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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

......................Mary Allison Janaky......................................

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......of the Drosophila melanogaster Embryo...............................

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF......Bachelor of Science in Biochemistry...........................

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CHARACTERIZATION OF THE MICROTUBULE PROTEIN SYSTEM

OF THE *DROSOPHILA MELANOGASTER* EMBRYO

BY

MARY ALISON CANADY

THESIS

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Microtubules play a wide range of functions in nearly every eukaryotic system. From the cytoskeleton to the cilia to the mitotic spindle, microtubules provide the framework and the driving force for many of the inter- and intracellular movements that cells must make. The importance of microtubule function has lent the protein to extensive biochemical and biophysical analyses which have revealed much about their structure and characteristics.

The functional unit of a microtubule is a dimer of two very similar proteins--α and β tubulin. Both have a molecular weight of approximately 50,000 daltons with α-tubulin being the larger by about five amino acids (1), and an average of 42% sequence identity between them. Both are highly acidic, especially near the C-terminus. Different isoforms of each tubulin protein have been documented, those differences occurring both interspecifically, inter- and sometimes intracellularly, and throughout development. In the *Drosophila* embryo, four distinct tubulins, α_1, α_2, β_1 and β_3, have been identified and characterized by Raff, *et al.* (2). It has even been proposed that tubulin, like antibodies, may have constant and variable regions (1). Nonetheless, tubulin is highly conserved throughout evolution, no doubt owing to its vital role in numerous basic cellular functions.

Associated with the microtubules (MTs) are an indefinite number of associated proteins known collectively as microtubule-associated proteins (MAPs). Operationally, a MAP is any protein that coassembles with microtubules during *in vitro* purification and shows a definite

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stoichiometric relation to the polymerized tubulin. While this is useful as a biochemical definition, it says nothing about the cellular function of a MAP. The challenge therefore remains to devise experimental systems that can elucidate the function of this potentially important class of proteins. *Drosophila* offers unique opportunities as a biological system, amenable to biochemical, cell biological, and genetic manipulations.

Biochemistry and Cell Biology of Microtubules

Inside a "typical" cell, cytoplasmic microtubules form a variety of supramolecular structures. These cytoskeletal arrays are organized by poorly defined regions in the cytoplasm known as microtubule organizing centers (MTOC). The MTOCs we know the most about are the centrosome and kinetochore. MTOCs are involved in defining the cell's during mitosis. Numerous studies have shown that MTs organize the cytoplasm. For example, drugs that specifically inhibit MT function have led to abnormal positioning and function of many organelles, suggesting that MTs function in anchoring and orienting the cytoplasm in this way (1). Nerve cells are particularly rich in MTs that are thought to organize and define the polarity of the nerve axon.

Because of the abundance (and importance) of MT in brain tissue, a primary source of tubulin for study has been bovine brain. Many advancements have been made in the study of the dynamics of microtubule polymerization as well as the identities, structures, and functions of several MAPs. Current work on bovine microtubules includes the mapping out of the binding regions of the known MAPs to the microtubule structures. Also, the literature abounds with the

Identification and characterization of MAPs from this and other systems.

How and why do microtubules assemble? Assembly requires the binding of GTP, reduced Ca^{2+} levels, the presence of Mg^{2+}, and a critical concentration of tubulin (typically about 0.1-0.2 mg/ml). Models have been proposed for the pathway of MT assembly and disassembly (3,4,5,6). These models are usually based upon solution kinetics of polymer assembly and disassembly that can provide only indirect information. The specifics of MT formation have yet to be fully identified for most systems.

A few MAPs have been characterized. MAP1 and MAP2 are the predominant proteins found in a group of high molecular weight (HMW) proteins which copurify with bovine brain tubulin. MAP2, a doublet of MW 286,000 and 271,000 daltons (1), is a long, L-shaped molecule, the shortest arm being linked to the MT, the longer arm extending almost perpendicularly from the MT. The structure and function of the 350,000 dalton MAP1 is less defined, but decorates MTs as "arms" as seen in the electron microscope (7). Both MAP1 and MAP2 are thought to stabilize MTs, each functioning differently for a given cell. In nerve cells, MAP1 has been suggested as a link between MT and cell membranes, while MAP2 is thought to be involved with the interactions of MT with actin and neurofilaments (15). Another group of MAPs are the *Tau* proteins, which consist of 5 proteins in the MW range of 55,000 and 77,000 daltons. In general, the functions of the *Tau* proteins are less defined than they are for MAP1 and MAP2.

Advantages of the *Drosophila* Embryonic System

As with MTs, MAPs have been seen to vary from species to species, as well as within the organism. As mentioned previously, the *Drosophila melanogaster* embryo provides several advantages for studying MAP structure and function.

1. Rapidly developing nervous system accounts for MT to be up to 2.5% of total cytoplasmic protein (see Tables 1 and 2). While this is less than the percentage found in vertebrate brain tissue it is certainly substantial enough to conduct studies upon.

2. Whole mount embryos can be labelled fluorescently using monoclonal antibodies (Mabs) to screen for potential MT or MAP antigenticity. This provides a quick screen. In addition, expression and distribution of a given MAP can be easily followed through development--often on the same slide mount. MAPs can also be characterized with respect to the cytoskeleton and the mitotic spindle, which is very useful in determining the function of a particular MAP. Karr, *et al.* have characterized MT in the cytoskeleton and mitotic apparatus of the *Drosophila* embryo (8), while Kellogg, *et al.* have studied MAPs (9). However, the developmental fates of the MAPs were not studied.

