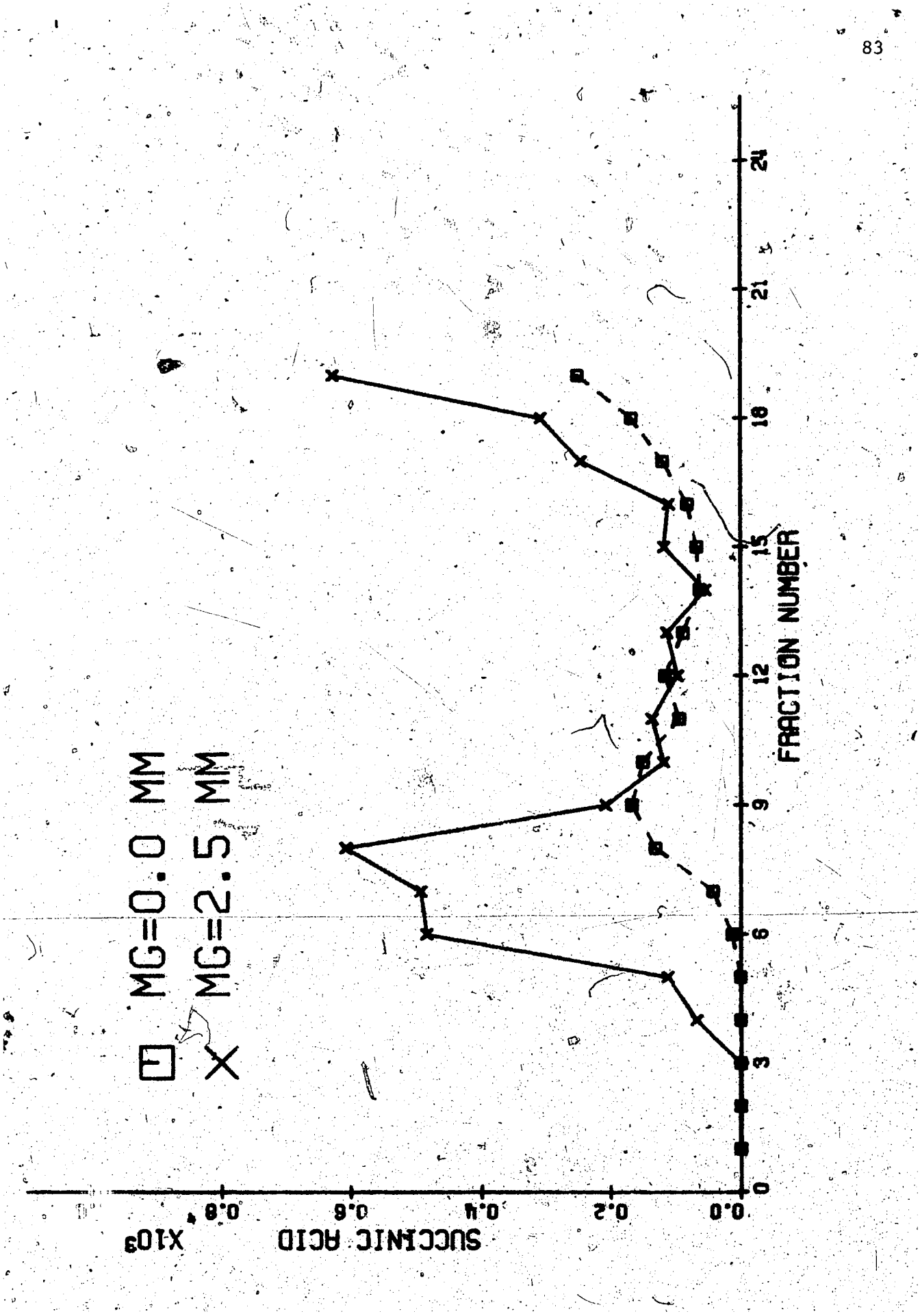
MG=0.0 MM
MG=2.5 MM
SUCCINIC ACID
X10³
0.0
0.2
0.4
0.6
0.8
0
3
6
9
12
15
18
21
24
FRACTION NUMBER

FIGURE 8

Sephadex fractionation of a 0.1 ml sample of the nerve preparation after incubation with 18,000 cpm colchicine (ring, C-methoxyl-^{3}H), with and without the presence of Mg^{++}. Other details as in Fig. 5.

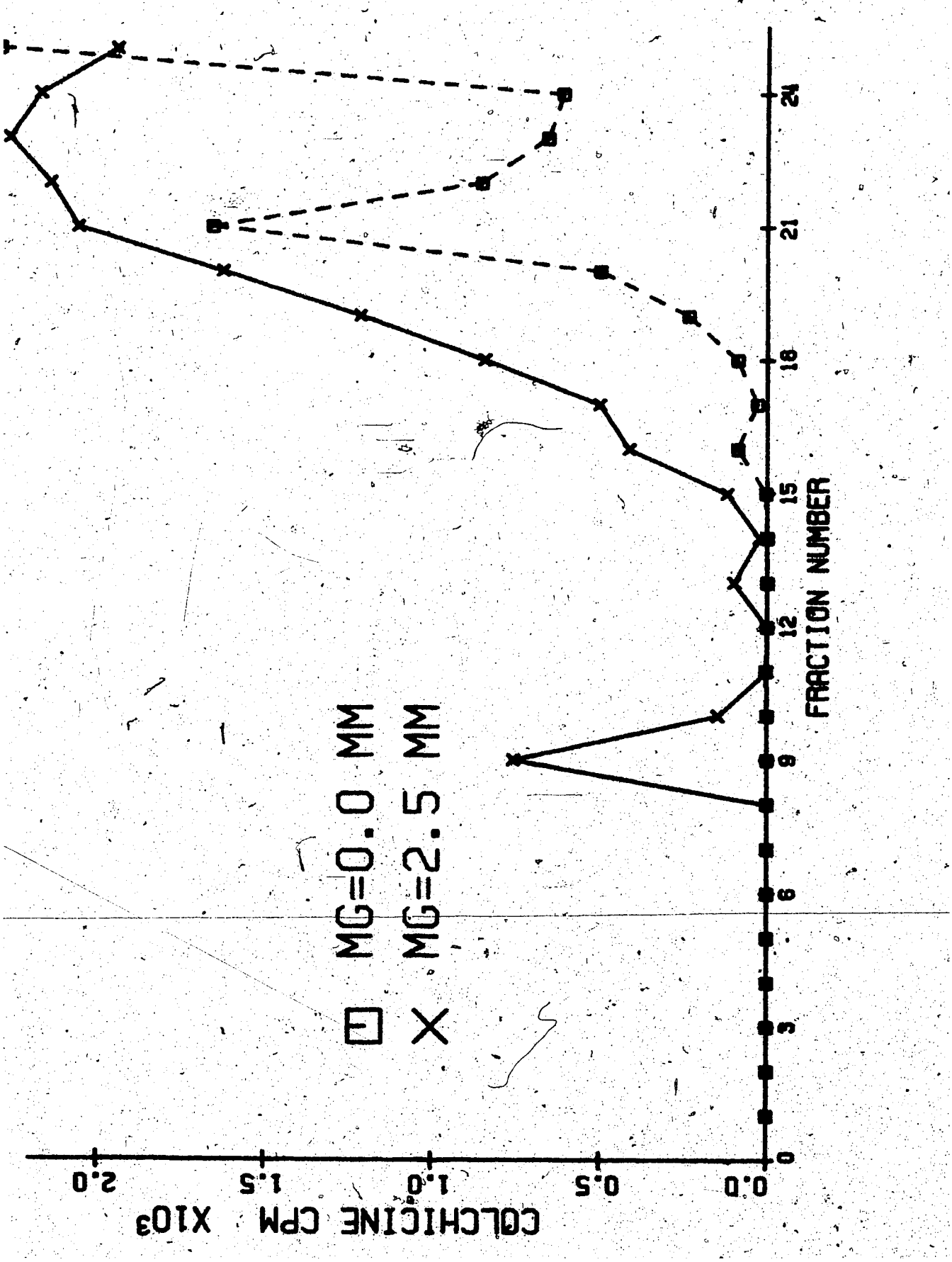
COLCHICINE CPM X10³
0.0
0.5
1.0
1.5
2.0
FRACTION NUMBER
0
3
6
9
12
15
18
21
24
□ MG=0.0 MM
X MG=2.5 MM

FIGURE 9

Standard leucine binding curve, over the range of 0-10 x 10^{-6} g leucine. Each sample in the upper line, represents the total leucine bound in fractions 1-16, under the conditions in Fig. 1, for 24 hours. The lower line, represents the binding of leucine to the peak 1 polymer only, under similar conditions. Each point represents the mean ± S.E. of three replicates.

