EFFECTS OF ACRYLAMIDE ON CYTOSKELETAL PROTEINS

M.Sc. Thesis by
Ceren EKE KOYUNCU

Department: Advanced Technologies in Engineering
Programme: Molecular Biology – Genetics & Biotechnology

JUNE 2005
EFFECTS OF ACRYLAMIDE ON CYTOSKELETAL PROTEINS

M.Sc. Thesis by
Ceren EKE KOYUNCU, B.Sc.

(707021003)

Date of submission: 9 May 2005
Date of defence examination: 30 May 2005

Supervisor (Chairman): Assist. Prof. Dr. Arzu KARABAY KORKMAZ
Members of the Examining Committee Prof. Dr. Turgut ULUTİN (İÜ)

Assist. Prof. Dr. Cenk SELÇUKİ (İTÜ)

JUNE 2005
ACKNOWLEDGEMENTS

I would like to thank Assistant Professor Arzu Karabay Korkmaz for invaluable guidance, advice, and also for her motivation and morale support at difficult times.

I would like to thank Eyser Kılıç for being understanding, tolerating and balancing me. She is a very good partner, as well as a very good friend. These three years would be very difficult and boring without her.

I would like to thank Ayça Gülçin Ülgen, Aygül Akar, Deniz Şahin, Gamze Çelikyilmaz, Hilal Yazıcı, M. Hale Öztürk, Şirin Korulu and Volkan Demir for their friendship and help.

I would like to thank Assoc. Prof. Seyhun Solakoğlu, Ebru Karabulut and Fadime Aktar from Istanbul University, Istanbul Faculty of Medicine, Department of Histology and Embryology for their technical support.

I would like to thank Demet Kaya and Department of Physics, Istanbul Technical University.

I would like to thank Kerem Teralı and Balca Mardin, undergraduate students, for their help.

I would also like to acknowledge the funding agencies. This study was supported by TUBİTAK and Turkish State Planning Organization (Molecular Biology – Genetics and Biotechnology Program as part of Advanced Technologies in Engineering Program).

I would like to thank Erkan Koyuncu for being the meaning of the life for me and giving me peace. His support and encouragement are very valuable for me.

Finally, I would like to thank my family for their endless love and support.

June 2005

Ceren EKE KOYUNCU
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABBREVIATIONS</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>ÖZET</td>
<td>xii</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>xiii</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1. Cytoskeleton</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1. Microtubules</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1.1. Microtubule Structures</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1.2. Microtubule Assembly and Disassembly</td>
<td>5</td>
</tr>
<tr>
<td>1.1.1.3. Dynamic Instability of Microtubules</td>
<td>8</td>
</tr>
<tr>
<td>1.1.1.4. Microtubule Motor Proteins</td>
<td>9</td>
</tr>
<tr>
<td>1.1.1.5. Microtubule Associated Proteins (MAPs)</td>
<td>12</td>
</tr>
<tr>
<td>1.2. Neuronal Cytoskeleton</td>
<td>13</td>
</tr>
<tr>
<td>1.2.1. Axonal Transport</td>
<td>14</td>
</tr>
<tr>
<td>1.2.1.1. Anterograde Axonal Transport</td>
<td>15</td>
</tr>
<tr>
<td>1.2.1.2. Retrograde Axonal Transport</td>
<td>16</td>
</tr>
<tr>
<td>1.3. Acrylamide</td>
<td>16</td>
</tr>
<tr>
<td>1.3.1. Structure</td>
<td>16</td>
</tr>
<tr>
<td>1.3.2. Chemical and Physical Properties</td>
<td>16</td>
</tr>
<tr>
<td>1.3.3. Production</td>
<td>17</td>
</tr>
<tr>
<td>1.3.4. Usage</td>
<td>18</td>
</tr>
<tr>
<td>1.3.5. Disposal and Environmental Fate</td>
<td>18</td>
</tr>
<tr>
<td>1.3.6. Exposure and Metabolism</td>
<td>18</td>
</tr>
<tr>
<td>1.3.7. Acrylamide Formation in Foods</td>
<td>19</td>
</tr>
<tr>
<td>1.3.8. Toxicity</td>
<td>20</td>
</tr>
<tr>
<td>1.3.8.1. Genotoxicity and Carcinogenicity</td>
<td>20</td>
</tr>
<tr>
<td>1.3.8.3. Male Reproductive Toxicant</td>
<td>21</td>
</tr>
<tr>
<td>1.4. Acrylamide neurotoxicity</td>
<td>22</td>
</tr>
<tr>
<td>1.4.1. Possible Mechanisms of Neurotoxicity</td>
<td>23</td>
</tr>
</tbody>
</table>
2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Buffers for Tubulin Purification

2.1.1.1. 5x PM Buffer
2.1.1.2. 1x PM Buffer
2.1.1.3. PM-4M Buffer
2.1.1.4. PM-8M Buffer
2.1.1.5. PMSF Solution

2.1.2. Buffers for Ion-Exchange Column Purification

2.1.2.1. NaOH Solution
2.1.2.2. HCl Solution
2.1.2.3. 1x PM Buffer containing GTP and DTT

2.1.3. Solutions for Determination of Protein Concentration

2.1.3.1. Bradford Reagent
2.1.3.2. Bovine Serum Albumine (BSA) Stock Solution

2.1.4. Buffers for Reactions

2.1.4.1. AB Buffer

2.1.5. Solutions for SDS-PAGE

2.1.5.1. Acrylamide Monomer Solution
2.1.5.2. 4x Running (Separating) Gel Buffer
2.1.5.3. 4x Stacking Gel Buffer
2.1.5.4. 10% Sodium Dodecyl Sulfate (SDS)
2.1.5.5. 10% Ammonium Persulfate (APS)
2.1.5.6. TEMED (N, N', N’ – Tetramethylethylene diamine)
2.1.5.7. 2xSample Buffer
2.1.5.8. Tank Buffer (Running Buffer)
2.1.5.9. Coomassie Blue Stain
2.1.5.10. Gel Destain

2.1.6. Stock Solutions

2.1.6.1. GTP Stock Solution
2.1.6.2. DTT Stock Solution
2.1.6.3. Taxol Stock Solution
2.1.6.4. Acrylamide Stock Solution
2.1.7. Phosphocellulose Resin 29
2.1.8. Solutions for Electron Microscopy 29
  2.1.8.1. Pioform Solution 29
  2.1.8.2. Gluteraldehyde 29
  2.1.8.3. Uranyl Acetate Solution 30
2.1.9. Protein Marker 30
2.1.10. Lab Equipments 30

2. 2. Methods 32
2.2.1. Tubulin Purification 32
  2.2.1.1. Bradford Assay 33
  2.2.1.2. Further Tubulin Purification 33
  2.2.1.3. Elution of MAPs from Phosphocellulose Column 36
2.2.2. Sedimentation Assay 36
  2.2.2.1. Semipurified Tubulin Reaction Conditions 36
  2.2.2.2. TMT Preparation 36
  2.2.2.3. SDS-PAGE 37
  2.2.2.4. Loading and Staining of the Polyacrylamide Gel 38
2.2.3. Fluorescence Spectrometer 39
2.2.4. Malachite Green Procedure 39
  2.2.5.1. Color Reagent Preparation 39
  2.2.5.2. Protein Reaction Mixture Preparation 40
  2.2.5.3. Deproteinization and Phosphate Determination 40
2.2.6. Electron Microscopy 40
  2.2.6.1. Membrane Coating of Grids 40
  2.2.6.2. Reaction Conditions 41
  2.2.6.2. Staining 41

3. RESULTS AND DISCUSSION 43
3.1. Tubulin Purification 44
3.2. Elution of MAPs from Phosphocellulose Column 45
3.3. Sedimentation Assay 46
3.5. Fluorescence Spectrometer 49
3.6. Malachite Green Procedure 53
3.7. Electron Microscopy 55
4. CONCLUSION
REFERENCES
RESUME
ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arp1</td>
<td>Actin-capping protein 1</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BRB</td>
<td>Bromophenol Blue</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie Brilliant Blue</td>
</tr>
<tr>
<td>Cc</td>
<td>Critical concentration</td>
</tr>
<tr>
<td>CENP-E</td>
<td>Centromere Protein E</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxiribonucleic Acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol bis (2-aminoethyl ether)-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosinediphosphate</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamine</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosinethreephosphate</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>KIF1A</td>
<td>Kinesin-like protein 1A</td>
</tr>
<tr>
<td>KIF1B</td>
<td>Kinesin-like protein 1B</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>MAPs</td>
<td>Microtubule-associated proteins</td>
</tr>
<tr>
<td>MBO</td>
<td>Membrane Bound Organelle</td>
</tr>
<tr>
<td>β-ME</td>
<td>β- mercaptoethanol</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger-ribonucleic acid</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule Organising Center</td>
</tr>
<tr>
<td>MTs</td>
<td>Microtubules</td>
</tr>
<tr>
<td>ncd</td>
<td>Nonclaret disjunctional protein</td>
</tr>
<tr>
<td>NF</td>
<td>Neurofilament</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl Methyl Sulphonyl Fluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N’ – Tetramethylethylene diamine</td>
</tr>
<tr>
<td>TMTs</td>
<td>Taxol-stabilized microtubules</td>
</tr>
<tr>
<td>γ-TuRC</td>
<td>γ-tubulin Ring Complex</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

| Figure 1.1 | General organization of microtubules in a typical cell | Page No
| Figure 1.2 | A) Tubulin dimer and microtubule structure B) A model of γ-TuRC. This complex is thought to nucleate microtubule assembly by presenting a row of γ-tubulin subunits, which can directly bind αβ-tubulin subunits | 2
| Figure 1.3 | Structure of tubulin monomers and GTP binding sites on α- and β-tubulin. The anticancer drug taxol (black) is used to stabilize the microtubule structure | 2
| Figure 1.4 | Microtubule structure. Free αβ-tubulin dimers associate longitudinally to form short protofilaments. These protofilaments are probably unstable and quickly associate laterally into more stable curved sheets. Eventually, a sheet wraps around into a microtubule with 13 protofilaments. The microtubule then grows by the addition of subunits to the ends of protofilaments composing the microtubule wall | 3
| Figure 1.5 | Arrangement of protofilaments in singlet, doublet and triplet microtubules | 4
| Figure 1.6 | Microtubule polarity due to head to tail arrays of tubulin dimers. (+) end is ringed by β-tubulin and (-) end is ringed by α-tubulin | 4
| Figure 1.7 | Orientation of cellular microtubules around MTOC in an interphase cell and a mitotic cell | 5
| Figure 1.8 | Cryoelectron microscopy of assembling (A) and disassembling (B) microtubules | 6
| Figure 1.9 | Temperature affects whether microtubules (MTs) assemble or disassemble | 7
| Figure 1.10 | Tubulin subunits add to one end and dissociate from the opposite end | 7
| Figure 1.11 | General model of kinesin- and dynein-mediated transport in a typical cell | 8
| Figure 1.12 | Vesicle or organelle transport in cytoplasm by kinesin and dynein on microtubule track | 9
| Figure 1.13 | A) A typical neuron with a long axon and dendrites. B) Microtubules and intermediate filaments are oriented longitudinally in axon and they are cross-linked by various proteins | 10
| Figure 1.14 | In nerve cells, the (-) ends of all axonal microtubules are oriented toward the base of the axon, but dendritic microtubules have mixed polarities | 13
| Figure 1.15 | Anterograde and retrograde transport in a typical neuron | 14
| Figure 1.16 | Chemical structure of acrylamide monomer | 15