3. The *Drosophila* genome has been extensively mapped, and mutants are readily available.

5. Although *Drosophila* are invertebrate, many protein systems are very similar to those of higher vertebrates. As stated earlier, tubulin is highly evolutionarily conserved, and probably has similar properties in the two systems. The developing *Drosophila* is being used by several

workers as a model for neurogenesis (10). In addition, studies involving *Drosophila* proteins will likely yield quicker and more informative results than those in higher vertebrates, because of the ability to manipulate the organism genetically

In Vitro Cycling

Cell extracts were prepared by collecting embryos using a 8.3 mM Triton X-100, 1.19 M NaCl solution. Embryos were dechorionated using a 2.63% sodium hypochlorite solution for 90 seconds. After rinsing extensively with Triton to remove sodium hyperchlorite, an equal volume (1 ml/g of embryos) of protease inhibitor cocktail in PEM buffer (80 mM Pipes, 1 mM $MgSo_4$, 1 mM EGTA) was added. Solution was homogenized using a motor driven teflon pestle then left on ice for twenty minutes. After this, homogenate is ultracentrifuged at 80K rpm in a Beckman TL100 for 10 minutes at 2°C. Supernatant is then spun at 100K for 20 minutes at 2°C. This supernatant defines the cell extract. The cell extract is then taken through two cycles of assembly/ disassembly. To the cell extract, 0.35X of glycerol is added, along with 0.02X of a 50 mM solution of GTP. This mixture is incubated for 45 minutes at 25°C (first warm assembly). Then, the mixture is spun at 100K rpm for 25 minutes at 25°C. Supernatant after this step is the H_1S fraction, while the pellet is the H_1P. The pellet is resuspended in PEM buffer to a protein concentration of 10-15 mg/ml. The resuspended pellet is then kept on ice for 30 minutes (first cold disassembly). Fraction is now spun at 75K rpm for 10 minutes. The supernatant is the C_1S fraction, while the pellet is the C_1P. GTP and glycerol are added in equivalent amounts as before, and the sample is taken through a second cycle of warm assembly followed by cold

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disassembly until the C$_2$S fraction is obtained. Note: the H$_2$P pellet should be resuspended to a protein concentration of about 5 mg/ml.

Ion Exchange Chromatography

Ion exchangers used in this study:

Phosphocellulose--Cellulose Phosphate Cation exchanger (Sigma, St. Louis, MO) used for columns and batch preparations. Before use, Cellulose Phosphate was washed with 0.5M KOH 3 times for 10 minutes each. It was then rinsed with distilled H$_2$0 until effluent had a pH of 8. 0.5M HCl was then washed over the matrix, again 3 times for 10 minutes each. Distilled water rinsed the beads again until the effluent was a pH of 8. Cellulose Phosphate was then ready for a column or batch preparation.

QAE Sephadex--Pharmacia (Piscataway, N. J.) QAE Sephadex A-50 Anion Exchanger was used, and the only preparation was to swell the beads with PEM buffer before use.

Two Dimensional Gel Electrophoresis

First dimension was run according to the Biorad (Richmond, CA) Mini-Protean II 2D protocol with some alterations. In short, the gel monomer solution consisted of 9.2M urea, 4% acrylamide, 4.0% Triton X-100, 0.67% each of "Pharmalyte" (Sigma, St. Louis, MO) Ampholytes of ranges 3-10, 6-8, and 4-6.5. Sample buffer consisted of 9.5M urea, 4.0% Triton X-100, 200mM Dithiothreitol (protocol called for 5% β-mercaptoethanol, but we found that the DTT produced fewer staining

artifacts), and Sigma "Pharmalyte" Ampholytes in the same amounts as in the gel monomer solution. Isoelectric focusing (IEF) was carried out with a 20mM NaOH upper chamber buffer and a 10mM H_3PO_4 lower chamber buffer, both degassed for at least 30 minutes before use. Noodles were electrophoresed first for 10 minutes at 500V, then for 3.5 hours at 750V. For Non-equilibration pH Gradient Electrophoresis (NEPHGE), the upper chamber buffer was a 10mM H_3PO_4 solution, while the lower chamber buffer was 20mM NaOH, with degassing as before. Electrophoresis was for the same voltage and duration as IEF, with the exception that the electrodes must be reversed. Thus, the anode (positive electrode) must be attached to the upper chamber, with the cathode (negative electrode) connected to the lower chamber. After extrusion of the noodles, the samples were stored at -70 C if the second dimension was not run immediately. Second dimension was run after a very brief equilibration (usually a minute) with an equilibration buffer consisting of 0.0625M Tris HCL, pH 6.8, 2.3% (w/v) SDS, 10% (w/v) glycerol, and 0.05% bromophenol blue. Noodles were run on 10% SDS Polyacrylamide gel electrophoresis (PAGE) (11).

Western Blotting

10% SDS Page gels (11) were run. The larger gels were run using the Hoefer SE600 Vertical Slab Unit. The smaller gels were run using Biorad (Richmond, CA) Mini-Protean II 2D gel apparatus (this was used for both the second dimension of the 2D gels and the 1D gels--with wells). For immunoblotting, (12), proteins were transferred to 0.45 μm nitrocellulose (Micron Separations Inc., Westboro, MA) using a capillary

technique   overnight in Towbin transfer buffer.   Blots were then
blocked using a 5% Nonfat Dry Milk solution in Tris buffered saline
(TBS--20mM Tris-HCl pH 7.5, 150mM NaCl) for >1 hour.   Staining was by
the Stratagene (La Jolla, CA) *pico* Blue Immunoscreening Kit, using
primary mouse, then secondary goat anti mouse conjugated with
alkaline phosphatase.   Color was developed using 5-bromo-4-chloro-3-
indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

EXPERIMENTAL SECTION

We have purified MTs using "*in vitro* cycling" of the *Drosophila* embryo cell extract (see methods) and then used the purified MTs and MAPs to generate Mabs in mice. These Mabs were then screened using whole-mount embryos. For a description of a similar characterization of 24 unique MAPS, see Kellogg, *et al.* (9). They estimate that 50 MAPs will bind to a microtubule affinity column, and this is consistent with our estimation of *in vitro* cycled MAPs (MAPs that will cycle with microtubules *in vitro*, see figures 10-12).