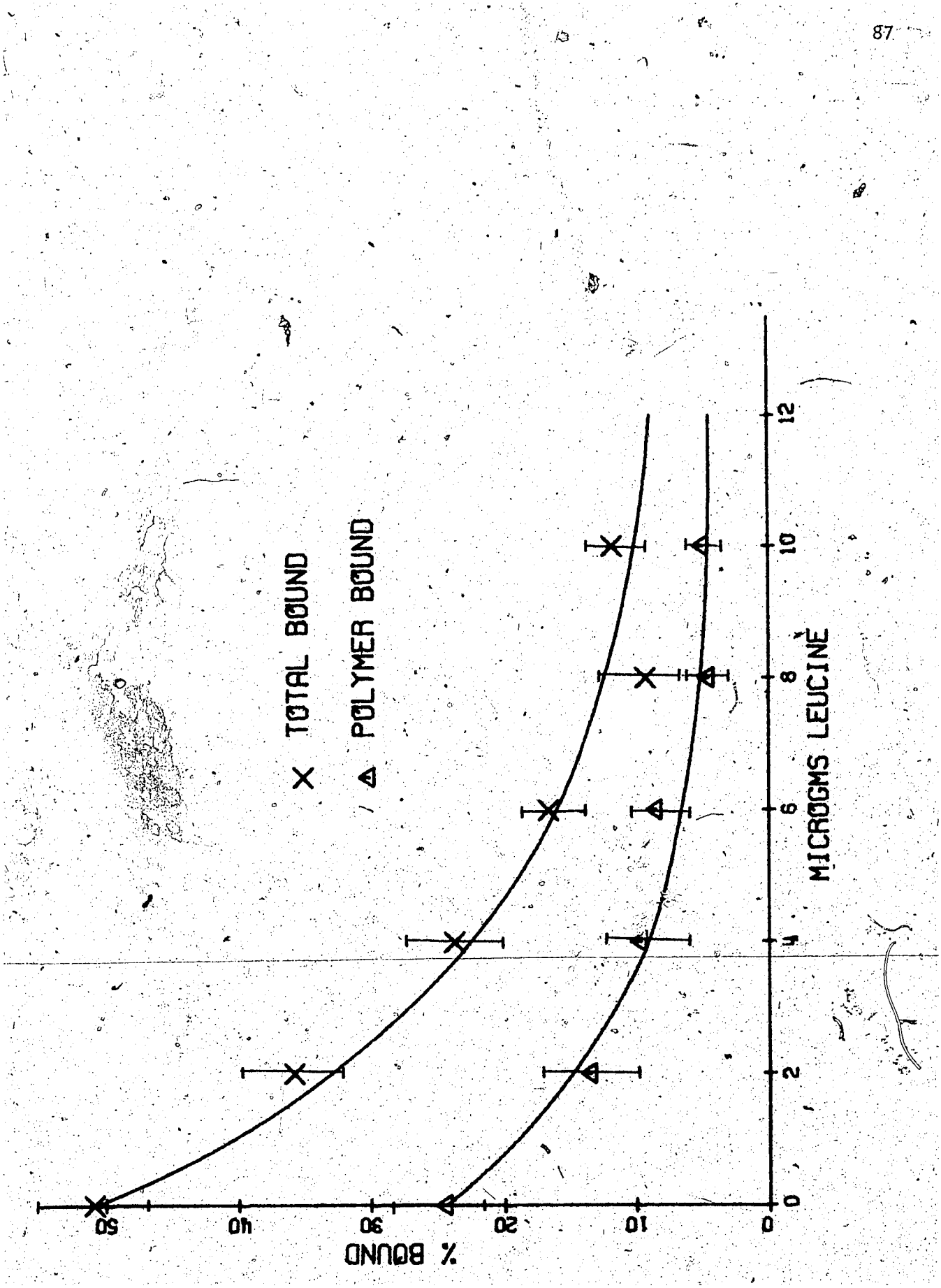
% BOUND
0
10
20
30
40
50
0
2
4
6
8
10
12
MICROGMS LEUCINE
X TOTAL BOUND
Δ POLYMER BOUND

FIGURE 10

G-25 Sephadex gel filtration of a 0.1 ml sample of the high speed (100,000 g) supernatant, after binding for 10 minutes under the conditions of Fig. 4. Varying amounts of nontritiated leucine have been added for comparison. The free leucine is eluted after fraction 14.

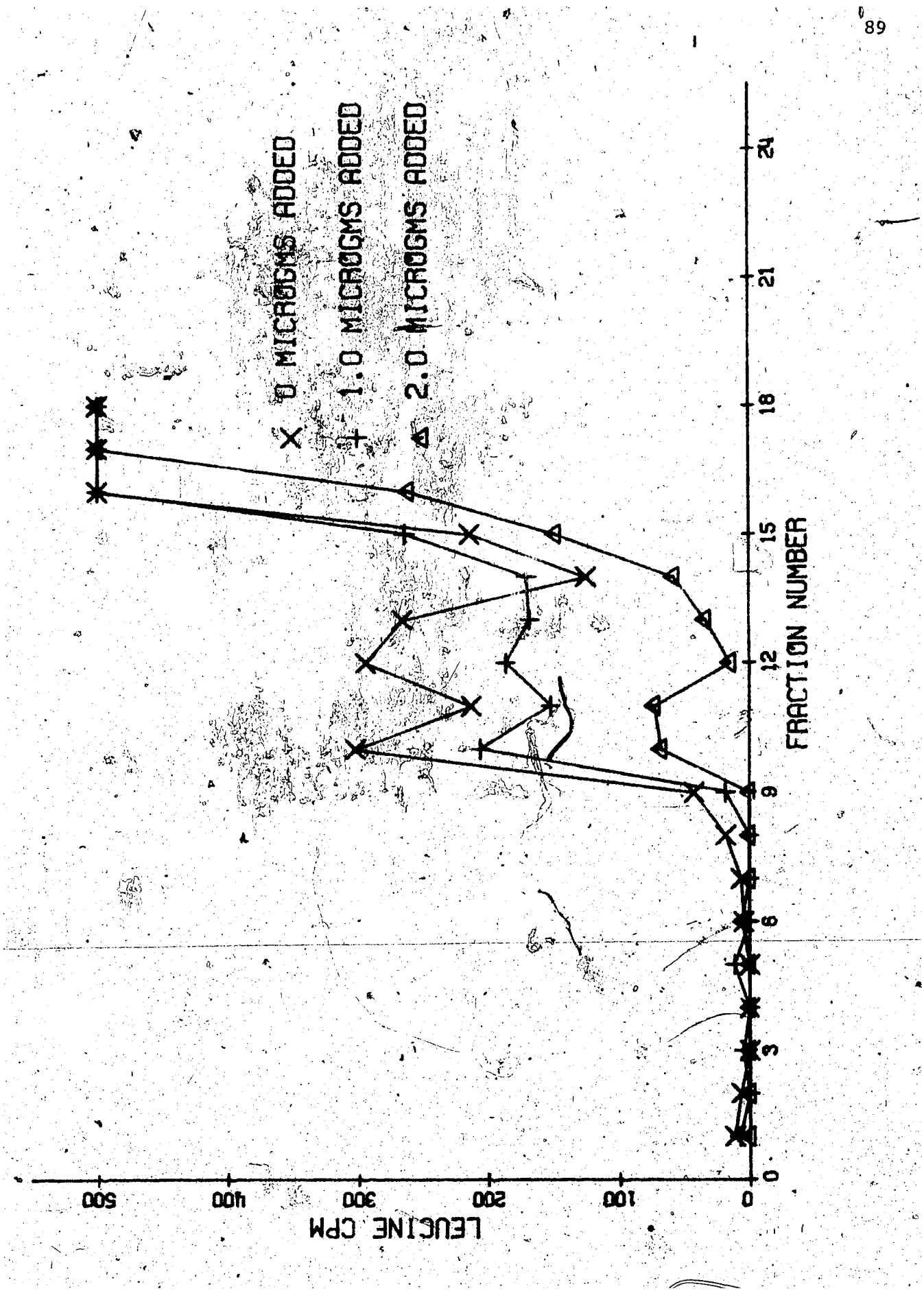
0 MICROGMS ADDED
1.0 MICROGMS ADDED
2.0 MICROGMS ADDED
LEUCINE CPM
0
100
200
300
400
500
FRACTION NUMBER
3
6
9
12
15
18
21
24

FIGURE 11

The ^{3}H-leucine activity after Sephadex fractionation of a sample diluted 1:1 with 1.3 M urea, prior to incubation under the conditions of Fig. 1.