ix
Figure 1.17 : Diagram of acrylamide formation by Maillard reaction between glucose and asparagine.......................................................... 20
Figure 2.1 : Scheme of tubulin purification.................................................. 35
Figure 3.1 : Polyacrylamide gel of purification steps. 1. column: protein marker. 2. column: semi-purified tubulin with MAPs. 3. column: purified tubulin. 4. column: pure tubulin (%99) which was purchased from Cytoskeleton as a control marker. ................. 45
Figure 3.2 : Polyacrylamide gel of MAPs fractions eluted from phosphocellulose column................................................................. 46
Figure 3.3 : Polyacrylamide gel of sedimentation assay with 5 µM semi-purified tubulin, 1mM GTP and 100 mM acrylamide.
1, 2, 3: Tubulin standards.
4, 5: Control reaction (Tubulin + GTP)
6, 7: 2. reaction (Tubulin + GTP → Acrylamide)
8, 9: 3. reaction (Tubulin + Acrylamide → GTP)
10, 11: 4. reaction (Tubulin + GTP + Acrylamide)
12, 13: 5. reaction (GTP + Acrylamide → Tubulin)......................... 47
Figure 3.4 : Polyacrylamide gel of GTP-ACR interaction reactions.
1, 2, 3: Tubulin standards
4, 5: Control reaction (GTP → Tubulin)
6, 7: Reaction with acrylamide (GTP + Acrylamide → Tubulin) 48
Figure 3.5 : Polyacrylamide gel of the TMT reactions with pure tubulin purchased from Cytoskeleton.
1, 2: Control reaction.
3, 4: Reaction with acrylamide....................................................... 49
Figure 3.6 : Polyacrylamide gel of the TMT reactions with semi-purified tubulin.
1, 2: Control reaction.
3, 4: Reactions with acrylamide...................................................... 49
Figure 3.7 : Graphic of fluorescence spectrometer showing maximum emission intensities at 336 nm and excitation at 295 nm............. 50
Figure 3.8 : Graphics of fluorescence spectrometer showing emission intensities versus time. In the absence of GTP A) without acrylamide and B) with acrylamide............................................. 51
Figure 3.9 : Graphics of fluorescence spectrometer for the reactions with acrylamide in the absence of GTP.A) Emission intensities versus time. B) Scattered light versus time. C) Corrected emission intensities versus time.............................................. 51
Figure 3.10 : Graphics of fluorescence spectrometer showing emission intensities versus time. In the presence of GTP: A) Without acrylamide B) With acrylamide................................................. 52
Figure 3.11 : Graphics of fluorescence spectrometer belonging the reaction with acrylamide in the presence of GTP: A) Emission intensities versus time. B) Scattered light versus time. C) Corrected emission intensities versus time............................................ 52
Figure 3.12: Graphic of phosphate determination using malachite green. First column indicates the absorbance of the control reaction containing GTP and tubulin. Second column indicates the absorbance of the reaction containing GTP, tubulin and acrylamide. 54

Figure 3.13: Transmission electron micrographs of control reaction1; Tubulin + GTP + Taxol. A) 50,000x and B) 100,000x. 55

Figure 3.14: Transmission electron micrographs of reaction 1a; Tubulin + GTP + Acrylamide + Taxol. A) 25,000x, B) 50,000x, C) 25,000x and D) 50,000x. 56
AKRİLAMİDİN HÜCRE İSKELET PROTEİNLERİ ÜZERİNE ETKİLERİ

ÖZET


Bu çalışmada, akrilamidin mikrotübüller üzerine in vitro etkileri farklı metodlar kullanarak araştırıldı. İlk olarak, tübülün proteinleri sığır beyninden tekrar döngü polimerizasyon ve depolimerizasyonu sonucu aksonopatik deneylerde multifokal paranodal şişkinlikler olarak başlayarak akson dejenerasyonu olarak düşünülmektedir. Akrilamid nörotoksitesinin semptomları ekstremitelerin güçsüzüğü, kilo kaybı ve koordinasyon bozulmuş yürümedir.

EFFECTS OF ACRYLAMIDE ON CYTOSKELETAL PROTEINS

SUMMARY

A further level of organization of cells was provided by the cytoskeleton, which consists of a network of protein filaments extending throughout the cytoplasm of all eukaryotic cells. The cytoskeleton is responsible for cell movements and general organization of cytoplasm. Microtubules are one of the major elements of cytoskeleton. They are long protein polymers which are composed of a globular protein, tubulin. Microtubules perform their tasks by polymerization and depolymerization of tubulin subunits or together with the actions of microtubule motor proteins. Microtubules are also one of the major elements of neuronal cytoskeleton and they have roles in axonal transport.

Acrylamide is an α,β-unsaturated carbonyl compound which is highly water soluble. It has broad industrial application; e.g., polymer production, ore processing, mineral processing, cosmetic additives etc. Acrylamide is a toxicant which has genotoxic, carcinogenic, male reproductive toxic effects as well as neurotoxic effects. Acrylamide causes a central – peripheral distal axonopathy. The morphological hallmark of this axonopathy is considered to be axon degeneration, which begins as multifocal paranodal swellings of preterminal distal fibers. The symptoms of acrylamide neurotoxicity are skeletal muscle weakness of the extremities, weight loss and uncoordinated gait.

In this study, in vitro effects of acrylamide on microtubules were investigated using different methods. Firstly, tubulin proteins were purified from bovine brain after repetitive cycles of polymerization and depolymerization steps and ion-exchange chromatography. To show the effects of acrylamide on microtubules, sedimentation assay, fluorescence spectrometer, malachite green procedure and transmission electron microscopy techniques were used. During sedimentation assays, tubulin was exposed to acrylamide in different conditions, and in some experiments taxol-stabilized microtubules were treated with acrylamide. Fluorescence spectrometer was used to show structural transition of microtubules in the presence of acrylamide through intrinsic fluorophore tryptophan residues in tubulin subunits. Pi amounts occurring during microtubule polymerization in the presence of acrylamide were measured by Malachite Green Procedure. In addition, to observe the structures that pelletted in the sedimentation assay and fluorescence spectrometer acylamaide-treated taxol-stabilized microtubules were examined by Transmission Electron Microscopy.
1. INTRODUCTION

1.1. Cytoskeleton

During evolution, a further level of organization of cells was provided by the cytoskeleton, which consists of a network of protein filaments extending throughout the cytoplasm of all eukaryotic cells. The cytoskeleton provides a structural framework for the cell, like serving as a scaffold that determines the cell shape and general organization of the cytoplasm. In addition to playing this structural role, the cytoskeleton is responsible for cell movements. These include not only the movements of entire cells, but also the internal transport of organelles and other structures such as mitotic chromosomes through the cytoplasm [1, 2].

Cytoskeleton is a dynamic structure that is continually reorganized as cells move and change shape. Three types of protein filaments form the cytoskeleton: actin filaments, intermediate filaments and microtubules. However, these filaments are held together and linked to subcellular organelles and the plasma membrane by a variety of accessory proteins [2].

1.1.1. Microtubules

Microtubules are long protein polymers which are involved in the cell motility and determination of cell shape (Figure 1.1). Microtubules are responsible for various cell movements like beating of cilia and flagella, the transport of membrane vesicles in the cytoplasm. These movements result from the polymerization and depolymerization of microtubules or the actions of microtubule motor proteins. However, some cell movements such as the alignment and separation of chromosomes during mitosis and meiosis involve both processes [2, 3].
1.1.1.1. Microtubule Structures

Microtubules are composed of a globular protein called “tubulin”. Tubulin is a dimer consisting of two 55 kDa polypeptides, α-tubulin and β-tubulin. These monomers are found in all eukaryotes and their sequences are highly conserved: 36-42 % identical and 63 % homologous. After polymerization of tubulin subunits, a cylindrical tube which is 24 nm in diameter occurs (Figure 1.2A). However, its length varies from a fraction of micrometer to hundreds of micrometers [1, 2].

In addition, a third type of tubulin which is not a part of microtubules exists, γ-tubulin. It is specifically localized to the centrosome, where it plays a critical role in the initiation of microtubule assembly and it is the part of γ-TuRC (γ-tubulin Ring Complex) (Figure 1.2B) [2].

The interactions holding α-tubulin and β-tubulin in a heterodimeric complex are strong enough that a tubulin subunit rarely dissociates under normal conditions.

Figure 1.1. General organization of microtubules in a typical cell.

Figure 1.2. A) Tubulin dimer and microtubule structure. B) A model of γ-TuRC. This complex is thought to nucleate microtubule assembly by presenting a row of γ-tubulin subunits, which can directly bind αβ-tubulin subunits.
Dimers dissociate into monomers with a $K_d$ that varies between 0.06 and 0.32 μM, depending on nucleotide and magnesium concentration. Magnesium at the N-site (or N-terminal of tubulin) controls the stability of the dimer, and its dissociation is followed by the release of bound nucleotide and functional inactivation [4, 5].

There are two GTP-binding sites on a tubulin dimer, one on α-tubulin and one on β-tubulin (Figure 1.3). The GTP-binding site which is located on α-tubulin, binds GTP irreversibly and does not hydrolyze it. The site which is on β-tubulin, binds GTP reversibly and hydrolyzes it. This site is also called exchangeable site, because GDP can be replaced by GTP. Magnesium increases the affinity of the β subunit for GTP with respect to GDP, and in its presence the binding constants are 0.062 μM (compared to 71 μM in the absence of magnesium) and 0.18 μM, respectively. A nucleotide in oligomeric tubulin or in microtubules does not exchange with the solution, except for terminal subunits at the microtubule ends [2, 5, 6].

![Figure 1.3](image_url)

**Figure 1.3.** Structure of tubulin monomers and GTP binding sites on α- and β- tubulin. The anticancer drug taxol (black) is used to stabilize the microtubule structure.

Head to tail arrays of tubulin dimers form protofilaments. Therefore, dimeric subunits repeat every 8 nm. 13 linear protofilaments assemble around a hollow core and form microtubules (Figure 1.4). Maintenance of the tubular form is provided by lateral and longitudinal interactions between tubulin subunits. In some cases, singlet microtubules contain more or fewer protofilaments; for example, certain microtubules in the neurons of nematode worms contain 11 or 15 protofilaments. However, there are doublet and triplet microtubules in specialized structures such as cilia and flagella (douplet microtubules) and centriols and basal bodies (triplet...
microtubules). Each of these contains one complete 13-protofilament microtubule and one or two additional tubules consisting of 10 protofilaments (Figure 1.5) [2, 3].

**Figure 1.4.** Microtubule structure. Free αβ-tubulin dimers associate longitudinally to form short protofilaments. These protofilaments are probably unstable and quickly associate laterally into more stable curved sheets. Eventually, a sheet wraps around into a microtubule with 13 protofilaments. The microtubule then grows by the addition of subunits to the ends of protofilaments composing the microtubule wall.