To begin our characterization of the microtubule-protein (MTP) system, *in vitro* cycles of warm assembly and cold disassembly were prepared (13). The fractions collected after each step of purification were run on an 8% SDS polyacrylamide gel (figure 1) for a qualitative analysis. Percent yield after each step was determined using a modified Lowry assay (BCA Reagent, Pierce Chemical Co, Rockford, IL). The final yield was determined as being the total mg protein present in the "C_2S" (Cold Supernatant--2nd cycle) compared to the total mg of cytoplasmic protein present in the original cell extract. After many such MTP preps were performed, a correlation was found between the age of the embryos and the percent yield obtained. In the older (~16 hours old) embryos, a 2-3% yield was normal, while a 0.5-0.8% yield was typical for the younger (~4 hours old). On comparing the young and old cell extracts, however, the amounts of tubulin present in the two fractions are nearly the same (figure 1). These results indicate that

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the difference(s) between the two samples may reflect the differences in MAP composition.

We wished to correlate the yields of MTP with the age of the embryos. This was necessary because the female *Drosophila* sometimes holds the fertilized embryo before laying, thus causing many of the embryos collected to be older than the actual collection period. To compensate for this phenomena, we began collections only after we had allowed the flies to lay their embryos on fresh plates for two hours. These so-called "clearing plates" were then discarded and the collection began with new plates. Thus, the flies would have ample opportunity to lay their embryos, and the chance of the flies withholding them is decreased (females prefer to lay their embryos in areas that are not overcrowded with other embryos).

What type or types of mechanisms are causing the observed difference in MTP yields? Two reasonable hypotheses can be generated: 1. There is (are) a MT stimulating MAP(s) present in older embryos but absent or dysfunctional in younger embryos. 2. There is (are) a MT inhibitory MAP(s) present in younger embryos but absent or dysfunctional in older embryos. We can use the examples of known MAPs in other systems to analyze the problem. MAP1 and MAP2 are found in many systems to stimulate MT assembly (14). MAP1 does this also, presumably by anchoring the MTs to structures intracellularly (15). To date, no gene products have been known to inhibit assembly, but this may be that these are hard to isolate, since the putative proteins would not be bound to assembled MTs and thus would be hard

to isolate. As in most developmentally regulated phenomena, the answer is probably not as simple as either of our hypotheses.

To test our hypotheses, we decided to begin by fractionating the cell extract using ion exchange chromatography. This method had been successfully used in the early studies of brain MTs. We were hoping that we would find a matrix that would help us to separate tubulin from putative MAPs and help us to further characterize these proteins.

Phosphocellulose was the first ion exchanger screened. Since tubulin is negatively charged at a neutral pH, this cation exchanger would be used to bind all of the positively charged proteins and thus give us a purer fraction containing tubulin for polymerization. On running the columns, we found that the flowthrough was indeed purified from several ~45,000 dalton yolk proteins. (10). This was interesting and perhaps useful--we decided to try to polymerize the tubulin in the flowthrough to determine if these proteins played a role in polymerization, along with the many other proteins that would bind to the phosphocellulose. Before doing this, we ran a preliminary phosphocellulose column and ran the fractions (or representative fractions) on 10% SDS gels and performed western blots. A sample gel can be seen in figure 2, and the corresponding western blot probed with anti α-tubulin in figure 3.

To determine the effect of phosphocellulose fractionation on MTP purification, we attempted *in vitro* cycling on the flowthrough. The flowthrough fractions seemed to be too dilute to perform *in vitro* cycling with. This dilution problem can be seen in the gel run on the *in*

vitro cycling fractions taken after a phosphocellulose preparation, figure 4.

A different type of exchanger was then used--one that would presumably concentrate the tubulin instead of diluting it. An anion exchanger would be such an exchanger, so we used QAE Sephadex. Columns were loaded with approximately 6 mg cytoplasmic protein (i.e. from cell extract) per ml column volume. After rinsing with at least 40 ml PEM, proteins were eluted from column with a 20 ml 0-1.0M KCl gradient. As can be seen in the western blot in figure 5, tubulin was eluted after about fourteen mls were collected after the salt gradient had been started. In addition, the tubulin bands seem to be much more concentrated than in the phosphocellulose preparations.

A QAE batch preparation was used to fractionate the cell extract, and then *in vitro* cycling was performed--results can be seen in figure 6. As can be seen by the protein content of the fractions leading up to the C_2S fraction, an abundance of yolk proteins prevail. The components of the QAE purified cell extract are not sufficient for MT preparation through *in vitro* cycling.

We decided to determine if there was a MT assembling stimulator present in the older embryos but absent in the younger embryos by doing a direct assay. This was done by adding a portion of an older embryo cell extract to a young cell extract, and then cycling the mixture. An increase in tubulin in the C_2S fraction of the "young + old" compared to the "young only" cell extracts would indicate that there is a assembly stimulating protein present in the older embryos.

The experiment was carried out. Both a young (~4 hour old embryos) and an old (~16 hour old embryos) cell extract were prepared. To the younger extract, volumes of the older extract that contained an estimated 8 and 10% of the total protein content of the younger extract were added. Then, each of the samples (Young Only, Young + 8% Old, Young + 10% Old, Old Only) were taken simultaneously through two cycles of assembly/disassembly. The results are shown on a 10% Page gel in figure 7. The C_2S lanes can be compared against the Marker (labelled "M"--contains a previous C_2S sample) for tubulin content. A significant enrichment of tubulin can be seen in the fractions which contain young extract plus old extract, especially in the "Young + 10% Old" experiment. But is this an enrichment because of the addition of a stimulator, or simply because of an increase in the tubulin pool? Qualitatively, it seems as though a stimulation has occurred. Unfortunately, quantitative information cannot be inferred from this data.