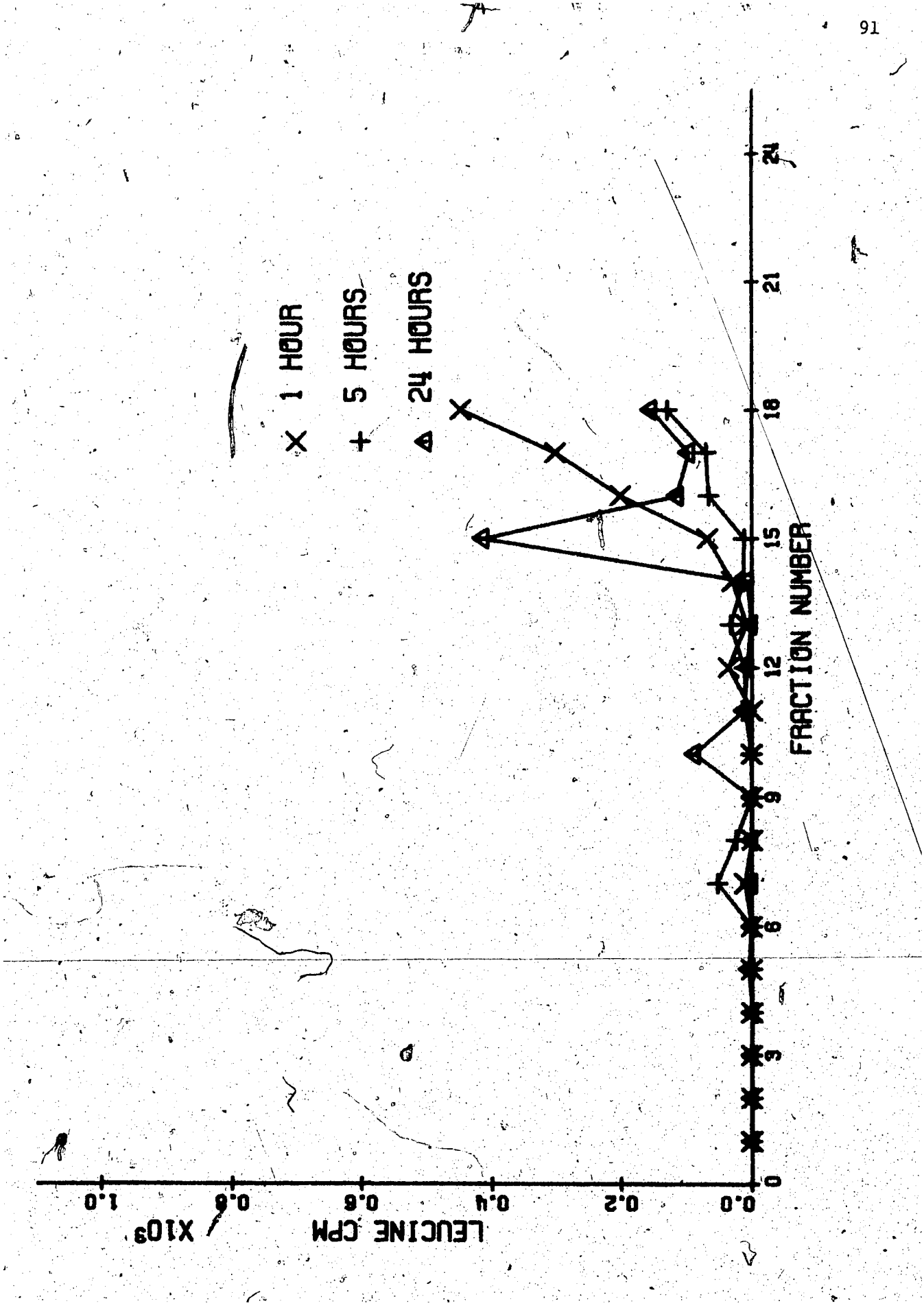
1 HOUR
5 HOURS
24 HOURS
LEUCINE CPM X10³
FRACTION NUMBER
0.0
0.2
0.4
0.6
0.8
1.0
0
3
6
9
12
15
18
21
24

FIGURE 12

G-200 Sephadex fractionation of a 0.1 ml sample of the 1000g supernatant, previously incubated with 12,000 cpm L-leucine, 2.5 mM Mg^{++}, and 0.25 mM colchicine, for 24 hours at 22° C.

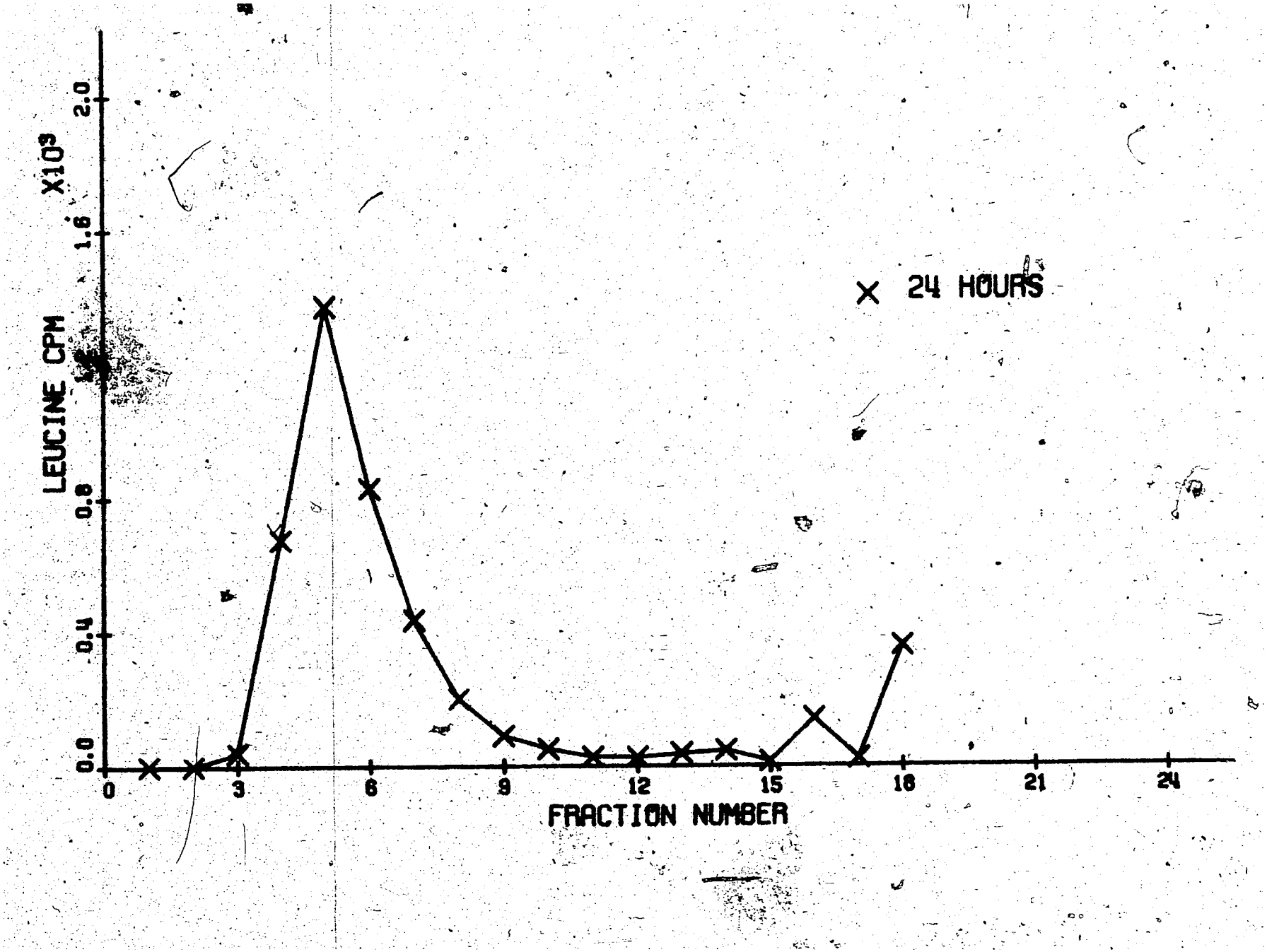
LEUCINE CPM X10³
2.0
1.6
0.8
0.4
0.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER
× 24 HOURS

FIGURE 13

G-200 Sephadex fractionation of a 0.1 ml sample of the 1000 g supernatant. Samples were incubated with 12,000 cpm L-leucine, RNAase, and 2.5 mM Mg^{++} for 4 hours at 37° C.

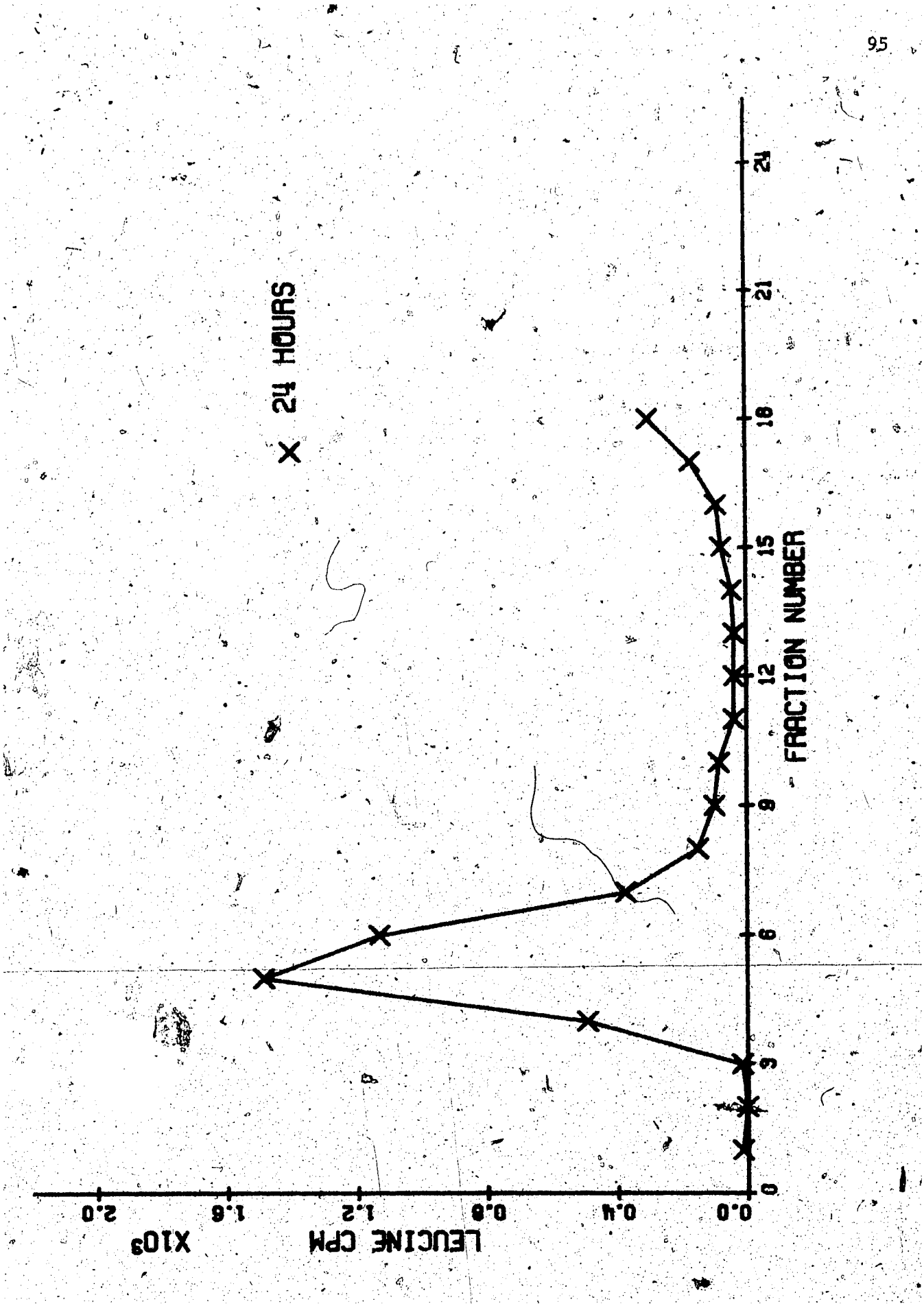
24 HOURS
LEUCINE CPM X10³
0.0
0.4
0.8
1.2
1.6
2.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER

FIGURE 14

The fractionation of the proteins present in a sample from the 1000g supernatant, as the time of incubation is increased from 2.5 to 15 hours. The incubation conditions are as described in Fig. 1, with Mg^{++} present.

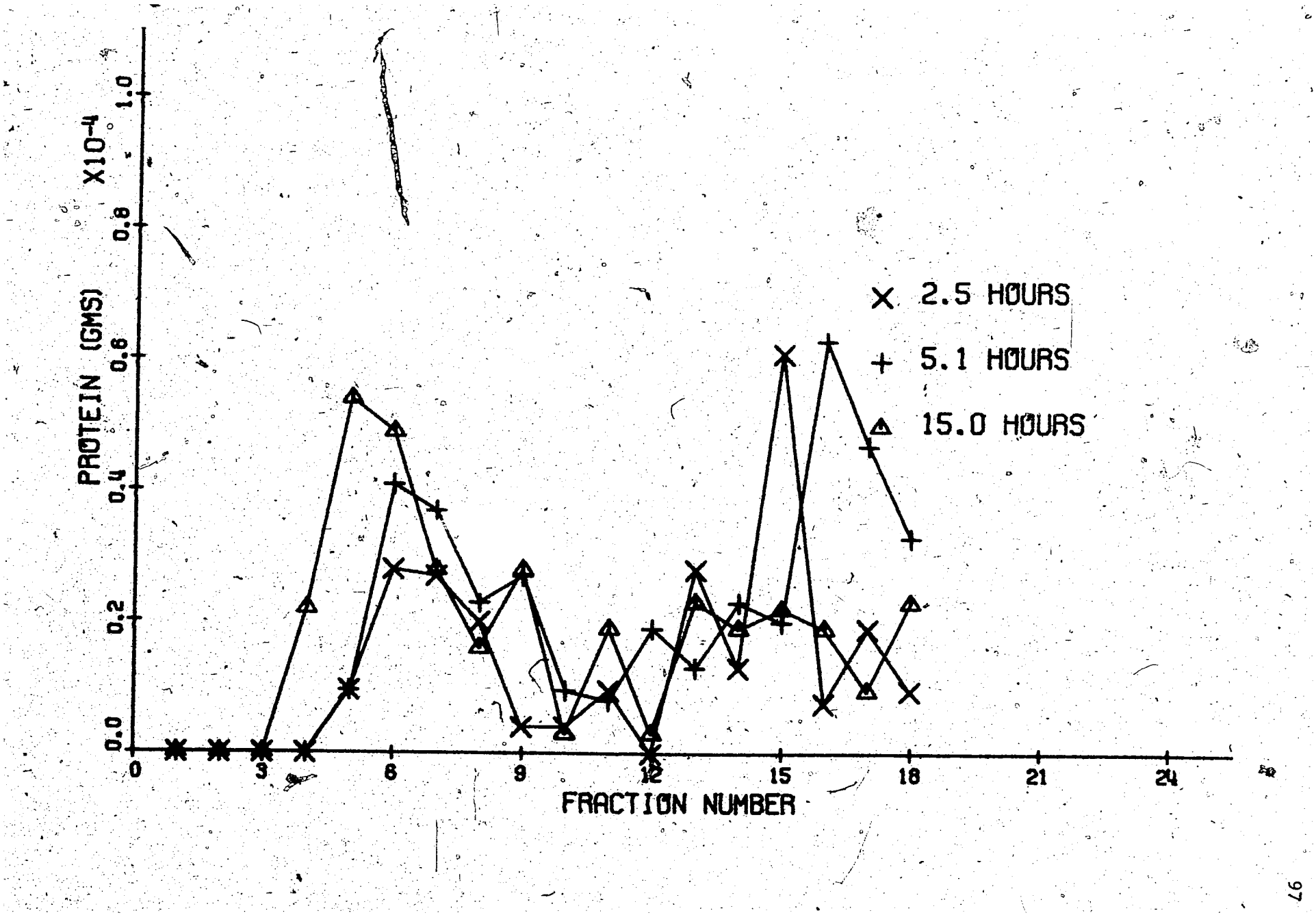
PROTEIN (GMS) X10-4
0.0
0.2
0.4
0.6
0.8
1.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER
2.5 HOURS
5.1 HOURS
15.0 HOURS

FIGURE 15

The fractionation of the proteins present in a sample from the 1000g supernatant, as the time of incubation increased from 3.5 to 16.5 hours. The conditions for incubation were as described in Fig. 2, without the presence of Mg^{++}.

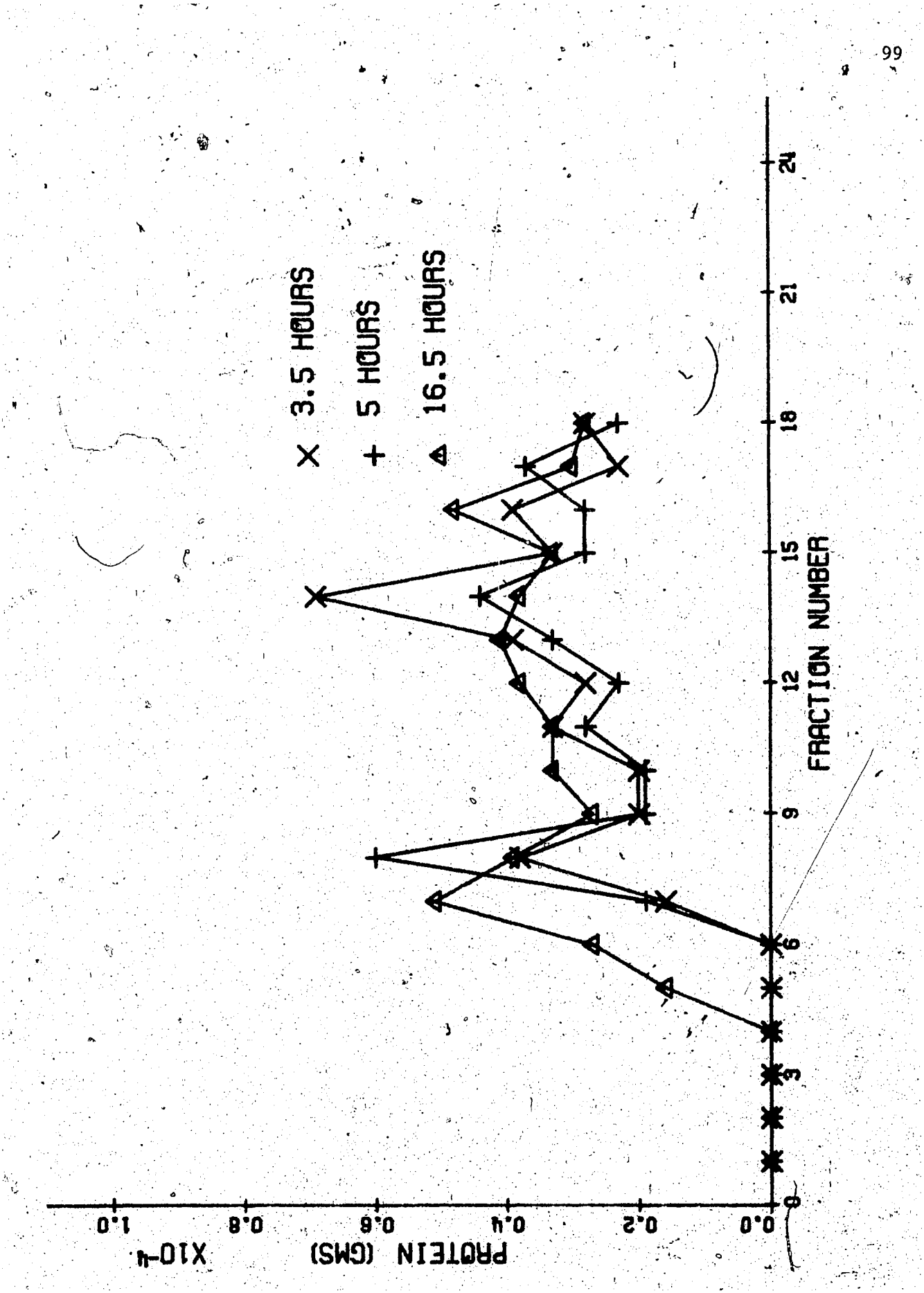
X 3.5 HOURS
+ 5 HOURS
Δ 16.5 HOURS
PROTEIN (GMS) X10-4
0.0
0.2
0.4
0.6
0.8
1.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER

FIGURE 16

The fractionation of the proteins present in a sample from the 1000g supernatant, as the time of incubation hours. The preparation was diluted 1:1 with 1.3 M urea, prior to incubation under the conditions as in Fig. 1, with Mg^{++} present.