**Figure 1.5.** Arrangement of protofilaments in singlet, doublet and triplet microtubules.

Microtubules are polar structures because of the head-to-tail arrangement of the α- and β-tubulin dimers in a protofilament. One end of a microtubule is ringed by α-tubulin, whereas the other end is ringed by β-tubulin because of the same orientation of all protofilaments. These two different ends are named as a fast-growing plus end and a slow-growing minus end (Figure 1.6). This polarity is an important consideration in determining the direction of movement along microtubules [7, 8].
Figure 1.6. Microtubule polarity due to head to tail arrays of tubulin dimers. (+) end is ringed by β-tubulin and (-) end is ringed by α-tubulin.

There are two populations of microtubules: stable, long-live microtubules and unstable, short-live microtubules. Some cells, usually non-replicating cells, contain stable microtubule-based structures, for instance, the axoneme in the flagellum of sperm and the marginal band of microtubules in most red blood cells and platelets and also in nerve cells. Unstable microtubules are found when cell structures composed of microtubules need to assemble and disassemble quickly such as mitotic cells [3, 9].

1.1.1.2. Microtubule Assembly and Disassembly

In most cells, microtubules extend outward from a microtubule-organizing center (MTOC). In this kind of organization, mostly the minus ends are anchored in these centers and plus ends extend toward the cell membrane so called radial localization. In animal cells, the MTOC is a centrosome, which is a pinwheel array of triplet microtubules and located adjacent to the nucleus near the center of interphase cells (Figure 1.7). Centriols are not present in plants and fungi and also in some epithelial cells and newly fertilized eggs from animals. In these cases, the associated proteins in an MTOC have the capacity to organize cytosolic microtubules. However, in vitro experiments showed that necessary concentration of dimers to permit spontaneous formation of microtubules is lower in the presence of MTOCs relatively [10, 11, 12].
MTOCs contain many proteins that are necessary for initiating the assembly of microtubules, and γ-tubulin is one of these. It has been shown that antibodies against γ-tubulin block microtubule assembly within the cells. Approximately 80% of the γ-tubulin in cells is part of a 25S complex, which has been isolated from extracts of frog oocytes and fly embryos. It is named as γ-tubulin ring complex (γ-TuRC) for its ringlike appearance in the electron microscope. *In vitro* experiments show that the γ-TuRC can directly nucleate microtubule assembly at subcritical tubulin concentrations (Figure 1.2B) [13].

The critical concentration \( (C_c) \) is the concentration of dimeric αβ-tubulin in equilibrium with microtubules. At dimer concentrations above the \( C_c \), tubulin subunits polymerize into microtubules, while at concentrations below the \( C_c \), microtubules depolymerize. However, the addition of fragments of flagellar or other microtubules to a solution of αβ-tubulin accelerates the initial polymerization rate by acting as nucleation sites [2, 14].

At αβ-tubulin concentrations higher than the \( C_c \) for polymerization, dimers add to both ends of a growing microtubule. Yet, the addition of tubulin subunits occurs preferentially at one end. This difference between the two ends of a growing microtubule is demonstrated by examining electron micrographs of microtubules that have assembled from the ends of nucleating flagellar fragments *in vitro*. The electron micrographs show a tuft of microtubules sprouting from both ends of the fragment, but one tuft is much longer than the other. The preferred assembly end is designated as the (+) end, and the end that assembles more slowly is the (-) end. When the tubulin concentration is diluted below the \( C_c \), the microtubules disassemble twice as rapidly at the (+) end as at the (-) end. Thus both assembly and disassembly occur preferentially at the (+) end [2, 14].

*Figure 1.7.* Orientation of cellular microtubules around MTOC in an interphase cell and a mitotic cell.
In the electron microscope, growing microtubules appear to have relatively smooth ends, although some protofilaments are longer than others, indicating that they elongate unevenly. The appearance of microtubules undergoing shortening is quite different, suggesting that the mechanism of disassembly differs from that of assembly. Under shortening conditions, the microtubule ends are splayed, as if the lateral interactions between protofilaments have been broken. Once frayed apart and freed from lateral stabilizing interactions, the protofilaments might depolymerize by endwise dissociation of tubulin subunits (Figure 1.8.) [2].

![Figure 1.8. Cryoelectron microscopy of assembling (A) and disassembling (B) microtubules.](image)

Once microtubules have assembled, their stability is temperature-dependent. For instance, if microtubules are cooled down to 4° C, they depolymerize into stable αβ-tubulin dimers. When warmed up to 37° C in the presence of GTP, the tubulin dimers polymerize into microtubules (Figure 1.9) [7].

![Figure 1.9. Temperature affects whether microtubules (MTs) assemble or disassemble.](image)
1.1.1.3. Dynamic Instability of Microtubules

Under favorable *in vitro* conditions, microtubules exhibit the ability to treadmill, in which subunits add to one end and dissociate from the opposite end (Figure 1.10). Furthermore, a single microtubule can oscillate between growth and shortening phases. In all cases, the rate of microtubule growth is much slower than the rate of shortening [15].

![Figure 1.10. Tubulin subunits add to one end and dissociate from the opposite end.](image)

Two conditions influence the stability of microtubules. First, the oscillations between growth and shrinkage *in vitro* occur at tubulin concentrations near \( C_c \). At tubulin concentrations above the \( C_c \), the entire population of microtubules grows, and at concentrations below the \( C_c \), all microtubules shrink. At concentrations near the \( C_c \), however, some microtubules grow, while others shrink [10, 15] .

The second condition affecting microtubule stability is whether GTP or GDP occupies the exchangeable nucleotide-binding site on β-tubulin at the (+) end of a microtubule. A microtubule becomes unstable and depolymerizes rapidly if the (+) end becomes capped with subunits containing GDP-β-tubulin rather than GTP-β-tubulin. This situation can arise when a microtubule shrinks rapidly, exposing GDP-β-tubulin in the walls of the microtubule, or when a microtubule grows so slowly that hydrolysis of GTP bound to β-tubulin converts it to GDP before additional subunits can be added to the (+) end of the microtubule [5, 16].

Before a shortening microtubule vanishes, it can be rescued and start to grow if tubulin subunits with bound GTP add to the (+) end before the bound GTP hydrolyzes. Thus, the one parameter that determines the stability of a microtubule is the rate at which GTP-tubulin subunits are added to the (+) end. Possible factors that switch a microtubule between growth and shrinkage have been identified. One is a microtubule-severing protein, katanin, which may generate nuclei at centrosomes. Another factor is Op 18, which increases the frequency of catastrophe, possibly by binding tubulin dimers [16].
1.1.1.4. Microtubule Motor proteins

Cells can accomplish a remarkable number of animated tasks due to the actions of proteins that convert chemical energy into force and motion (Figure 1.11). The eukaryotic motors hydrolyse nucleotides and harness the derived energy to move unidirectionally along cytoskeletal polymers. Motor proteins are mostly categorized according to the type of cytoskeletal polymer with which they interact. Myosins, a large protein family, move along actin filaments. Motors that utilize intermediate filaments as tracks have not been described. On the other hand, microtubules interact with two types of force-generating motors: dyneins and kinesins.[17, 18].

Figure 1.11. General model of kinesin- and dynein-mediated transport in a typical cell.

Related motor proteins are categorized as superfamilies such as myosins, kinesins and dyneins. The membership sign for belonging to a motor superfamily is amino acid identity within the motor domain, a region of the polypeptide that is responsible for force generation. The non-motor regions or tail domains can differ considerably in size, structure and amino acid sequences. These diverse regions confer unique self-assembly properties as well as binding interactions [19,20].
**Kinesin**

Kinesin moves toward the (+) end of the microtubules so it is called (+) end-directed microtubule motor protein. It is a dimer of two heavy chains, each complexed to a light chain, with a total molecular weight of 380 kDa. The molecule is organized into three domains, a pair of large globular head domain connected by a long central stalk to a pair of small globular tail domain, which associates with the light chains (Figure 1.12) [20].

A 340 amino acid conserved sequence in the heavy chain, part of the globular head domains, possesses sequences for microtubule binding and ATP hydrolysis. The remainder of the heavy chains associate together as a α-helical coiled-coil to form a stalk. A natural disruption in the typical heptad repeat of the coiled-coil results in a kink in this filamentous portion, presumably endowing flexibility between the globular heads and the filamentous tails. Light chains are associated with the amino terminal ends of the heavy chain forming the tail. Each domain carries out a particular function: the head domain, which binds microtubules and ATP, is responsible for the motor activity of kinesin, and tail domain is responsible for binding to membrane vesicles. In light of the transport function of kinesin, a bound membrane vesicle is often referred to as kinesin’s cargo [20, 21].

The ATP-dependent reversible binding of kinesin to microtubules and conformational change between the head and the rest of the molecule permits locomotion of kinesin along the microtubule [20, 21].

---

**Figure 1.12.** Vesicle or organelle transport in cytoplasm by kinesin and dynein on microtubule track.
To date, more than 12 different family members have been identified. Each member transports a specific cargo. All kinesins contain the kinesin motor domain, but differ in their tail domains and several other properties. In most kinesins, the motor domain is at the N-terminus (N-type), but in some, the motor domain is central (M-type) or at the C-terminus (C-type). Both N- and M-type kinesins are (+) end-directed motors, whereas C-type kinesins are (-) end-directed motors. In addition, some kinesins are monomeric; however, most are dimeric. These two types of kinesins, differing in quaternary structure, may move along a microtubule by different mechanisms [22].

Kinesins can also be divided into two broad functional groups, cytosolic and spindle kinesins, based on the nature of the cargoes that they transport. The functional differences between kinesins may be related to their unique tail domains. Cytosolic kinesins are involved in vesicle and organelle transport; they include the classic axonal kinesin, implicated in transport of lysosomes and other membranous organelles. However, some cytosolic kinesins are responsible for transport of one specific cargo. For example, KIF1B transports mitochondria, and its relative KIF1A transports synaptic vesicles to the nerve terminal. In contrast, spindle kinesins participate in spindle assembly and chromosome segregation during cell division. This group comprises numerous proteins, including the kinetochore-associated protein CENP-E; the spindle pole protein BimC; and a (-) end-directed motor protein called ncd [22].

**Dynein**

Dynein is a (-) end-directed motor protein. Dyneins are exceptionally large, multimeric proteins, with molecular weights exceeding 1,000 kDa. They are composed of two or three heavy chains (MW 470 -540 kDa) complexed with a poorly determined number of intermediate and light chains (Figure 1.12). The dyneins are divided into two functional classes: cytosolic dynein, which is involved in the movement of vesicles and chromosomes, and axonemal dynein, which is responsible for the beating of cilia and flagella [20].

Like kinesin, cytosolic dynein is a two-headed molecule, with two identical or nearly identical heavy chains forming the head domains. However, unlike kinesin, dynein cannot mediate transport by itself. Rather, dynein-related motility requires a large complex of microtubule-binding proteins that link vesicles and chromosomes to
microtubules but by themselves do not exert force to cause movement. The best-characterized complex is dynactin, a heterocomplex of at least eight subunits, including a 150 kDa protein called Glued, the actin-capping protein Arp1, and dynamatin [23].