To further refine the procedure, we decided to first purify the old cell extract by taking it through two cycles of *in vitro* cycling and adding a portion of this C_2S fraction to the younger cell extract, and then taking the young cell extract + old C_2S through two cycles. Presumably, if there were an assembly stimulator in the older embryos, it should be present in the purified fraction. Adding purified C_2S rather than crude cell extract eliminates the possibility of nonspecific stimulation of assembly. The results of this experiment are shown on a 10% Page gel in figure 8. Again, an increase in tubulin assembly can be

seen in comparing the C_2S fractions of the "Young Only" and "Young + Old C_2S" preparations.

We wanted to compare the proteins present in younger and older embryos by using 2D gel electrophoresis. Non-equilibrium pH Gradient Electrophoresis or NEPHGE) is a variation of isoelectric focusing in which the upper and lower chamber buffers are reversed (16). This method was said to be better in resolving basic proteins, which the proteins that we were working with (MAPs) were presumed to be. An example of these NEPHGE 2D gels can be seen in figure 9. The proteins focused over the same length and we were able to compare proteins present in both. However, we found that many proteins in the samples did not enter the first dimension noodle. We're not sure why this happened, possibly because the sample buffer we used was meant for use in an IEF system, and was not compatible with the change in buffer for the upper chamber.

IEF 2D gels were run at this point. Examples of these silver stained gels can be found in figure 10. Resolution of the MAPs was high and the proteins entered the noodle almost completely. The proteins from the different samples focused over the same distance, and a qualitative comparison of protein content was possible. However, the silver staining can be deceiving because some proteins may have a high affinity for Ag and stain very heavily, while others may have a low affinity and stain lightly or not at all. Quantitative results are not accurate because there is not a linear relationship between the intensity of stain and the amount of protein present. A stain such as Coomassie, however, does stain linearly with the amount of protein

present, but is not sensitive enough to stain the 10-20 µg of protein loaded on a 2D gel. An alternative is the colloidal blue method, where Coomassie stain is in the form of colloids, which bind to proteins in the gel. This method was very successful with 20 µg protein loaded per gel. See figures 11 and 12. In determining the identity of MAPs, we must be careful to consider that only proteins that show a definite stoichiometric relation to tubulin may be called MAPs (1).

We have observed a consistent difference in percent yields between two sets of data. In the first set of data, there is a 5-15 fold difference in the protein content of the young and old embryo C_2S fractions, while in the second set, a 2-3 fold difference is found. Compare these quantitations in tables 1 and 2. The origin of these discrepancies is unknown, but is thought to be some sort of systematic error. A variable as simple as the pH of the buffer could be the source, as has been seen when MAPs are cosedimented with taxol. Percent yield of MAP protein rose from 0.5 to 1.2 percent of total cytoplasmic protein when the pH of the buffer was raised from 6.8 to 7.6 (9). On looking at our system, in which we are trying to quanititate the amount of MT protein when 50 other MAPs are present, perhaps we should use a better method than determining the total mg protein present.

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Figure 1. 10% SDS Page gel run w th fractions from *in vitro* cycling
experiments performed on cell extrcts from young (4 hr old) and old
(16 hr old) embryos. Notice that boi young and old embryo cell
extracts are very similar in tubulin content, but the C_2S fractions
differ considerably in tubulin content, as well as in the amounts of
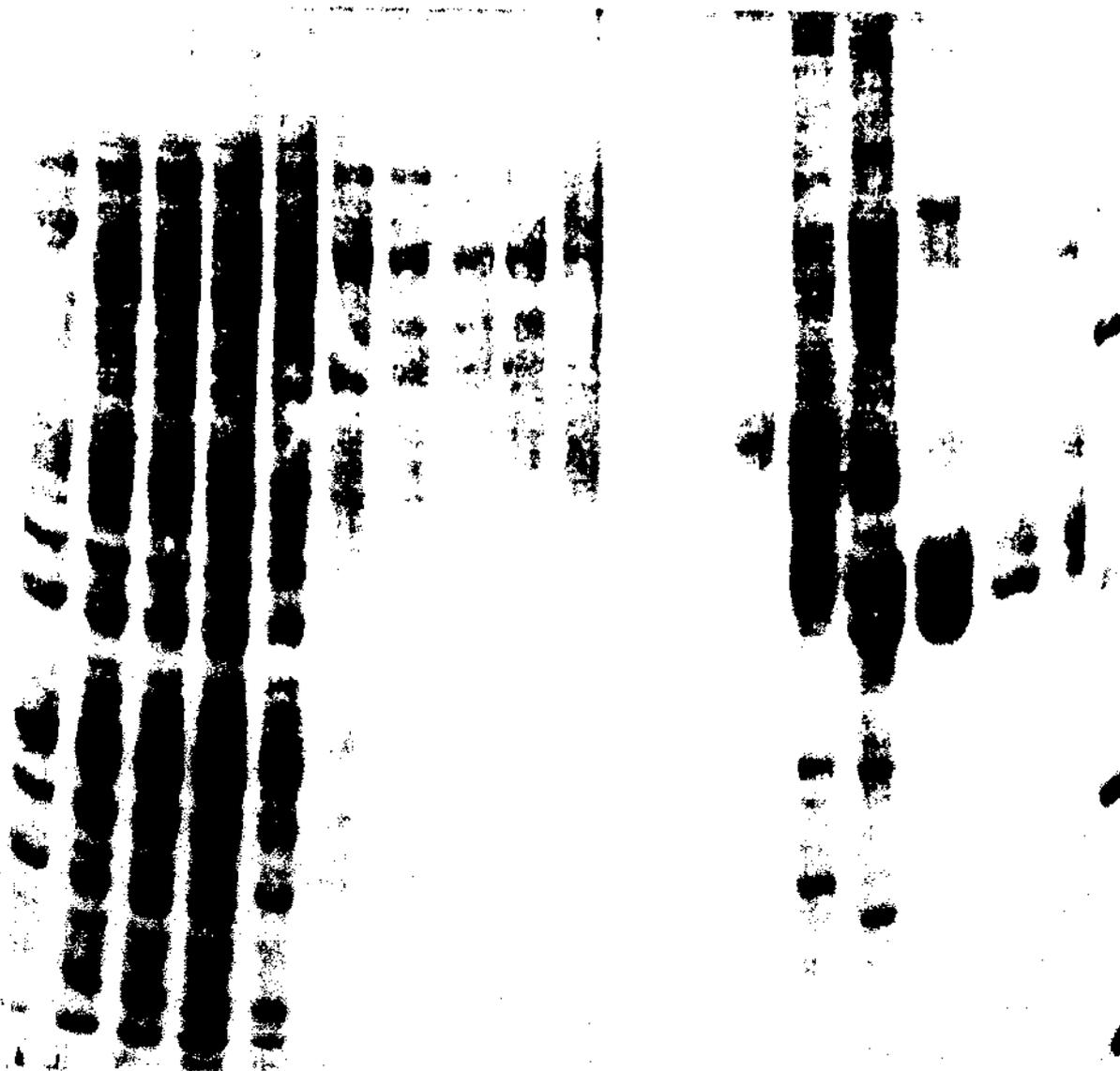other proteins (MAPs) present.