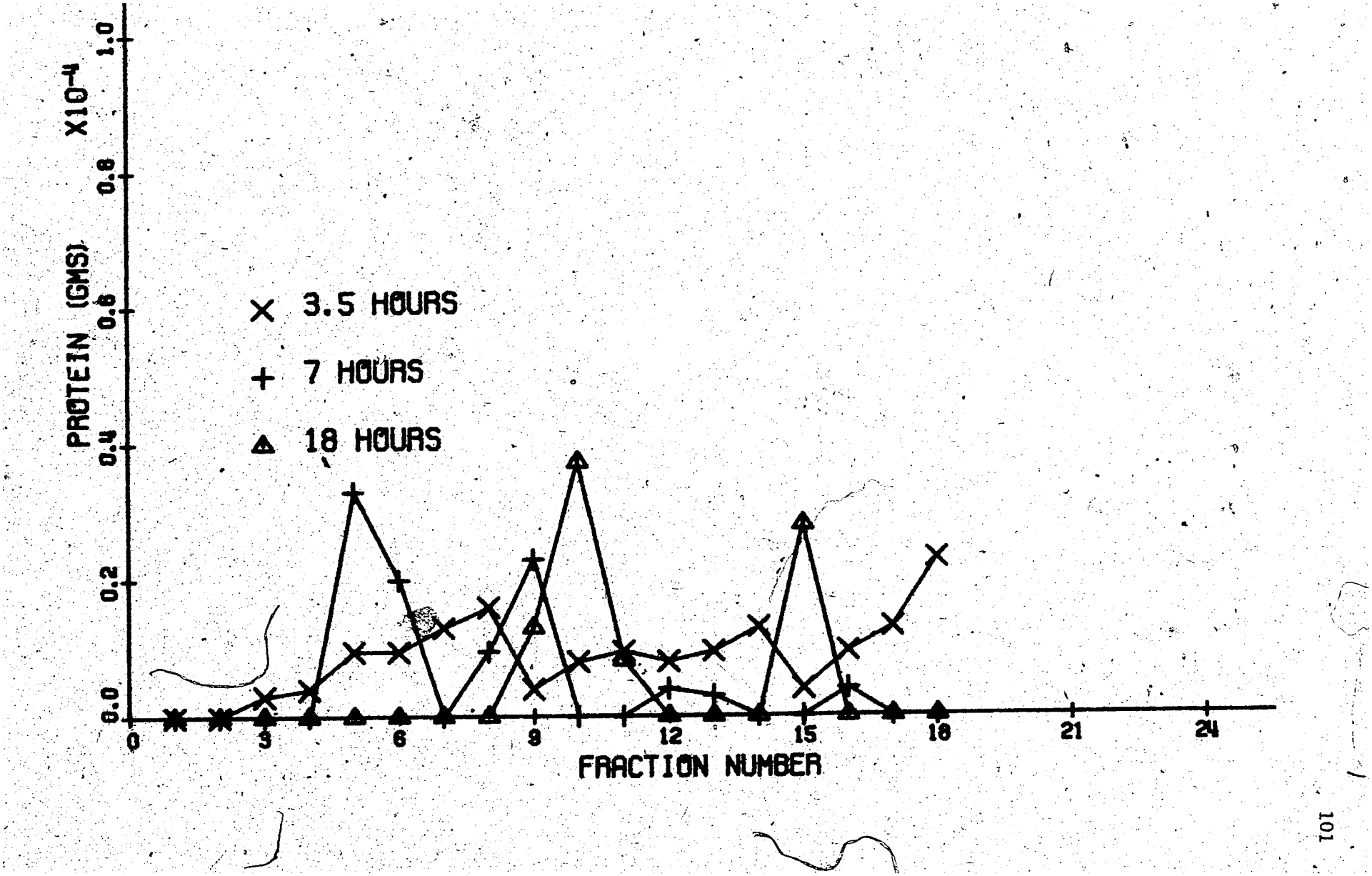
PROTEIN (GMS) X10⁻⁴
1.0
0.8
0.6
0.4
0.2
0.0
× 3.5 HOURS
+ 7 HOURS
△ 18 HOURS
0
3
6
9
12
15
18
21
24
FRACTION NUMBER

FIGURE 17

Protein concentration of the Sephadex fractionations from a sample previously incubated with 0.25 mM colchicine and Mg^{++}, under the conditions of Fig. 12.

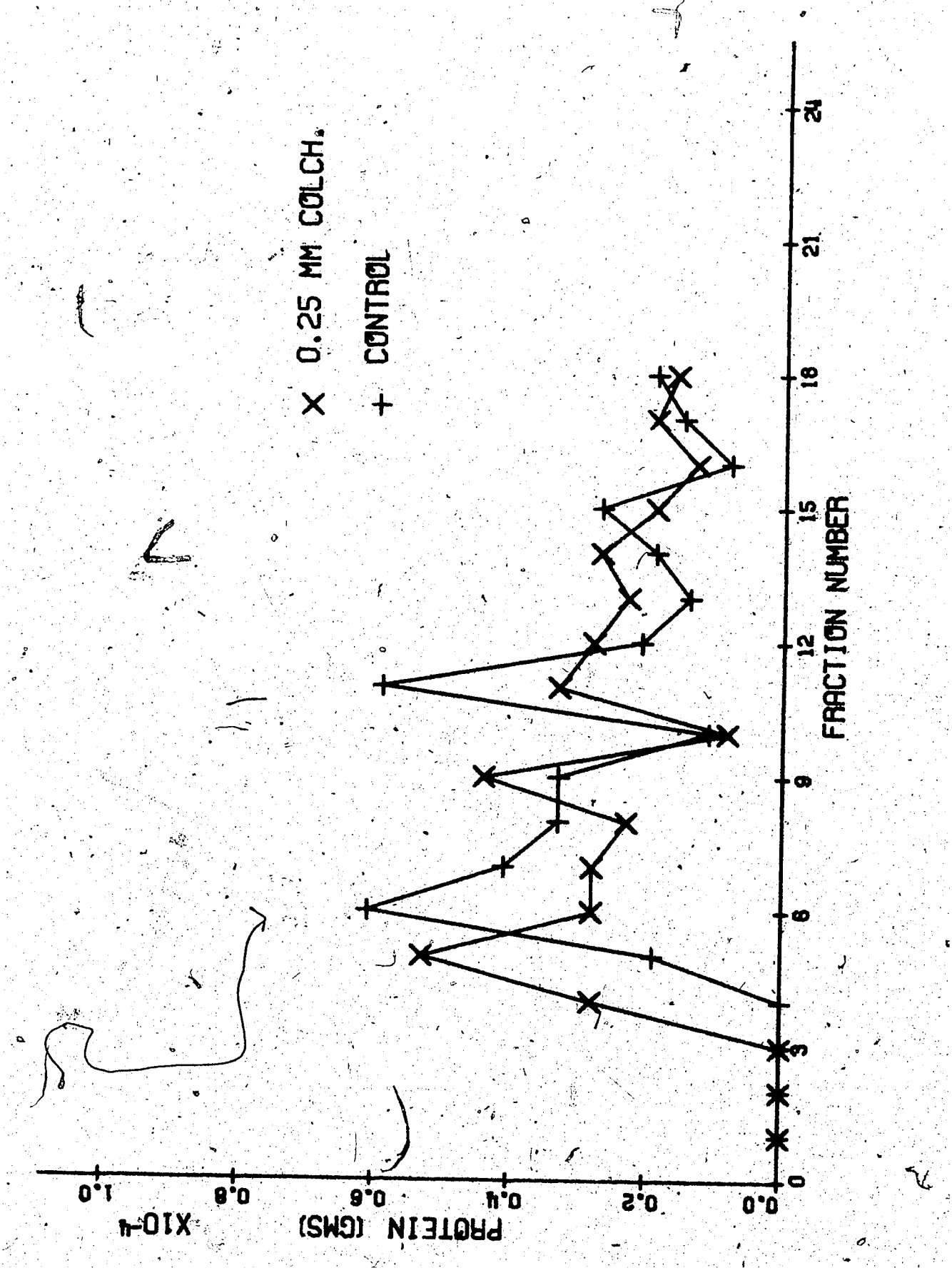
X 0.25 MM COLCH.
+ CONTROL
PROTEIN (GMS) X10-4
0.0
0.2
0.4
0.6
0.8
1.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER

FIGURE 18

The protein concentration of the Sephadex fractions from a sample previously incubated with 2.5 mM colchicine and 2.5 mM Mg^{++}, under the conditions of Fig. 12.