1.1.1.5. Microtubule Associated Proteins (MAPs)

Highly enriched tubulin preparations obtained after several assembly-disassembly cycles still contain small amounts of other proteins, which maintain their quantitative ratio to α- and β-tubulin through successive cycles. Co-purification of these proteins with tubulin suggested that they are not nonspecific contaminants but rather molecules that interact specifically with microtubules [2].

One major family of MAPs, called assembly MAPs, is responsible for cross-linking microtubules in the cytosol. These MAPs are organized into two domains: a basic microtubule-binding domain and an acidic projection domain. In the electron microscope, the projection domain appears as a filamentous arm that extends from the wall of the microtubule. This arm can bind to membranes, intermediate filaments, or other microtubules and its length controls how far apart microtubules are spaced [24].

Based on sequence analysis, assembly MAPs can be grouped into two types. Type I MAPs, MAP1A and MAP1B, contain several repeats of the amino acid sequences Lys-Lys-Glu-X, which is implicated as a binding site for negatively charged tubulin. This sequence is postulated to neutralize the charge repulsion between tubulin subunits within a microtubule, thereby stabilizing the polymer. MAP1A and MAP1B are large, filamentous molecules found in axons and dendrites of neurons and also in non-neuronal cells. Each of these proteins is derived from a single precursor polypeptide, which is proteolytically processed in a cell to generate one light chain and one heavy chain [24, 25].

Type II MAPs include MAP2, MAP4 and Tau. These proteins are characterized by the presence of four repeats of an 18-residue sequence in the microtubule-binding domain. MAP2 is found only in dendrites, where it forms fibrous cross-bridges between microtubules and also links microtubules to intermediate filaments. MAP4, the most ubiquitous of all MAPs, is found in neuronal and non-neuronal cells. It is thought to regulate microtubule stability during mitosis. Tau, which is much smaller
than most other MAPs, is present only in the axons. This protein exists in four or five forms derived from alternative splicing of tau mRNA. The ability of Tau to cross-link microtubules into thick bundles may contribute to the stability of axonal microtubules [24, 25].

When MAPs coat the outer wall of a microtubule, tubulin subunits are unable to dissociate from the ends of a microtubule. Although the rate of microtubule disassembly is generally dampened by bound MAPs, the assembly of microtubules is affected to varying degrees: some MAPs, like MAP4 and Tau, stabilize microtubules, whereas other MAPs do not [25].

1.2. Neuronal Cytoskeleton

Neurons have two distinct types of processes extending from a cell body; axons and dendrites (Figure 1.13A). The neuronal cytoskeleton is comprised of two major elements: neurofilaments and microtubules. Both axons and dendrites are supported by stable microtubules together with the neurofilaments (Figure 1.13B) [26].

![Figure 1.13](image.png)

**Figure 1.13.** A) A typical neuron with a long axon and dendrites. B) Microtubules and intermediate filaments are oriented longitudinally in axon and they are cross-linked by various proteins.

In axons, neurofilaments and microtubules are aligned parallel to the longitudinal axis of the axon with the neurofilaments forming cross-bridged complexes with themselves as well as neurotubules [26]. However, the microtubules in axons and dendrites are organized differently and associate with distinct MAPs. In axons, the microtubules are all oriented with their plus ends away from the cell body, similar to the general organization of microtubules in other cell types. The minus ends of most
of the microtubules in axons, however are not anchored in the centrosome. Instead, both plus and minus ends of these microtubules terminate in cytoplasm of the axon. In dendrites, the microtubules are oriented in both directions; some plus ends point toward the cell body and some point toward the cell periphery (Figure 1.14). These distinct microtubule arrangements are paralleled by differences in MAPs: Axons contain tau proteins, but no MAP-2, whereas dendrites contain MAP-2, but no Tau proteins [27, 28, 29].

![Image of nerve cell showing microtubule orientations](image)

**Figure 1.14.** In nerve cells, the (-) ends of all axonal microtubules are oriented toward the base of the axon, but dendritic microtubules have mixed polarities.

### 1.2.1. Axonal Transport

The axon is dependent upon the soma for production of vital macromolecules. In some instances, the volume of the axon may exceed that of the cell body by 1000-fold, placing a massive metabolic demands on the cell body. In addition, an intracellular transport system is required to function efficiently for delivery of *de novo* synthesized macromolecules to the various axonal and dendritic domains [26]. However, substances which consist mainly of old membrane from the synaptic terminals, are destined to be degraded in lysosomes in the cell body. Therefore, axonal transport can be classified as anterograde and retrograde axonal transport (Figure 1.15) [30].
1.2.1.1. Anterograde Axonal Transport

Nerve impulses are transmitted from a neuron by release of neurotransmitters from the terminal of the axon, the very long process that extends from the cell body. The neuron must constantly supply new materials to the terminal to replenish those lost by exocytosis at the synapse. Ribosomes are mainly present in the cell body and the dendrites of nerve cells. Therefore, proteins and membranes must be synthesized in the cell body and then transported down the axon, which can be up to several meters in length, to the synaptic regions [30].

The anterograde axonal transport proceeds from the cell body to the synaptic junctions and is associated with axonal growth and the renewal of synaptic vesicles. It is also divisible into fast and slow components. In addition to different rates of movement, different materials are conveyed in the two components. The slow component is defined as movement of the cytoskeleton at rates of 0.5-5 mm per day [24]. Fast anterograde axonal transport represents a spectrum of rates ranging from 200 to 400 mm per day. Conveyed in this system is a variety of proteins, glycoproteins, and glycolipids destined for the axolemata, mitochondria and synaptic vesicles including neurotransmitters and the enzymes associated with neurotransmitter generation and/or regeneration. These components are moved along microtubules by the motor protein kinesin. The anterograde direction of kinesin transport is the combined result of polarized microtubules, the dedicated movement
of kinesin toward the faster growing plus end and the cellular distribution of microtubules with their plus end distally [30, 31].

1.2.1.2. Retrograde Axonal Transport

Retrograde axonal transport operates at approximately half the rate of fast anterograde transport. The mechanism is similar to fast anterograde transport; membrane bound organelles (MBOs) are conveyed along microtubules with a motor protein [30].

One difference between anterograde and retrograde transport is that the vesicles conveyed by retrograde transport are larger than those conveyed in the fast transport system. In addition to mitochondria, multivesicular or lysosomal types of vesicles are often observed. A second difference is the use of the motor protein, cytoplasmic dynein. The locomotion is directed toward the minus or slow-growing end. Microtubule orientation dictates dynein-directed movement in the retrograde direction [26].

1.3. Acrylamide

1.3.1. Structure

Acrylamide is an α,β-unsaturated carbonyl compound. It is a highly water-soluble, solid substance at room temperature with a molecular weight of 71.08. Monomeric acrylamide readily participates in radical-initiated polymerization reactions [32].

![Figure 1.16. Chemical structure of acrylamide monomer.](image)

1.3.2. Chemical and Physical Properties

Acrylamide is a colorless to white, odorless crystalline solid. When further purified with benzene, it precipitates as a leaf of flake-like crystal. Its solubility in various solvents at 30°C is as follows: water 215.5 g/100 ml, methanol 155 g/100 ml, ethanol 86.2 g/100 ml, acetone 63.1 g/100 ml, ethyl acetate 12.6 g/100 ml, chloroform 2.66
g/100 ml, benzene 0.346 g/100 ml and heptane 0.0068 g/100 ml. Acrylamide has a density of 1.122 g/cm$^3$ at 30°C, a melting point of 84.5°C and boiling point of 125°C at 25 torr. It has a vapor pressure of 0.007 torr at 25°C and relative vapor density (air=1) of 2.45. Acrylamide (solid) is stable at room temperature, but polymerizes on melting or exposure to ultraviolet radion [33].

1.3.3. Production

Acrylamide was first produced in 1893 by Moureu in Germany. In 1952, Hercules started making research quantities and by 1954 began commercial production [33]. Acrylamide is formed from the hydration of acrylonitrile with sulfuric acid monohydrate at 90-100°C. From the resulting sulfate solution acrylamide is extracted by neutralization with ammonia and subsequent cooling to isolate the crystalline monomer. The reaction of acrylonitrile with sulfuric acid and water is highly exothermic, and produces by-products of polyacrylamide and acrylic acid. The addition of copper salts can suppress side reactions leading to these by-products. In 1971, a direct catalytic conversion of acrylonitrile to acrylamide was developed, thereby eliminating by-products. By this method, an aqueous solution of acrylonitrile is passed over a fixed bed of copper or copper-metal admixtures at 25-200°C. Unreacted aqueous acrylonitrile can be recycled by subsequent oxidation reaction. [33, 34].

Acrylamide monomer is used primarily for the production of polyacrylamides with widely different physical and chemical properties. These high-molecular-weight polymers can be modified to have non-ionic, anionic or cationic properties for specific uses. Polymerization (homopolymerization and copolymerization) is brought about through a free-radical mechanism in aqueous solution. The solubility and polyelectrolyte characteristics of these polymers are imparted by residual carboxyl groups or hydrolysed amide groups. Water-soluble cationic polymers can be produced by copolymerization with unsaturated quaternary ammonium compounds such as diallyldimethyl ammonium chloride and vinylbenzyltrimethyl ammonium chloride. Anionic polyacrylamides are formed by copolymerization with carboxylic or sulfonic acids [32, 33].
1.3.4. Usage

Acrylamide polymers have broad industrial application; e.g., polymer production, ore processing, mineral processing, concrete processing, cosmetic additives, soil and sand treatment, coating applications, oil production process, dye and fiber synthesis, water purification, soil stabilization, pulp and paper, fabric, textile industries and also are used in molecular biology laboratories as laboratory electrophoresis gels [32, 35, 36, 37, 38].

1.3.5. Disposal and Environmental Fate

Acrylamide may be disposed of by polymerization in landfills, dissolved in large quantities of water, hydrolysed with hot sodium or calcium hydroxide or incinerated, provided that there is provision for scrubbing of nitrogen oxide from flue gases [33]. Acrylamide and its monomeric analogues are readily leachable in soil and may travel great distances in the ground-water of deep rock aquifers. Bioconcentration and accumulation of acrylamide in food chain organisms are unlikely because of its higher solubility in water when compared to $n$-octanol, an in vitro indicator of fat solubility and membrane transversibility. Thus, acrylamide will not accumulate in fatty tissues. Furthermore, a wide variety of microbes possess the ability to degrade acrylamide under light or dark, anaerobic or aerobic conditions [33].

Acrylamide is not a normal substrate used by microorganisms and they require a period of time to adjust. Thus, a lag period of several days, weeks or months may occur before any significant degradation is observed. In laboratory experiments, acrylamide was not absorbed by sewage sludge, natural sediments, clays, peat, or synthetic resins over the pH range of 4-10. Thus, acrylamide in contaminated water cannot be removed by water treatment [32].

1.3.6. Exposure and Metabolism

Humans are exposed to acrylamide during its manufacture, in grouting operations, by leaking of acrylamide from polyacrylamide used in the purification of drinking water, and in laboratories using acrylamide for the preparation of chromatographic gels. In addition, humans consuming carbohydrate-rich foods processed at high temperatures are exposed to acrylamide [33, 39].
Acrylamide is absorbed by all routes of exposure, such as oral, intravenous, intraperitoneal, subcutaneous, dermal or inhalation. Once absorbed, acrylamide is rapidly distributed throughout the body, metabolized and excreted. In autoradiographic studies of mice, Marlowe et al. (1986) found that radioactivity from acrylamide is evenly distributed among tissues, although 9 days after treatment the label was found to have accumulated in the reproductive tract. The tissues with the highest amounts of radioactivity were muscle, skin, blood and liver. By contrast, the lowest concentrations were found in nervous tissues [32, 40].