Figure 2. 10% SDS Page gel on fractions collected off phosphocellulose column. It's not clear from this gel where the tubulin is, but it's clear that the flowthrough and salt eluant fractions contain different proteins. Also, it can be seen that column was effectively washed by looking at the later flowthrough fractions and the earlier salt elution fractions.
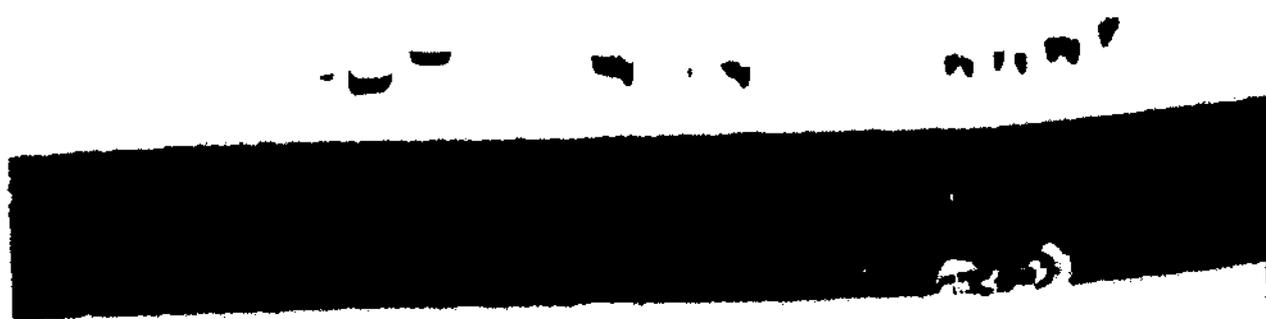
Figure 3. Western blot done on fractions collected from phosphocellulose column, probed with anti α-tubulin monoclonal antibody. Tubulin can be found in fractions 8-11 and 13-17 of the flowthrough and in fractions 6-8 of the salt eluant

Figure 4. 10 % Page gel for C_2S preparation using batch phospho-
cellulose method. Objective was to rid cell extract of 45 kd yolk
proteins to better the MT preparation, but tubulin was too diluted by
the procedure to be able to be polymerized. Lanes 1-8 are fractions
from the preparation done without phosphocellose, 9 and 10 are the
first two fractions of the phosphoceliulose preparation (other
fractions were too dilute to load). First lane on right side is the
marker lane (previous C_2S fraction was loaded here), lane 2 is the
original cell extract, 3 is the cell extract after phosphocellulose
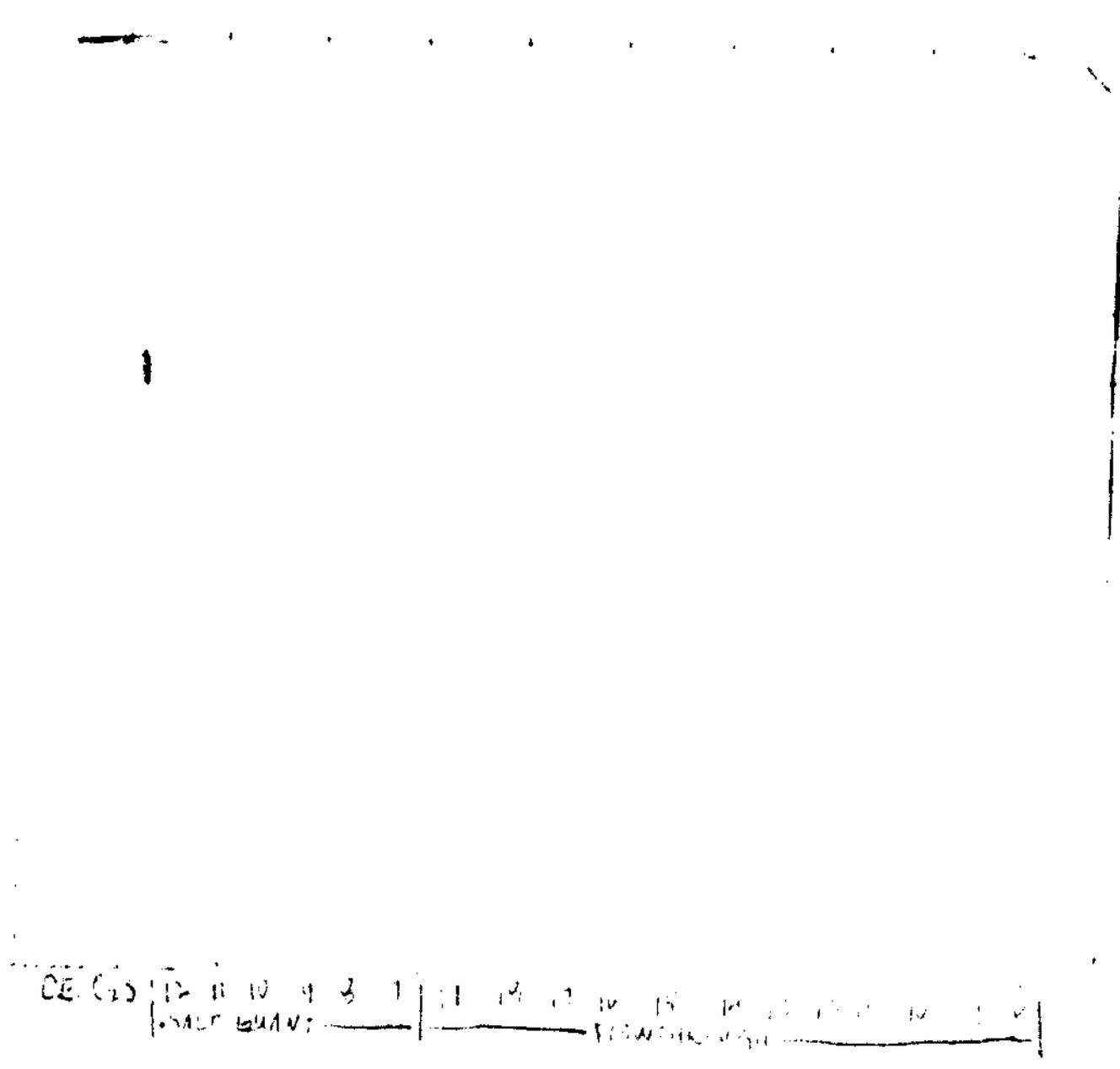preparation, and in lane 4 are the molecular weight markers.