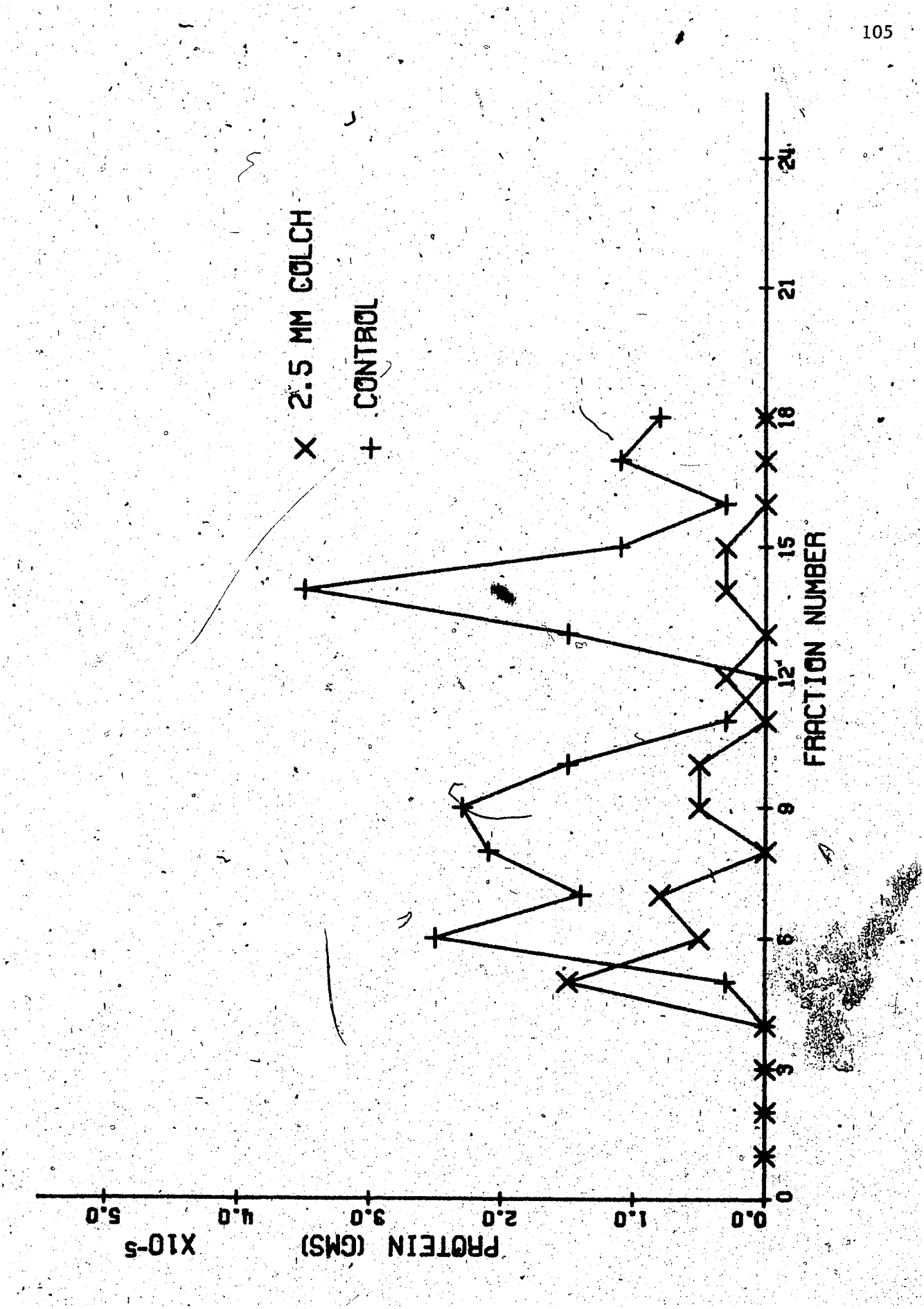
X 2.5 MM COLCH
+ CONTROL
PROTEIN (GMS) X10-5
0.0
1.0
2.0
3.0
4.0
5.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER

FIGURE 19

The protein concentration of each Sephadex fraction, from samples previously incubated with 0.25 mM colchicine, both with and without the addition of 3 mM ATP. A leucine control is added for comparison. The preparations were incubated for 4 hours at 37° C, with 2.5 mM Mg^{++} present.

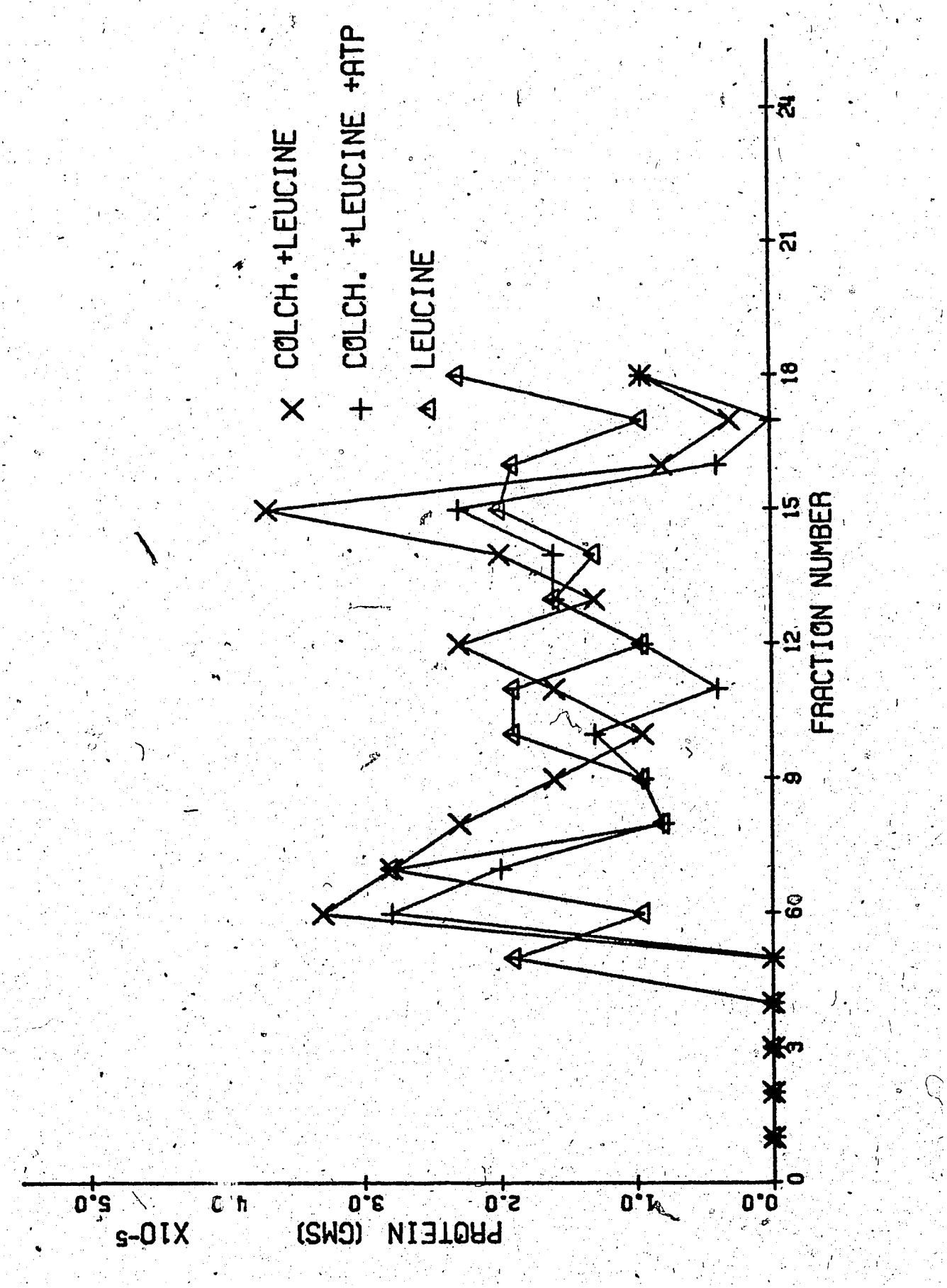
COLCH.+LEUCINE
COLCH. +LEUCINE +ATP
LEUCINE
PROTEIN (GMS) X10-5
FRACTION NUMBER
0.0
1.0
2.0
3.0
4.0
5.0
0
3
6
9
12
15
18
21
24
60

FIGURE 20

The protein concentration of each Sephadex fraction from a sample previously incubated with RNAase , under the conditions of Fig. 19. The control sample was incubated under similar conditions.