Following uptake, acrylamide may be conjugated with glutathion to take place in the bile and the liver. It is then excreted in the urine as the mercapturic acid N-acetyl-S-(2-carbamoylethyl) cysteine, or it may be oxidized to glycidamide. Acrylamide has also been reported to be excreted unchanged in the urine. The conjugation of acrylamide to glutathion is mediated by glutathion transferase. Also it is observed that the metabolic conversion of acrylamide to glycidamide is mediated by the cytochrome P<sub>450</sub> system. The metabolite glycidamide either reacts with glutathion, or is excreted intact or as the hydrolysis product of glyceramide. However glyceramide may undergo further oxidation to substances with a labile 1-2 carbon bond, resulting in the release of carbon dioxide [32].

1.3.7. Acrylamide formation in foods

Although foods have been cooked at the same way for a significant amount of time, it was not known that the chemical would turn up in food until 2002. When Swedish researchers were conducting studies on the health risks to workers who had been exposed to acrylamide while on the job, they discovered the connection to food. The control group for the study, people who had not been exposed to acrylamide at work, had high levels of acylamide in their bodies, leading researchers to look for other sources of exposure such as diet [41].

The researchers found, and the U.S. Food and Drug Administration (FDA), the U.K. Food Standards Agency and many other countries confirmed, significant levels of acrylamide in a wide range of foods as a result of baking or frying, and it is likely that the chemical is also produced by grilling and roasting food. Mottram and colleages reported that acrylamide is formed by Maillard reaction between
asparagine and glucose and that this reaction might happen during high-temperature (above 120°C) cooking and food processing (Figure 1.17) [42, 43, 44].

![Diagram of acrylamide formation by Maillard reaction between glucose and asparagine.](image)

**Figure 1.17.** Diagram of acrylamide formation by Maillard reaction between glucose and asparagine.

Interest in human exposure to acrylamide has recently regained notoriety due to its formation from asparagine in carbohydrate-rich foods such as French fries and potato chips, when cooked at high temperatures. Raw or even boiled potatoes test negative for the chemical. Acrylamide is also reported to be present in biscuits crackers, toast, soft bread, breakfast cereals and coffee powder. While the levels of exposure from consumption of these food sources may be carcinogenic, they appear well below what is necessary to cause neurotoxicity. Nevertheless, repeated exposures to acrylamide produce in both humans and experimental animals a distal axonopathy [42, 45, 46, 47, 48].

### 1.3.8. Toxicity

Acrylamide monomer has been known as a toxicant for more than for decades. It has been identified as genotoxic, carcinogenic, and a male reproductive toxicant, as well as a neurotoxicant [49, 50, 51].

#### 1.3.8.1. Genotoxicity and carcinogenicity

EU Risk Assessment Report indicates that acrylamide is carcinogenic in animals producing increased incidences in a number of benign and malignant tumours in a variety of organs. The tumour types observed show a possible relationship with disturbed endocrine function and raise the possibility of a hormonal mechanism. There is also a suggestion of acrylamide-induced tumours in brain and spinal cord.
International Agency for Research on Cancer (IARC) reports that acrylamide induces gene mutation, structural chromosomal aberrations, sister chromatid exchange and mitotic disturbances in mamalian cells \textit{in vitro} in the presence or absence of exogenous metabolic systems [49, 50]. Acrylamide and its metabolite glycidamide form covalent adducts with DNA in mice and rats. It induces structural chromosomal aberrations \textit{in vivo} in both somatic and germ-line cells. Treatment with acrylamide \textit{in vivo} also caused somatic mutation in the spot test, heritable translocation and specific locus mutations in mice, and dominant lethal mutations in both mice and rats in several studies. Acrylamide induces unscheduled DNA synthesis in rat spermatocytes \textit{in vivo} but apparently not in rat hepatocytes. It was also reported to induce transformation in cultured mammalian cells and sex-linked recessive lethal and somatic mutations in \textit{Drosophila} [52, 53].

1.3.8.3. Male Reproductive Toxicity

There are no human data available on reproductive toxicity of acrylamide. However, over the past 10 years, there have been a large number of studies investigating the effects of acrylamide on rodent reproductive performance to conclude that acrylamide is a reproductive toxicant in male rats and mice bred to untreated females. In addition, acrylamide does not affect female reproduction in rats and mice. These rodent data are assumed relevant to humans [54].

Male reproductive toxicity was manifested as impaired delivery of sperm to the female genital tract and reduction of live litter size due to increased post implantation loss. However, alterations in sperm function and motility, low hormone levels and testicular atrophy have been observed at high doses when the animals are already being intoxicated by the peripheral or central neurotoxicity, and/or by the clastogenic effects which appear to result from the interaction of acrylamide (or its metabolite glycidamide) with protamines during spermatogenesis in the seminiferous tubules or testes. In addition, there are sufficient data to conclude that acrylamide induces transmissible genetic damage in male germ cells of mice in the form of reciprocal translocations and gene mutations. Such effects can lead to genetic disorders and infertility in subsequent generations [51, 54].
Subsequently, the mechanisms of action of acrylamide reproductive toxicity appear to be:

1. Acrylamide and/or glycidamide binding to spermatid protamines results in dominant lethality due to induced clastogenesis and effects on sperm morphology.
2. Acrylamide interference with molecular transport between the neuron cell body and axon resulting in distal axonopathy, and therefore, hindlimb weakness, perhaps also anesthesia to the penis, resulting in effects on mounting and intromission and effects on sperm motility due to interference with flagellar function [54].

1.4. Acrylamide neurotoxicity

The burgeoning commercial use of acrylamide during the 1950s was accompanied by growing concern over potential toxicity. There have been case reports in monomer and polymer manufacturing sectors of worker peripheral neurotoxicity. Early studies indicated that exposure of humans and laboratory animals to acrylamide produced skeletal muscle weakness and ataxia. Subsequent researches suggested this behavioral neurotoxicity was related to nerve damage classified as a central-peripheral distal axonopathy [55, 56].

The morphological hallmark of this axonopathy is considered to be axon degeneration, which begins as multifocal paranodal swellings of preterminal distal fibers. Axonal swellings contain an abundance of neurofilaments, tubulovesicular profiles and degenerating mitochondria [57].

More recent studies have suggested axonal atrophy also is a morphologic component of acrylamide peripheral neuropathy. Axonal atrophy and segmental demyelination have been observed frequently and are considered as secondary phenomena. Because of the retrograde progression of degeneration, this type of toxic nerve injury was termed a “dying back” neuropathy by Cavanagh at 1964. Clinically, toxic axonopathies are associated with skeletal muscle weakness of the extremities, weight loss and uncoordinated gait (ataxia) [57, 58].

The acrylamide has been placed in a group of substances showing an organophosphorus pattern of degeneration. It is one of the recognized prototypical agents among the numerous compounds that produce this type of neuropathy and a
majority of studies investigating mechanisms of distal axonopathies have focused on this chemical [31, 59].

Acrylamide produces a retrograde axonal degeneration in nerve fibers of Peripheral Nervous System and Central Nervous System. In intoxicated animals, acrylamide was reported to impair either the slow phase or the rapid phase of the axonal transport of proteins in spinal roots and in long peripheral nerves. Short axons have been previously found to be less affected than long axons in acrylamide intoxicated animals [60].

1.4.1. Possible Mechanisms of Neurotoxicity

Although acrylamide neurotoxicity has been known for four decades, the exact mechanism of toxicity is still unknown. Based upon the time course of recovery of transport, the target must be one that is rapidly replenished within the axon. Identification of the molecular site of action is complicated by simultaneous compromise of both anterograde and retrograde transport systems by agents regarded as specific for one. Four targets have received the most attention; enzymes of intermediary metabolism, neurofilaments, motor proteins and microtubules.

1-In 1979, Spencer et al. first proposed the energy hypothesis as a mechanism of acrylamide neurotoxicity. Covalent binding of acrylamide to glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which had been shown vital to axonal transport, was suggested to cause reduced axonal transport by depletion of the required levels of ATP. In support, numerous enzymes were identified as inhibited by acrylamide such as enolase and phosphofructokinase [61]. In addition, LoPachin et al. focused their studies on effects of acrylamide on Na⁺/K⁺-ATPase activity. They proposed reduced Na⁺/K⁺-ATPase activity as an initiating event in ion disruption associated with swollen and, possibly, atrophied regions of distal axons. They measured rubidium (Rb⁺) transport as an index of enzyme function. X-ray microanalysis was used to quantify elemental Rb uptake and accumulation in internodal myelinated axons, mitochondria, Schwann cells and myelin of rat tibial nerve cryosections. Their results demonstrated impairment of Rb uptake in tibial axons [34].
2-The neurofilament (NF) hypothesis suggests that the neurotoxicant covalently binds to and modifies the NF subunits. In the case of acrylamide, the primary reactive group would be protein sulfhydryls. The modified NF either inappropriately interacts with other cytoskeletal elements and/or accumulates within the axon. The improper function and/or distribution of NFs are proposed to compromise fast transport, reducing delivery of vital macromolecules to the distal axon [61].

In 1985, Howland et al. assayed the activity of protein kinase in neurofilament preparations from spinal cords of rats treated with acrylamide. They observed significantly increased total phosphorylation of neurofilament preparations. According to these results they suggested that one possible cause of abnormally arranged neurofilaments in acrylamide induced neuropathy was related to an altered phosphorylation of these cytoskeletal elements [62].

3-The potential effect of acrylamide on the motor protein kinesin is consistent with the characteristics of reduced fast anterograde transport by this toxicant. Kinesin is one the candidates due to the dependence of kinesin function on sulfhydryl groups and the binding of acrylamide to protein sulfhydryls.

Sickles et al. evaluated kinesin as a molecular site of action using an in vitro microtubule motility assay. Their results demonstrated that pretreatment of kinesin with acrylamide adversely affected both the number and rate of microtubule moving in an in vitro motility. However, they observed decreased numbers of microtubules gliding over the kinesin-adsorbed coverslip and frequent dissociation of gliding microtubules. They suggested that this effect resulted from a direct and irreversible binding of acrylamide to kinesin [63].

4-The dependence of fast axonal transport upon the axonal microtubule railway system and the compromise of transport by microtubules poisons suggested this cytoskeletal element as a potential site of action. Radio-labeled acrylamide has been showed to bind tubulin and acrylamide reduced the binding of radio-labeled colchicine [34].

Some studies resulted in no change in microtubule assembly/disassembly following acrylamide. However, in 1997, Abou-Donia et al. reported an increased rate of polymerization and a decrease in time to $V_{max}$ following acrylamide treatment [64].
In addition, Sickles et al. observed that microtubule motility was adversely affected when tubulin was preincubated with acrylamide due to doubled microtubule detachment rate during \textit{in vitro} microtubule motility assay [63].