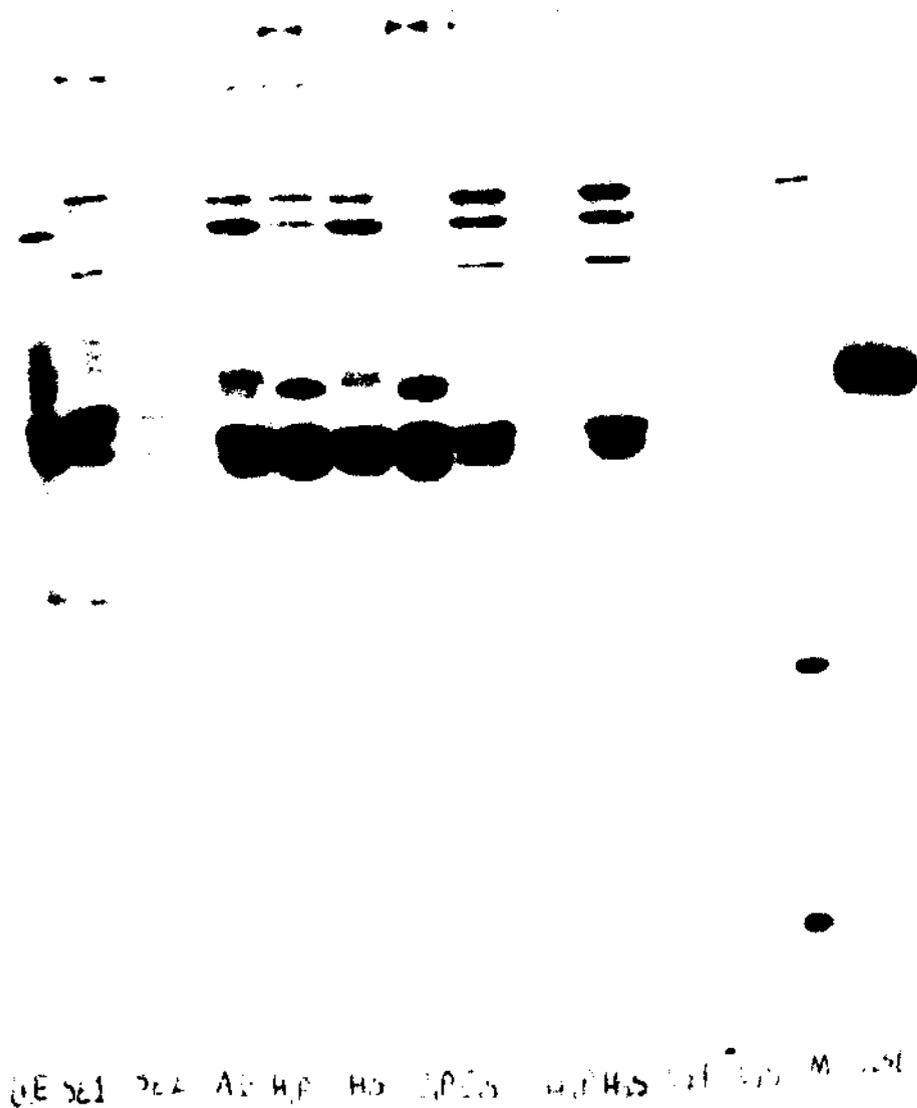Figure 5. Western Blot probed with anti α-tubulin. Fractions 7, 8 and 9 of the salt eluant contain tubulin.