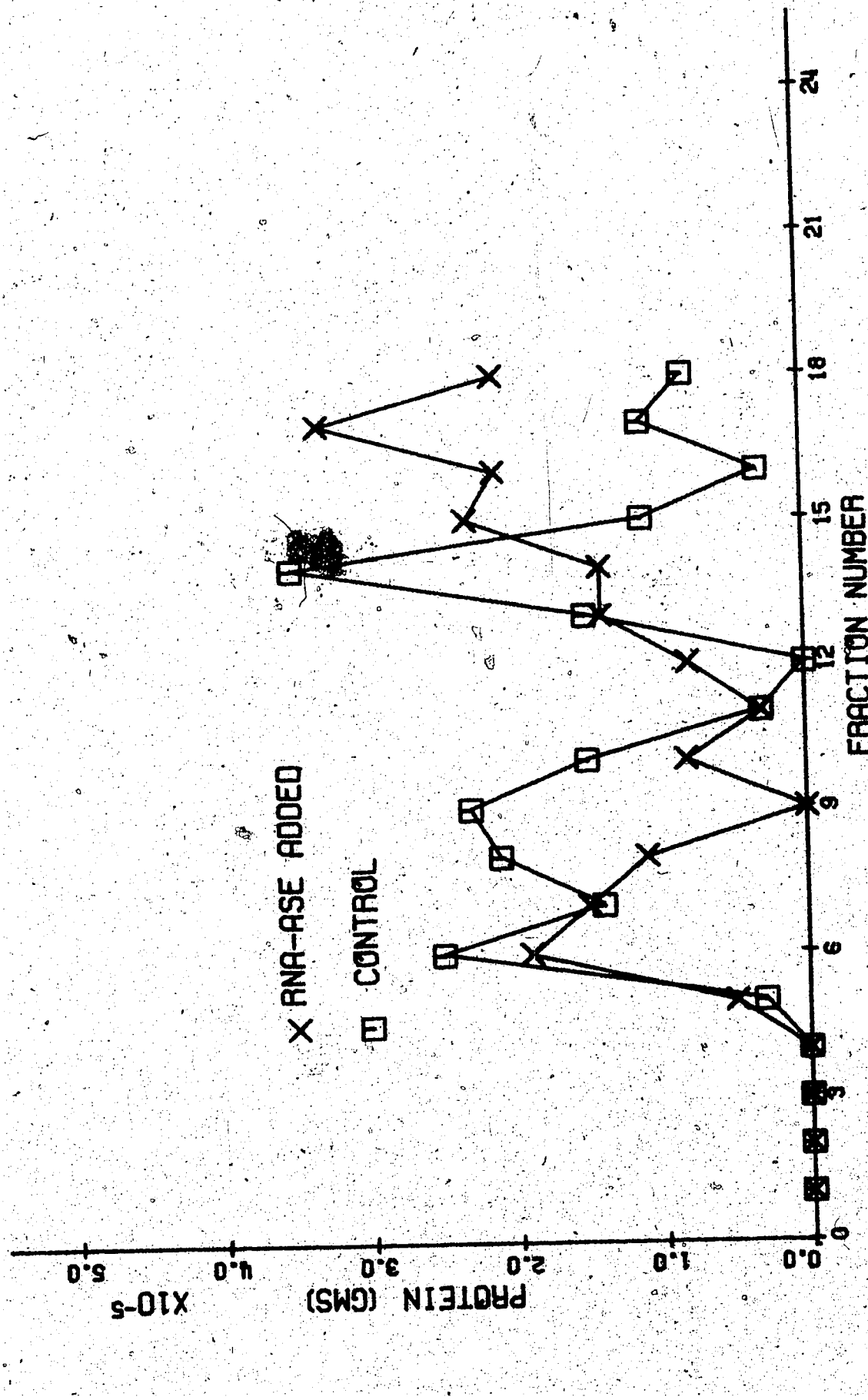
X RNA-ASE ADDED
□ CONTROL
PROTEIN (GMS)
X10-5
0.0
1.0
2.0
3.0
4.0
5.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER

FIGURE 21

The protein concentration following fractionation of a sample incubated with 3 mM ATP, under the conditions of Fig. 1.

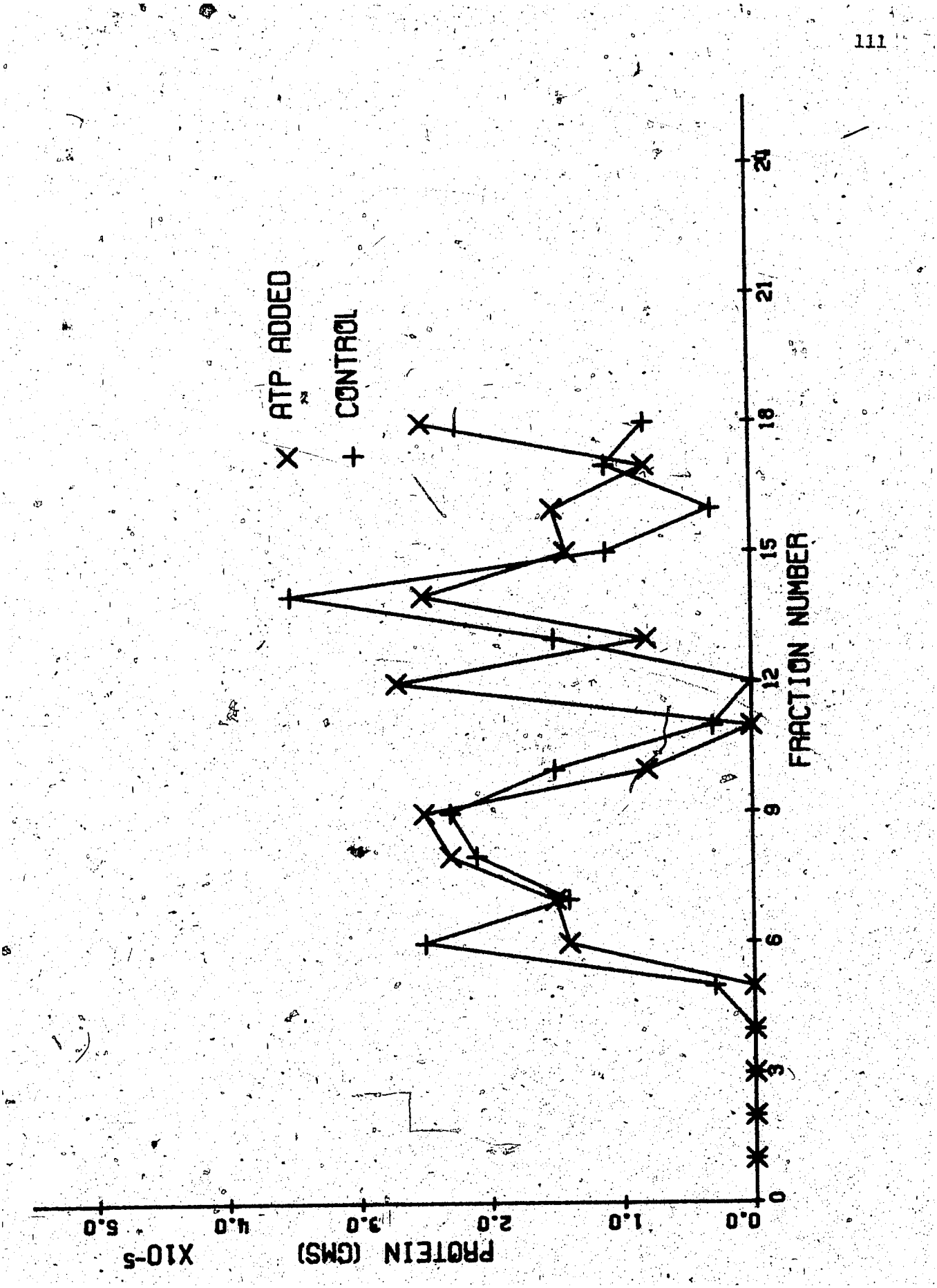
ATP ADDED
CONTROL
PROTEIN (GMS)
X10-5
0.0
1.0
2.0
3.0
4.0
5.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER

FIGURE 22

Sephadex gel filtration of a 0.1 ml sample of rat muscle and plasma proteins incubated for 24 hours, with ^{3}H-leucine. The conditions are as stated for Fig. 1.

RAT MUSCLE
RAT BLOOD
LEUCINE CPM
X10³
0.0
0.2
0.4
0.6
0.8
1.0
0
3
6
9
12
15
18
21
24
FRACTION NUMBER

FIGURE 23

Arrhenius plot of the temperature effect on the binding of ^{3}H-leucine, for a 24 hour incubation period, with 2.5 mM Mg^{++} present. The upper line represents the total leucine bound to fractions 1-16. The lower line represents the leucine bound to peak 1 polymer only. Each point represents the mean ± S.E. of three replicates.