In this thesis study, we investigated whether microtubules are the site of action for neurotoxic effects of acrylamide. In previous studies it has been shown that axon degeneration, which begins as multifocal paranodal swellings of preterminal distal fibers is the morphological hallmark of axonopathy. Axonal swellings contain an abundance of neurofilaments, tubulovesicular profiles and degenerating mitochondria. Since microtubules are the major elements of axonal transport, they are one of the candidates as the responsible ones for impaired axonal transport. However, time recovery of neurotoxicity is one of the reasons to show microtubules as targets of acrylamide neuropathy.

We used microtubule sedimentation assay, fluorescence spectrometer and malachite green procedure to evaluate the effects of acrylamide on polymerization kinetics of microtubules. In addition, transmission electron microscopy has been used to examine the structures that pelleted in the sedimentation assay and fluorescence spectrometer.
2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Buffers for Tubulin Purification

2.1.1.1. 5x PM Buffer

500 mM Pipes (Roche), 10 mM EGTA (Sigma) and 5 mM Mg SO$_4$7H$_2$O (Riedel-de Haen) were dissolved in de-ionized water up to 1 lt and pH was adjusted to 6.9 with NaOH. The solution was stored at 4°C.

2.1.1.2. 1x PM Buffer

5x PM buffer was diluted with de-ionized water 5 times and pH was adjusted to 6.9. It was stored at 4°C.

2.1.1.3. PM-4M Buffer

4 M glycerol (Carlo Erba) was dissolved in 1 lt 1x PM buffer and stored at 4°C.

2.1.1.4. PM-8M Buffer

8 M glycerol (Carlo Erba) was dissolved in 1 lt 1xPM buffer and stored at 4°C.

2.1.1.5. PMSF Solution

100 mM PMSF (Sigma) was dissolved in 10 ml isopropanol (Merk) and stored at room temperature.

2.1.2. Buffers for Ion-Exchange Column Purification

2.1.2. Ion-Exchange Column Preparation

2.1.2.1. NaOH Solution

0.5 M NaOH (Riedel-de Haen) was dissolved in de-ionized water up to 1.5 lt and stored at 4°C.
2.1.2.2. HCl Solution

0.5 M HCl (37 %) (Merk) was dissolved in de-ionized water up to 1.5 lt and stored at 4°C.

2.1.2.3. 1x PM Buffer containing GTP and DTT

0.1 mM GTP (Sigma) and 2 mM DDT (Sigma) were dissolved in 1 lt 1xPM buffer and stored at 4°C.

2.1.3. Solutions for Determination of Protein Concentration

2.1.3.1. Bradford Reagent

Bradford reagent (Sigma) was used as purchased and stored at 4°C.

2.1.3.2. Bovine Serum Albumine (BSA) Stock Solution

14.5 g BSA (Sigma) was dissolved in 10 ml de-ionized water and stored at -20°C.

2.1.4. Buffers for Reactions

2.1.4.1. AB Buffer

20 mM Pipes (Roche), 1 mM MgCl$_2$ (Riedel-de Haen) and 1 mM EGTA (Sigma) were dissolved in 1 lt de-ionized water and pH was adjusted 6.9. The solution was stored at 4°C.

2.1.5. Solutions for SDS-PAGE (Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis)

2.1.5.1. Acrylamide Monomer Solution (30% T/ 2.7% C$_{bis}$)

58.4 g acrylamide (Sigma) and 1.6 g bis-acrylamide (Sigma) were dissolved in de-ionized water up to 200 ml. Solution was stored in the dark and at 4°C.

2.1.5.2. 4x Running (Separating) Gel Buffer (1.5 M Tris-Cl)

36.3 g tris (Merk) was dissolved in de-ionized water up to 200 ml. pH was adjusted to 8.8 with HCl. Solution was stored at 4°C.
2.1.5.3. 4x Stacking Gel Buffer (0.5 M Tris-Cl)

3 g tris (Merk) was dissolved in 50 ml de-ionized water and pH was adjusted to 6.8. Solution was stored at 4°C.

2.1.5.4. 10% Sodium Dodecyl Sulfate (SDS)

10 g SDS (99 %) (Sigma) was dissolved in 100 ml de-ionized water and solution was stored at room temperature.

2.1.5.5. 10% Ammonium Persulfate (APS)

0.1 g APS (Sigma) was dissolved in 1 ml de-ionized water. Solution was stored at 4°C, had a two week life.

2.1.5.6. TEMED (N, N, N’, N’ – Tetramethylethylene diamine)

TEMED (Farmitalia Carlo Erba S.p.A.) was used as purchased and stored at 4°C.

2.1.5.7. 2xSample Buffer

2.5 ml 4X Stacking Gel Buffer (0.125 M Tris-HCl, pH 6.8), 4 ml 10% SDS solution (4% SDS), 2 ml Glycerol (20% glycerol) (Carlo Erba), 1 ml β- mercaptoethanol (10% β-ME) (Merk), 0.05% Bromophenol Blue (BRB) (0.5 mg/ml) (Sigma) were dissolved in 10 ml de-ionized water. Solution was stored at -20°C as aliquots of 1.5 ml.

2.1.5.8. Tank Buffer (Running Buffer)

3 g tris (0.025 M Tris) (Merk), 14.4 g glycine (0.192 M Glycine) (Merk), 10 ml SDS solution (1 g/lt) (0.1% SDS) were dissolved in 1 lt de-ionized water. Solution was stored at 4°C.

2.1.5.9. Coomassie Blue Stain

1 g CBB R-250 (0.1%) (Fluka), 500 ml methanol (50%) (Merk) and 100 ml acetic acid (10%) (Merk) were dissolved in de-ionized water up to 1lt. The solution was stored at room temperature.
2.1.5.10. Gel Destain

50 ml methanol (%5) and 100 ml acetic acid (10%) were mixed in 1 lt de-ionized water. The solution was stored at room temperature.

2.1.6. Stock Solutions

2.1.6.1. GTP Stock Solution

200 mM GTP (Sigma) was dissolved in 1 ml de-ionized water and stored at -20°C.

2.1.6.2. DTT Stock Solution

1 M DTT (Sigma) was dissolved in 1 ml de-ionized water and stored at -20°C.

2.1.6.3. Taxol Stock Solution

10 mM Taxol (Paclitaxel, Sigma) was dissolved in DMSO (dimethyl sulfoxide) (Riedel-de Haen) and stored at -20°C.

2.1.6.4. Acrylamide Stock Solution

1 M acrylamide was dissolved in 50 ml de-ionized water and stored in the dark, at 4°C.

2.1.7. Phosphocellulose Resin

Cellulose Phosphate Cation Exchanger (Whatman) was used for ion-exchange column preparation.

2.1.8. Solutions for Electron Microscopy

2.1.8.1. Pioloform Solution

250 mg pioloform (Agar Scientific) was dissolved in 100 ml chloroform (Merk) and stored at 4°C, in the dark.

2.1.8.2. Gluteraldehyde

40 μl % 25 Gluteraldehyde (Merk) was mixed with de-ionized water to make 4 % (v/v) and stored at room temperature.
2.1.8.3. Uranyl Acetate Solution

Uranyl acetate (Sigma) was dissolved in de-ionized water as %2 (w/v). Dye solution was stored in dark at room temperature.

2.1.9. Protein Marker

Benchmark Prestained Protein Ladder (Gibco) was used as a standard to analyze the SDS-PAGE gels. It consists of 10 proteins ranging in molecular weights of 10, 15, 20, 30, 40, 60, 70, 90, 130, and 220 kDa.

2.1.10. Lab Equipments

Autoclave : 2540 ML benchtop autoclave, Systec GmbH Labor-Systemtechnik.
            : NuveOT 4060 Vertical Steam Sterilizer, Nuve.
Balances : Precise BJ 610C, order# 160-9423-050, Presice Instruments AG Dietikon.
          : Precise XB 220A, order# 320-9204-001, Presice Instruments AG Dietikon.
            : Optima LE 80 K, Beckman Coulter.
            : Optima Max, Beckman Coulter.
            : Microfuge 18, Beckman Coulter.
Centrifuge rotors : MLA 80, Beckman Coulter.
                  : JLA-16250, Beckman Coulter.
                  : 70Ti, Beckman Coulter.
                  : TS-5.1-500, Beckamn Coulter.
                               : 2031 D deep freezer, Arçelik.
                               : 1061 M refrigerator, Arçelik.
Electron Microscope : Jeol Jem 1011.
<table>
<thead>
<tr>
<th>Equipment Type</th>
<th>Model/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoresis equipments</td>
<td>EC 120 Mini Vertical Gel System, Thermo EC.</td>
</tr>
<tr>
<td>Fluorescence Spectrometer</td>
<td>LS-50, Perkin Emler.</td>
</tr>
<tr>
<td>Fraction Collector</td>
<td>Model 2110, Bio-Rad.</td>
</tr>
<tr>
<td>Gel documentation system</td>
<td>UVIpro GAS7000, UVIttech Limited.</td>
</tr>
<tr>
<td>Gel Dryer</td>
<td>Bio Rad Model 583</td>
</tr>
<tr>
<td>Glassware</td>
<td>Technische Glaswerke Ilmenau GmbH.</td>
</tr>
<tr>
<td>Grids</td>
<td>300 mesh size copper grids, Sigma.</td>
</tr>
<tr>
<td>Homogenizer</td>
<td>Potter S, B. Braun Biotech International</td>
</tr>
<tr>
<td>Ice Machine</td>
<td>AF 10, Scotsman.</td>
</tr>
<tr>
<td>Incubator</td>
<td>EN400, Nuve.</td>
</tr>
<tr>
<td>Laminar Flow Cabinet</td>
<td>Ozge.</td>
</tr>
<tr>
<td>Magnetic stirrer</td>
<td>AGE 10.0164, VELP Scientifica srl.</td>
</tr>
<tr>
<td>Microwave</td>
<td>MD582, Arçelik.</td>
</tr>
<tr>
<td>Peristaltic pump</td>
<td>Econo Pump, Bio-Rad.</td>
</tr>
<tr>
<td>pH meter</td>
<td>MP 220, Metler Toledo International Inc.</td>
</tr>
<tr>
<td></td>
<td>Inolab pH level 1, order# 1A10-1113, Wissenschaftlich-Technische Werkstätten GmbH &amp; Co KG.</td>
</tr>
<tr>
<td>Pipettes</td>
<td>0.1-2.5 μl, 500-5000 μl, Mettler Toledo.</td>
</tr>
<tr>
<td></td>
<td>10 μl, 20 μl, 200 μl, 1000 μl, Gilson.</td>
</tr>
<tr>
<td>Pipette Tips</td>
<td>1-1000 μl, 1-200 μl, Axygen.</td>
</tr>
<tr>
<td></td>
<td>1-10 μl, Molecular Bioproducts.</td>
</tr>
<tr>
<td>Power supply</td>
<td>EC 250-90, E-C Apparatus.</td>
</tr>
<tr>
<td>Pure water systems</td>
<td>USF Elga UHQ-PS-MK3, Elga Labwater.</td>
</tr>
<tr>
<td>Shaker</td>
<td>Heidolph, Duomax 1030</td>
</tr>
<tr>
<td>Spectrophotometers</td>
<td>DU530 Life Science UV/Vis, Beckman.</td>
</tr>
<tr>
<td></td>
<td>UV-1601, Shimadzu Corporation.</td>
</tr>
<tr>
<td></td>
<td>Lambda 25 UV/Vis, Perkin Emler.</td>
</tr>
<tr>
<td>Spectrophotometer cuvettes</td>
<td>Delta</td>
</tr>
<tr>
<td>Test Tubes</td>
<td>1.5 ml tubes, Eppendorph.</td>
</tr>
<tr>
<td>Vacuum Pump</td>
<td>262 BR, Bio-Rad.</td>
</tr>
</tbody>
</table>
2. 2. Methods