Figure 6. 10% SDS Page gel on C_2S preparation using a QAE sephadex batch method. Lanes are as follows: CE: original cell extract. SE1 & SE2: first and second salt eluant fractions, respectively. AD: eluant fractions after dialysis. H_1P . . .C_2S: fractions taken at different steps of *in vitro* cycling (see Materials and Methods). M: Molecular weight markers. OLD C_2S: fraction from previous experiment, used as a marker for tubulin.
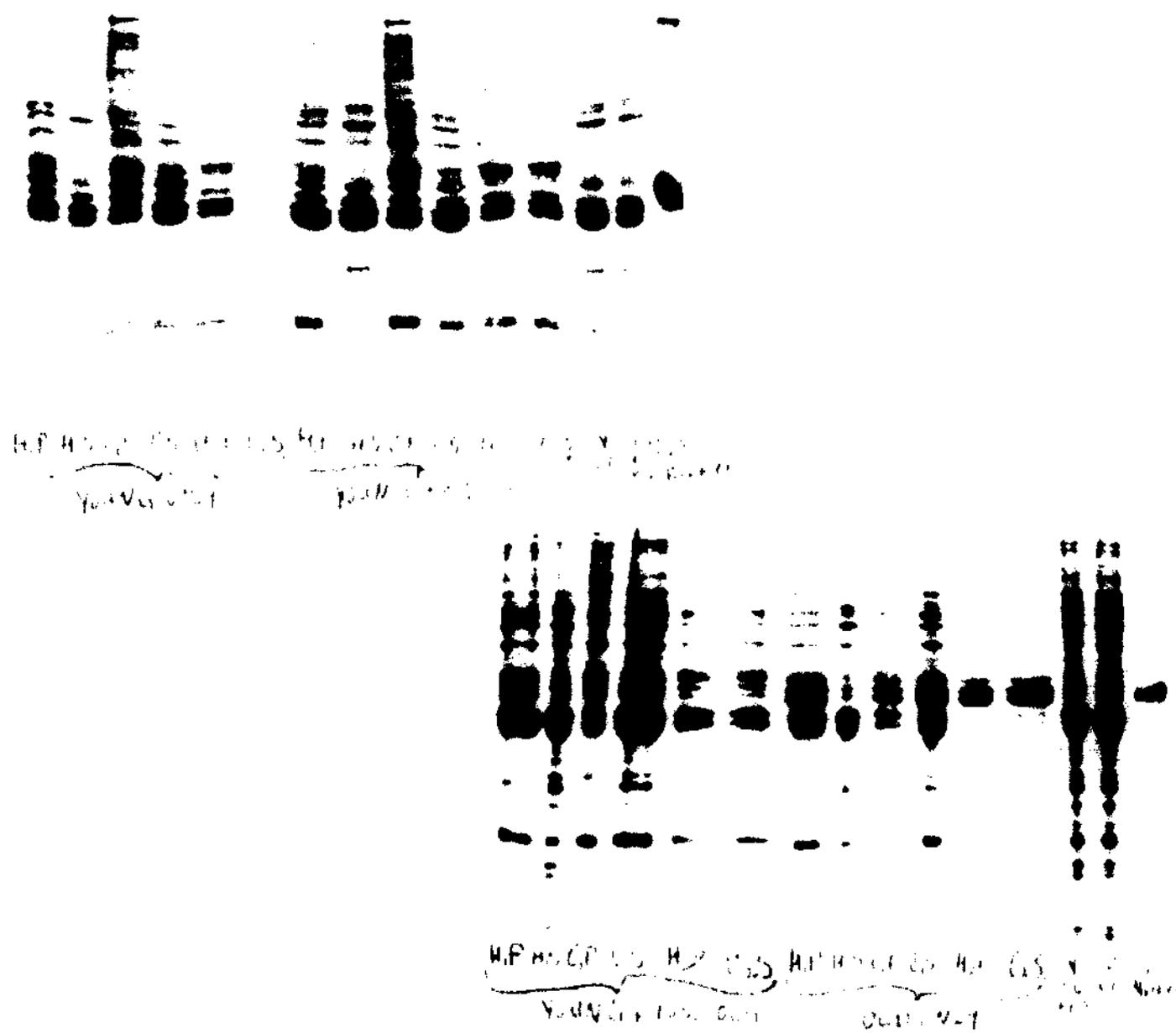
Figure 7. 10% SDS Page gels run with fractions of *in vitro* cycling experiment done by adding portions of an old embryo cell extract to a young embryo cell extract. (Left) Fractions from "Young Only" C_2S preparation, then "Young + 8% Old" (8% protein from old embryo cell extract preparation added), followed by the respective cell extracts. "M" is a previous C_2S sample. (Right) In the same way as in the gel on the left, fractions from the "Young + 10% Old" and "Old Only" C_2S preparations, followed by the cell extracts and the C_2S marker have been run.

Figure 8. C$_2$S preparation done using young embryo cell extract and an added 5.0% volume of an old embryo C$_2$S fraction. M is a previous C$_2$S used as a marker for tubulin.
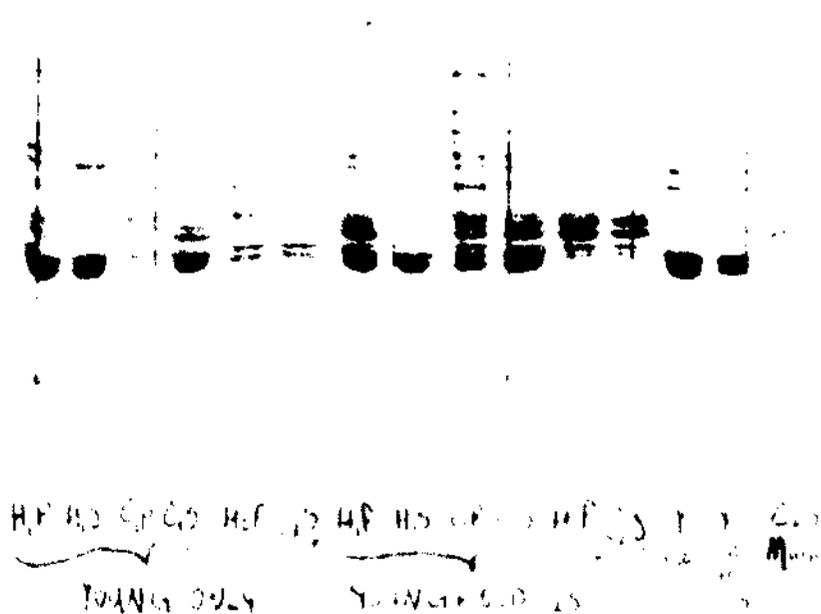