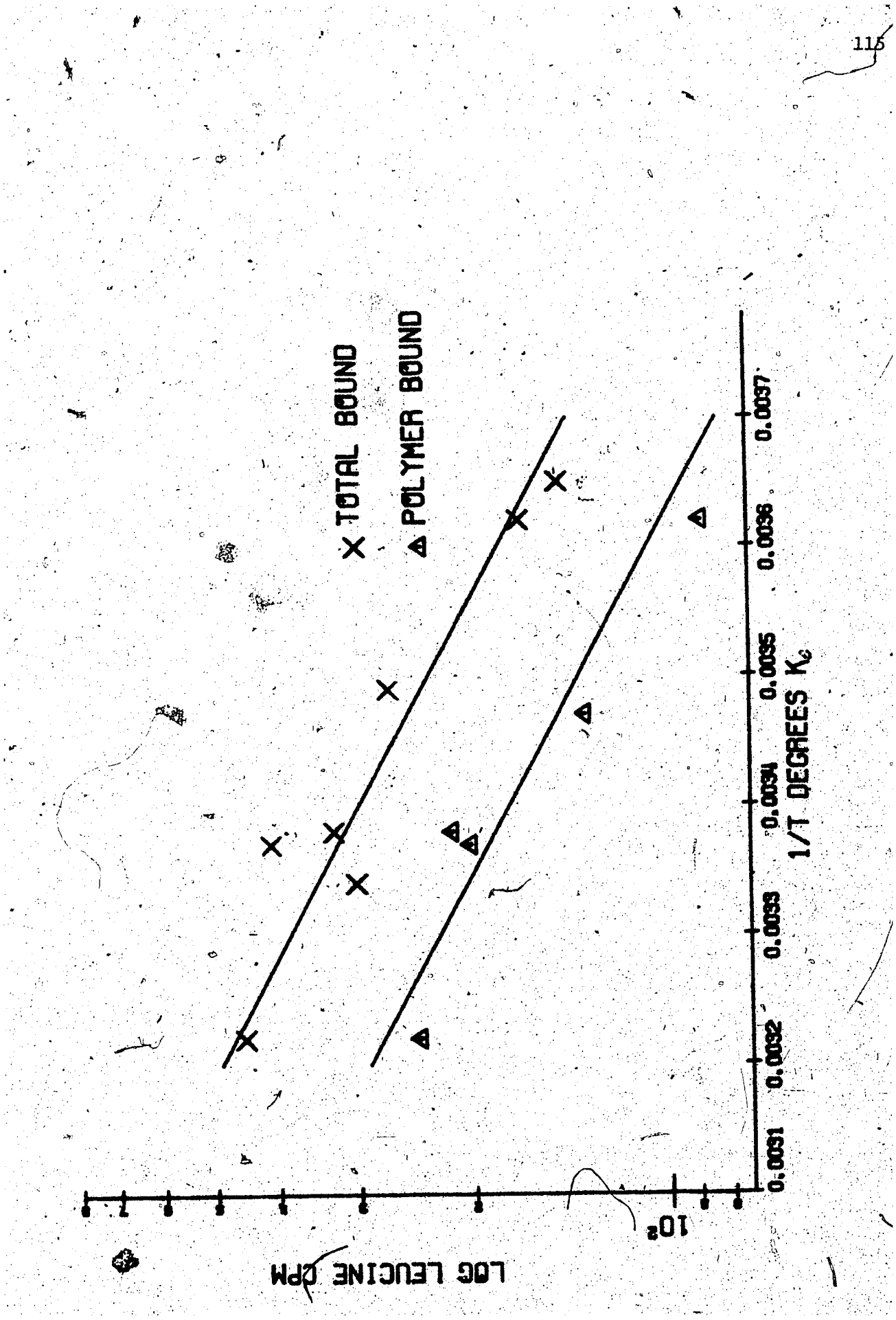
LOG LEUCINE CPM
10²
1/T DEGREES K
0.0031
0.0032
0.0033
0.0034
0.0035
0.0036
0.0037
POLYMER BOUND
TOTAL BOUND

FIGURE 24

The ATPase activity of single sample of fractionated 1000 x g supernatant proteins, incubated at 22° C for various times, with and without Mg^{++} present. The activity is expressed as meq H^{+}/ min. /g protein.

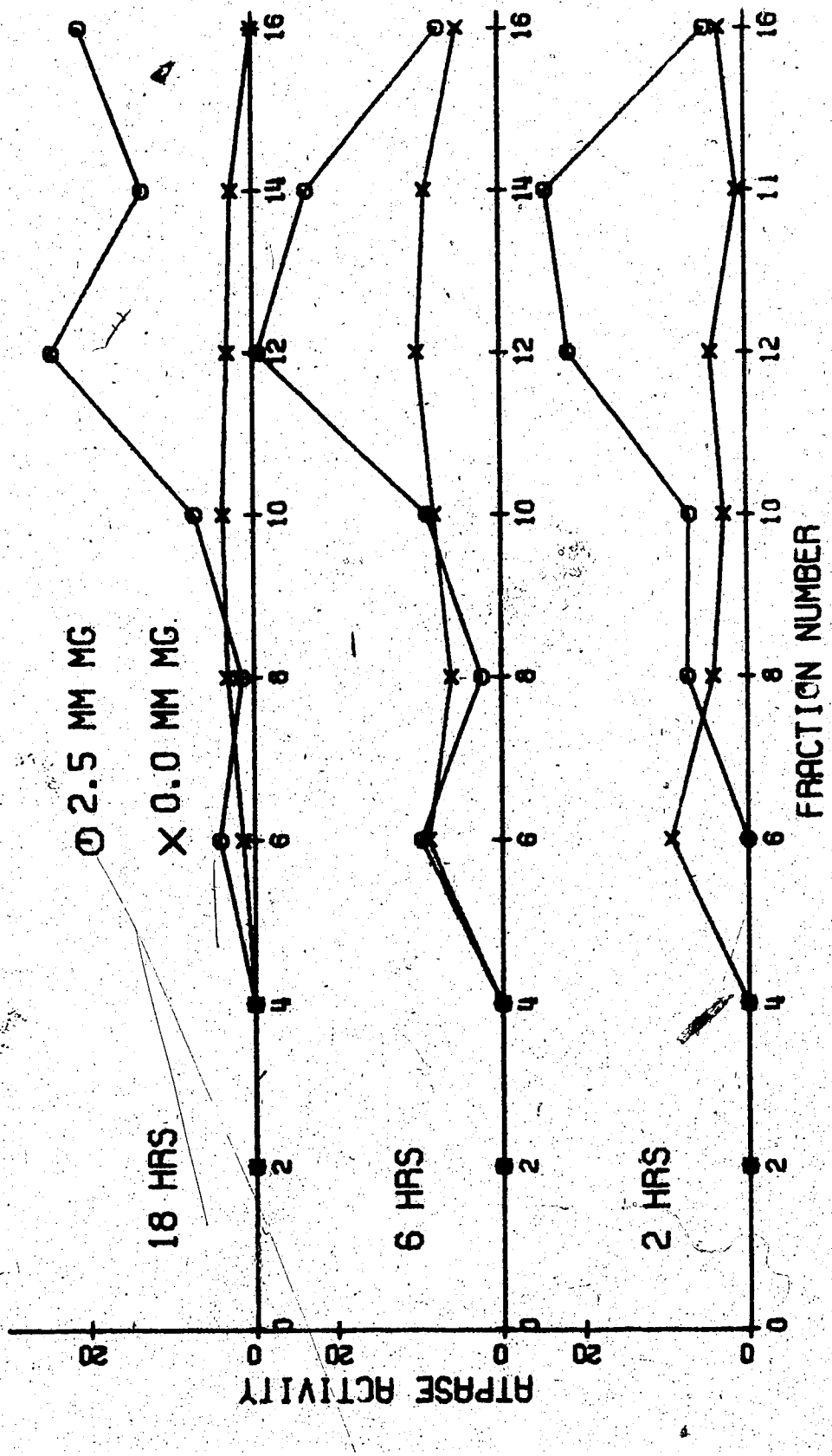
ATPASE ACTIVITY
FRACTION NUMBER
18 HRS
6 HRS
2 HRS
Θ 2.5 MM MG
X 0.0 MM MG

TABLE 1

Percent increase in the amount of each protein per Sephadex fraction, as the time of incubation increases from 1 to 6 hours. The conditions of incubation are as stated for Fig. 1, with Mg^{++} present.

TABLE 1 is continued on the next page.

TABLE 1

SDS GEL PROTEINS	SEPHADEX FRACTIONS #3,4	#5,6	#7,8	#9,10
PA		-3.2	-22.7	-51.9
PB	-11.4	0.4	35.8	52.0
PC	41.9	20.9	10.2	-1.6
PD		-6.2	-2.3	-1.3
PE			-1.8	1.0
PF		-27.9	23.9	-12.1
PG	-33.3	8.0	4.6	13.9

TABLE 1

PROTEINS	#11,12	#13,14	#15,16	#17,18
PA	-65.0	-50.8	-49.7	-42.9
PB	43.9	37.0	41.3	32.3
PC	0.5	2.6	1.3	0
PD	-1.1	-0.5	-0.4	4.5
PE	-1.6	1.0	-3.8	1.3
PF	-6.7	-1.2	-3.1	-3.7
PG	30.0	12.0	14.2	8.4

TABLE 2

Percent increase in bound ^{3}H-leucine, as the incubation time increases from 1 to 4.5 hours and from 1 to 6 hours. The conditions of incubation are as stated for Fig. 1.

TABLE 2 is continued on the next page.

TABLE 2

TIME INTERVAL	SEPHADEX FRACTIONS #3,4	#5,6	#7,8	#9,10
1 to 4.5 (hours)	13.0	4.6	-11.2	-3.7
1 to 6.0 (hours)	6.6	19.2	-6.8	-2.0

TABLE 2

TIME	SEPHADEX FRACTIONS			
INTERVAL	#11,12	#13,14	#15,16	#17,18
1 to 4.5 (hours)	-2.4	-3.2	7.0	9.9
1 to 6.0	-0.7	-1.9	-1.2	1.1

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