2.2.1. Tubulin Purification

- One bovine brain was collected and weighed as approximately 250 g
- The brain was kept on ice all the time, cerebellum and meninges were removed
- The brain was cut with scissors
- For each 100 g of brain, 75 ml PM-4M buffer containing 1 mM PMSF and 1 mM DDT were added into stainless steel blender and mixed
- The homogenate was centrifuged with Beckman Avanti J-30I centrifuge, JLA-16250 rotor, 250 ml tubes at 30,000xg and 4°C for 30 minutes
- Supernatants were collected and centrifuged with Beckman Optima LE80K centrifuge, 70Ti rotor, 35 ml tubes, at 183,960xg (50,000 rpm) and 4°C for 40 minutes
- Supernatants were collected as the total volume \( V_1 \)
- 200 mM GTP was added and resulting mixture was incubated at 37° C for 30 minutes
- The mixture was centrifuged with Beckman Optima LE80K centrifuge, 70Ti rotor, 26.3 ml tubes, at 183,960xg (50,000 rpm) (and Beckman Optima Max Ultracentrifuge, MLA 80 rotor, 10 ml tubes, at 184,262xg (60,000 rpm) at 37° C for 30 minutes
- Supernatants were decanded and pellets were taken onto ice and resuspended with 0.25x\( V_1 \) ml of 1xPM buffer containing 1 mM PMSF and 1 mM DDT
- The resuspended solution was homogenized with Potter S Homogenizator on ice and chilled on ice for 30 minutes
- The homogenate was centrifuged with Beckman Optima Max Ultracentrifuge, MLA 80 rotor, 10 ml tubes, at 184,262xg (60,000 rpm) and 4° C for 30 minutes
- The supernatants were collected and named as \( V_2 \)
- Equal volume (\( V_2 \)) of PM-8M buffer was added; total volume= 2x\( V_2 \)
• 1 mM PMSF, 1 mM DDT and 2 mM GTP were added into total volume
• Mixture was incubated at 37° C for 30 minutes
• Mixture was centrifuged with Beckman Optima Max Ultracentrifuge, MLA 80 rotor, at 184,262xg (60,000 rpm) and 37° C for 30 minutes
• Supernatants were decanted and pellets were resuspended with 0.25x V2 ml of PM buffer
• The resuspended solution was homogenized homogenizator on ice and incubated at 4° C for 30 minutes
• The homogenate was centrifuged with Beckman Optima Max Ultracentrifuge, MLA 80 rotor, at 184,262xg (60,000 rpm) and 4° C for 30 minutes
• Supernatants were collected, named as semipurified tubulin and kept at -80°C as aliquots
• The concentration of protein was measured by Bradford Assay

2.2.1.1. Bradford Assay
• 14.5 mg BSA was dissolved in 10 ml de-ionized water
• Stock standart solutions were prepared as 20 μg/ml, 40 μg/ml, 60 μg/ml, 80 μg/ml, 100 μg/ml, 120 μg/ml, 140 μg/ml, 160 μg/ml, 180 μg/ml, 200 μg/ml, 220 μg/ml, 240 μg/ml, 260 μg/ml, 280 μg/ml and 300 μg/ml
• From each stock standart solution, 100 μl was added to tubes for standart preparation
• Then 900 μl Bradford reagent was added into each tube (total 1 ml)
• Sample preparation: sample amount was completed up to 100 μl with de-ionized water
• Then 900 μl Bradford reagent was added to each tube (total 1 ml)
• The absorbance was measured at 595 nm with spectrophotometer

2.2.1.2. Further Tubulin Purification

Whatman Phosphocellulose Ion-Exchanger Column Preparation
• The volume of glass column was measured and named as V
• V/4 g of phosphocellulose resin was weighed and put into a baker
• 25xV/4 ml 0.5 M NaOH was added and stirred kindly, and left for 5 minutes
The supernatant was decanded and washed with de-ionized water until the filtrate pH was 11.0 or below

25xV/4 ml 0.5 M HCl was added and stirred gently, and left for 5 minutes

The supernatant was decanded and washed with de-ionized water until the filtrate pH was 3.0 or above

The supernatant was decanded completely and 25xV/4 ml 1x PM buffer was added

The pH was adjusted to 6.9 with NaOH

The resin was poured into the column

When all slurry was added, the cap of the column was attached

1x PM buffer was run through the column until the column bed height was constant

150 ml 1x PM buffer containing 0.1 mM GTP and 2 mM DDT was run through the column

The pH of the eluate was checked to be 6.9

The column was left overnight at 4° C

All the steps were completed at 4° C

Further Tubulin Purification Through Column

The fraction collector was attached with peristaltic pump connected to the outlet of the column

Proper amount of semipurified tubulin solution was loaded to the column

Approximately V/2 ml of 1x PM buffer was passed through the column with the flow rate of 2.5 ml/min

1 to 12 fractions were collected as 2.5 ml and rest of the fractions were collected as 0.6 ml

10 μl samples were taken from each fraction and analyzed by Bradford Assay to determine which fractions contained protein

The fractions containing protein were pooled and the concentration was measured by Bradford Assay
Figure 2.1. Scheme of tubulin purification.

- Homogenized
- Centrifuged:
  - 2 x 37°C, 30 min
  - 2 x 4°C, 30 min
- Purified tubulin (4 µg/ml)
- MAPs (1 µg/ml)
- Semi-purified tubulin (13 µg/ml)
- Phosphocellulose ion-exchange column
2.2.1.3. Elution of MAPs from Phosphocellulose Column

- After tubulin proteins were obtained, microtubule-associated proteins were eluted from the column.
- 200 ml 1X PM buffer containing 1 M NaCl was run through the column and the fractions were collected.
- 10 μl samples were taken from each fraction and analyzed by Bradford reagent to determine which fractions contained protein.
- To determine the MAP fractions 7% SDS-PAGE gel was run and the fractions containing protein were pooled.
- Then MAPs were centrifuged with Beckman Allegra 25R, TS-5.1-500 rotor, Amicon-Centriplus centrifugal filter devices at 1396 x g, at 4°C for several hours.
- The filtered buffer was decanted and centrifugation was continued until MAPs were concentrated to 1/5 of its starting volume.
- MAPs were washed three times with 1X PM buffer to decant from salt.
- Then MAPs were collected by centrifugation.
- The concentration of MAPs was measured by Bradford Assay.

2.2.2. Sedimentation Assay

2.2.2.1. Semipurified Tubulin Reaction Conditions:

1- 5 μM semi-purified Tb + 1 mM GTP (37°C–60’)
2- 5 μM semi-purified Tb + 1 mM GTP (37°C–60’) → 100 mM Acr (37°C–30’)
3- 5 μM semi-purified Tb + 100 mM Acr (37°C–60’) → 1 mM GTP (37°C–30’)
4- 5 μM semi-purified Tb + 1 mM GTP + 100 mM Acr (37°C–60’)
5- 1 mM GTP + 100 mM Acr (37°C–60’) → 5 μM semi-purified Tb (37°C–30’)

2.2.2.2. TMT preparation:

- 10 μM semipurified tubulin, 1 mM GTP, 100 μM Taxol were mixed in AB buffer in a volume of 100 μl
- Mixture was incubated at 37°C for 30 minutes
- Microtubule sedimentation assays were carried out in AB buffer.
In a reaction volume of 100 μL, tubulin (5 μM final) or taxol-stabilized microtubules (TMT) were incubated with or without GTP (1 mM) in the presence of acrylamide (100 mM).

1. 5 μM TMT
2. 5 μM TMT + 100 mM acrylamide

Control reactions contained no acrylamide.

After incubations for 30 and 60 minutes at 37° C, reactions were centrifuged at 50,000xg (127,960 rpm) for 30 minutes at 37° C.

The supernatants were transferred into tubes containing equal amounts of 2x SDS sample buffer, and the pellets were resuspended in 100 μL of 1x SDS sample buffer.

Supernatant and pellet fractions were then analyzed by SDS-PAGE.

For quantitative analysis, the amount of supernatant and pellet samples loaded onto the gels was optimized to ensure a linear relationship between the amount of protein loaded and the intensity of the Coomassie blue stained protein bands.

The amount of MT proteins in the supernatant and the pellet fractions was quantified by measuring the protein band intensities relative to standards with documentation and analysis system.

Prior to performing MT polymerization experiments, in particular reactions tubulin was incubated for 30 minutes with taxol and GTP to be assembled into MTs.

2.2.2.3. SDS-PAGE

Supernatants and pellets were separated

For each 100 μl of supernatant, 100 μl 2x sample buffer was added and mixed with vortex

For each pellet, 100 μl 1x sample buffer was added and mixed with vortex

5 μl samples from pellet solutions and 10 μl samples from supernatant solutions were loaded onto SDS-PAGE gels using micropipette and loading tips
Assembly of Gel Apparatus

- Glass plates and spacers were cleaned with ethanol
- They were assembled in gel holder on casting stand
- Upper and lower screws were tightened and bottom edges of gel plates were checked
- Smaller plate was marked 1.5 cm from top

%10 Running (Separating) Gel Preparation

- 1.67 ml %30 acrylamide, 1.25 ml 4x running gel buffer, 1.97 ml de-ionized water, 50 μl %10 SDS, 30 μl %10 APS and 10 μl TEMED were mixed and poured between the glass plates up to the mark
- When the gel was polymerized, the top of the gel was rinsed with tank buffer
- Remaining water was absorbed with filter paper

%4 Stacking Gel Preparation

- 0.27 ml %30 acrylamide, 0.5 ml 4x stacking gel buffer, 1.9 ml de-ionized water, 20 μl %10 SDS, 10 μl %10 APS and 5 μl TEMED were mixed and poured onto the running gel up to the top of the front plate
- A comb was placed into the stacking gel quickly
- Gel was left for polymerization
- After polymerization the comb was removed carefully
- Lanes were washed with tank buffer and dried with filter paper

2.2.3.4. Loading and staining of the polyacrylamide gel

- 5 μl samples from pellet solutions and 10 μl samples from supernatant solutions were loaded onto polyacrylamide gels using micropipette and loading tips
- Gel was run at 200 mV for 45 minutes with power supply
- The glass plates were separated and the gel was placed in Coomassie Blue stain in a clean box
- The gel was microwaved at 750 Watt for 40 second
- Then the gel was placed on shaker for 2 minutes
- The stain was poured off and the gel was rinsed with gel destain
The gel was covered with gel destain and microwaved at 750 Watt for 50 seconds.

The gel was placed on shaker for 15 minutes.

Then destain was poured off and replaced with fresh destain solution.

The gel was left overnight on shaker.