Figure 9. Two dimensional NEPHGE gel, silver stained. Notice the large amount of protein that did not enter the noodle as seen by the heavily stained vertical line on the right.
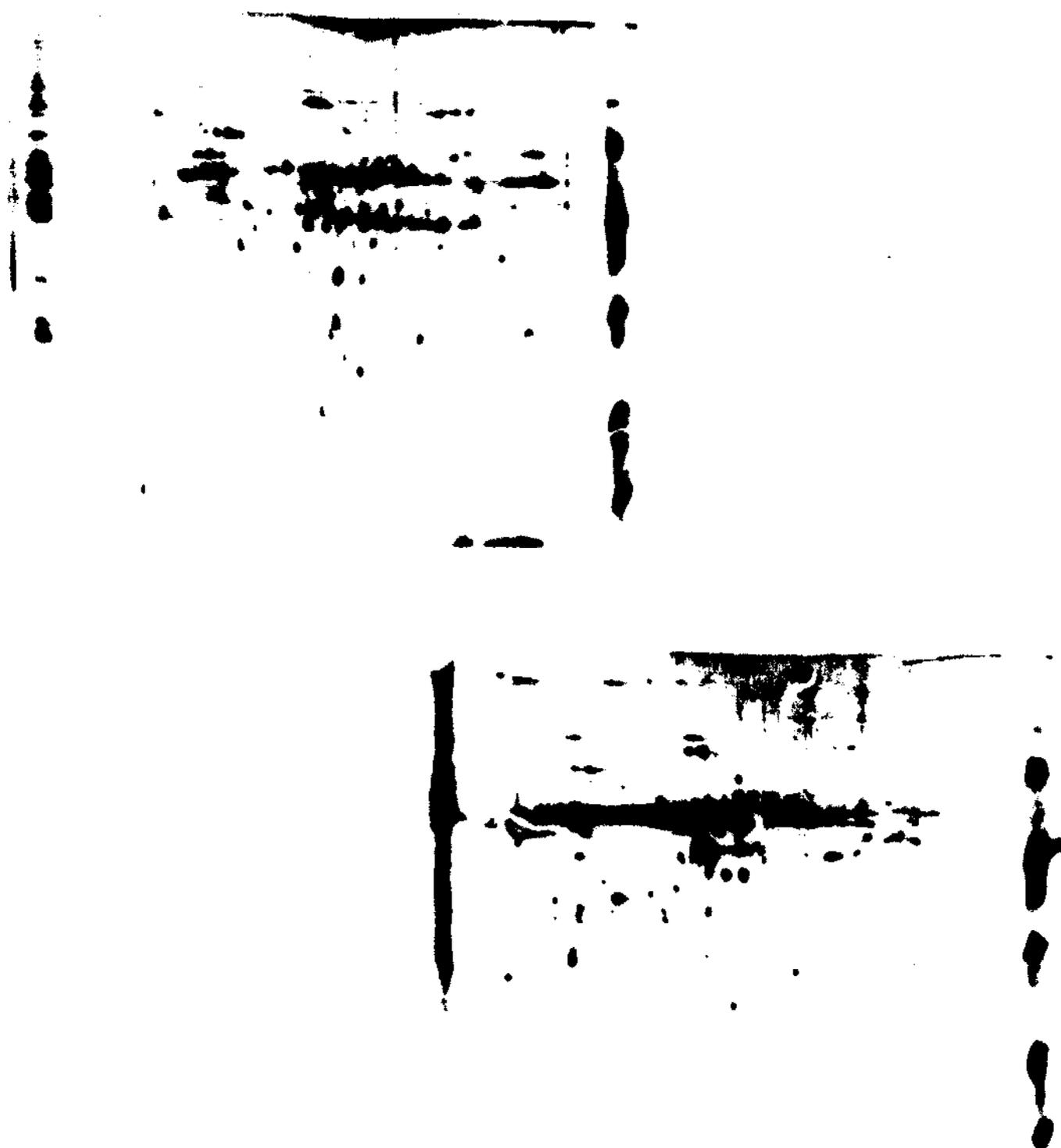
Figure 10. 2D IEF gels silver stained. (Left) Four hour old embryo C$_2$S fraction. (Right) Sixteen hour old embryo C$_2$S fraction. Notice the difference in number and amounts of MAPs present in the two ages. Also notice the difference in MT content. Immunoblots were probed with anti α-tubulin, which confirmed these results (data not shown).

Figure 11. Two dimensional IEF gel colloidally stained. 20 μg of a four hour old embryo C_2S is shown. First dimension focusing from left to right, basic-acidic. Second dimension SDS Page from top to bottom.

Figure 12. Two dimensional IEF gel colloidally stained. 20 μg if a sixteen hour old embryo C_2S was loaded. First dimension focusing from left to right, basic-acidic, top of noodle at left. Compare amounts of tubulin and the amounts and identities of MAPs with the four hour C_2S preparation in figure 11.

Table 1. First set of MTP yield data, which suggest a large change in percent yield between young and old embryos.

DATE	AGE OF EMBRYOS	PERCENT MTP YIELD
12/9/88	16 hours	1.1
12/14/88	16 hours	1.1
1/3/89	16 hours	1.1
2/8/89	16 hours	1.2
2/25/89	16 hours	1.1
12/11/88	4 hours	0.20
12/29/88	4 hours	0.35
2/7/89	4 hours	0.05
2/9/89	4 hours	0.065
2/10/89	4 hours	0.12

AVERAGE 16 HOUR MTP YIELD: 1.13%

AVERAGE 4 HOUR MTP YIELD: 0.15%

AVERAGE FOLD DIFFERENCE: 7.19

Table 2. Second set of data, implying a decreased change in MTP yields
between the two ages of embryos

DATE	AGE OF EMBRYOS	PERCENT MTP YIELD
1/31/90	16 hour	2.0
2/3/90	16 hour	1.5
3/2/90	16 hour	2.9
2/6/90	4 hour	0.69
2/28/90	4 hour	0.83
3/1/90	4 hour	1.38

AVERAGE 16 HOUR MTP YIELD: 2.13%

AVERAGE 4 HOUR MTP YIELD: 0.97%

AVERAGE FOLD DIFFERENCE: 2.2