The gel was dried with gel drier for storage.

After scanning of dry gel, it was analysed with gel documentation system.

2.2.4. Fluorescence Spectrometer

Reaction conditions:

1- 10 μM tubulin + 1 mM GTP

2- 10 μM tubulin + 1 mM GTP + 100 mM acrylamide

Spectrometric measurements were performed in AB buffer using the Model LS-50 spectrometer of Peklin-Elmer, equipped with temperature controller.

In reaction volume of 3 mL, tubulin (5-10 μM) protein was mixed with acrylamide (100 mM) and/or GTP (1mM).

Samples were excited at 295 nm and variation in the fluorescence spectra and emission intensity of the tryptophan residues in tubulin were monitored at 336 nm as a function of polymerization time during 1 hour incubation at 37°C.

2.2.5. Malachite Green Procedure

2.2.5.1. Color reagent preparation:

Concentrated sulfuric acid, H₂SO₄ (60 ml, d = 1.84 g/liter) was slowly added to 300 mL of water.

The solution was cooled to room temperature.

0.44 g of malachite green was added.

The resulting orange solution was stable for at least 1 year at room temperature.

On the day of use, 0.3 g ammonium molybdate and 22 μl Tween-20 were added to 10 ml of the dye solution.
2.2.5.2. **Protein reaction mixture preparation:** (10 μM ptb and 10 mM GTP)

- First, 100 mM GTP (stock: 200 mM) was exposed to 100 mM acrylamide (stock:1000 M)
  1- GTP alone mix= 50 μl GTP + 50 μl AB buffer
  2- GTP + acrylamide mix= μl GTP + 10 μl acylamide + 40 μl AB buffer
- These two reactions were incubated at 37° C for 30 minutes
- 10 μM purified tubulin (stock: 14 μM) was exposed to these two mixtures (final GTP and acrylamide concentrations became 10 mM)
  1- 643 μl ptb + 90 μl GTP alone mix + 167 μl AB buffer = 190 μl
  2- 643 μl ptb + 90 μl GTP + acrylamide mix + 167 μl AB buffer = 190 μl
- These two reactions were incubated at 37° C for 30 minutes

2.2.5.3. **Deproteinization and Phosphate determination:**

- Deproteinization with perchloric acid was performed by adding 1 volume of 70 % (w/w) perchloric acid, HClO₄ to 22 volume of protein reaction mixture (40.9 μl HClO₄ + 900 μl protein solution)
- After standing for 10 minutes at 0° C, the mixture was centrifuged for 2x 3 minutes at 13,000xg (~14,000xrpm) and the supernatant was used for phosphate determination
- The color reagent (200 μl) was mixed with 800 μl of the supernatant and incubated for 10 minutes at room temperature
- The absorbance at 630 nm was measured using spectrophotometer
- Blanks contained AB buffer and the color reagent (800 μl AB buffer + 200 μl color solution)
- Note: Absorbance measurements were never exceeded 10 minutes.

2.2.6. **Electron Microscopy**

2.2.6.1. **Membrane coating of grids:**

- 0.25 gram Pioloform was dissolved in 100 ml chloroform and incubated at 4°C in a dark bottle for 10 days
- Grids were rinsed with absolute alcohol and then chloroform and left for air drying
A clean glass block was plunged into pioloform solution and left for approximately 2 minutes for air drying
Pioloform membrane on the glass block was cut from the sides of the glass block
Glass block was plunged into water so that pioloform membrane swimmend on water
Then grids were put onto membrane as their shiny sides on top
A glass slight was put onto pioloform membrane with a 45° angle
Glass slight with grids coated with pioloform membrane was put into a glass petri and incubated at 37°C for 24 hours

2.2.6.2. Reaction Conditions:
Control 1: 10 μM semi-purified tubulin + 5 mM GTP + 100 μM Taxol
Control 2: 10 μM semi-purified tubulin + 5 mM GTP
Reaction 1a: 10 μM semi-purified tubulin + 5 mM GTP + 100 μM Taxol + 100 mM acrylamide
Reaction 1b: 10 μM semi-purified tubulin + 5 mM GTP + 100 mM acrylamide

All reactions were completed up to 100 μl with AB buffer in 1.5 ml eppendorf tubes
The tubes were incubated at 37°C in water bath for 1 hour
They were centrifuged with Beckman Optima Max Ultracentrifuge, MLA-80 rotor, at 127,960xg (50,000 rpm), 37°C for 30 minutes
The supernatants were decanded
20 μl %4 glutharaldehyde was added onto pellets and left for 15 minutes
Pellets were resuspended with micropipette one-two times gently
Grids were plunged into tubes and waited for 20 seconds
Then grids were washed by plunging into distelled water three times
Grids were left for air-dry for 30 minutes and stained

2.2.6.2. Staining:
One drop of %2 uranylacetate for each grid was put onto parafilm
Grids were placed on drops upsite down and left for 2 minutes
Grids were washed by plunging into distilled water two times
- Excess water was removed with filter paper
- Grids were left for air-dry
- Grids were analysed with transmission electron microscope
3. RESULTS AND DISCUSSION

Since the announcement by a Swedish research group in April 2002 regarding the presence of acrylamide in potato and grain-based foods such as potato chips and breakfast cereals, there has been a renewed interest in the toxic actions of this chemical. Acrylamide is a prototypic chemical in elucidating molecular mechanisms leading to distal axonopathy. Elucidation of the mechanisms is essential to prevention, treatment and repair of neurotoxic damage produced by acrylamide and similar agents. In addition to this, identification of toxicants’ mechanisms provides insight into molecular mechanisms of human diseases. These agents are also useful tools in understanding of normal cellular function and structural elements of the cells.

Because of the abundance of cytoskeleton and its many related functions in the nervous system, it has been difficult in many cases to identify whether alterations in cytoskeleton represent primary toxic effects or secondary effects caused by other changes in the cell.

The morphological hallmark of this axonopathy is considered to be “axon degeneration”, which begins as multifocal paranodal swellings of preterminal distal fibers. Axonal swellings contain an abundance of neurofilaments, tubulovesicular profiles and degenerate mitochondria. In addition, it has been shown that microtubule poisons adversely affect axonal transport. Several studies have identified reduced delivery of macromolecules to the distal axon by acrylamide. In this study, we have studied *in vitro* effects of acrylamide on microtubules purified from bovine brain to investigate the molecular mechanism of neurotoxic damage.

Tubulin obtained by two cycles of polymerization-depolymerization and ion-exchange chromatography was investigated for polymerization dynamics in the presence of acrylamide using different methods.

In sedimentation assays, purified or semi-purified tubulin was treated with acrylamide under different conditions to show the effect of acrylamide on
polymerization. In addition, taxol-stabilized microtubules (TMTs) were exposed to acrylamide to show the effect of acrylamide on stabilized microtubules. Also, GTP exposed to acrylamide was used in sedimentation assay to show whether acrylamide had an effect on GTP.

Structural transitions such as polymerization cause an increase in emission spectrum of fluorescence. Fluorescence spectrometric measurements were performed to show whether acrylamide causes inhibition of polymerization of tubulin dimers into microtubules in the presence or absence of GTP.

Since GTP is hydrolyzed into GDP and Pi during microtubule polymerization, and the produced Pi is well-proportioned to the number of tubulin subunits which join to the microtubule structure, we measured the produced Pi amount during microtubule polymerization using malachite green. Malachite green reacts with phosphomolybdate, and this reaction results in the appearance of an intense absorbance band at 620-650 nm.

Then, to examine the structures that pelleted in the sedimentation assay and fluorescence spectrometer, tubulin samples were observed by Transmission Electron Microscopy. Effects of acrylamide on microtubule structure were examined.

### 3.1. Tubulin Purification

A fresh bovine brain was taken to the laboratory on ice immediately. The cerebellum, meninges and blood vessels were removed. The brain was weighed and homogenized. Then, the homogenate was subjected to repetitive cycles of polymerization-depolymerization. At the end of these cycles, tubulin proteins were purified as associated with microtubule associated proteins (MAPs) and this elute was called semi-purified tubulin. The protein concentration was measured by the Bradford protein assay using bovine serum albumin (BSA) as a standard.

Afterwards, the pelleted solution from cycles of polymerization-depolymerization was further purified using phosphocellulose ion-exchange column to remove contaminating microtubule-associated proteins. Ion-exchange chromatography separates proteins based on their different charges. Since tubulin is charged negatively and MAPs are charged positively, MAPs remained attached to the resin in the column and tubulin passed through the column.
All the fractions collected from the column were analyzed qualitatively using Bradford reagent to examine the presence of tubulin. Coomassie Brilliant Blue G-250 in Bradford reagent binds to tubulin and causes a change in the color of the protein solution. Therefore, fractions containing protein were pooled and called purified tubulin. To confirm the purity of tubulin, the fractions from different steps of purification were loaded to polyacrylamide gel (Figure 3.1).

![Figure 3.1. Polyacrylamide gel of purification steps. 1. column: protein marker. 2. column: semi-purified tubulin with MAPs. 3. column: purified tubulin. 4. column: pure tubulin (%99) which was purchased from Cytoskeleton as a control marker.](image)

3.2. Elution of MAPs from phosphocellulose column

Positively charged MAPs remained attached to the resin during elution of tubulin. 1 M NaCl was run through the column to elute MAPs from the column. The eluted buffer containing MAPs was collected as fractions. Samples were taken from each fraction and analyzed qualitatively using Bradford reagent to detect the presence of proteins. Subsequently, samples from fractions containing protein were loaded to polyacrylamide gel (Figure 3.2).
3.3. Sedimentation Assay

Microtubule sedimentation assay was used to evaluate the effect of acrylamide on polymerization dynamics of microtubules. Semi-purified and purified tubulin proteins (5 μM) were subjected to acrylamide (100 mM) in the presence or absence of GTP (1 mM), and then subjected to centrifugation to separate unpolymerized tubulin in the supernatant from polymerized microtubule-containing pellet fractions.

Semi-purified tubulin was exposed to acrylamide under different conditions shown below. The reason for choosing these different reaction conditions was to evaluate on which steps acrylamide had possible effects. Acrylamide was added into reactions at different steps to see whether the effect was on tubulin polymerization or polymerized microtubules, or both.

1. Tubulin + GTP
As a control reaction, tubulin was incubated with GTP at 37°C to provide microtubule polymerization.

2. Tubulin + GTP → Acrylamide
To see the effect of acrylamide on previously polymerized microtubules, tubulin was incubated with GTP at 37°C, then acrylamide was added and incubated at 37°C.

3. Tubulin + Acrylamide → GTP
Tubulin before polymerization into microtubules (in the absence of GTP) was treated with acrylamide at 37°C and then GTP was added and incubated at 37°C.
4. Tubulin + GTP + Acrylamide
Tubulin, GTP, and acrylamide were altogether incubated at 37°C, to see tubulin polymerization into microtubules due to GTP in the presence of acrylamide.

5. GTP + Acrylamide → Tubulin
GTP was exposed to acrylamide at 37°C to see whether acrylamide has an effect on GTP. Then, tubulin was added and incubated at 37°C.

Afterwards, all reaction mixtures were centrifuged and separated as supernatant and pellet fractions. Samples from supernatants and pellets for each reaction were analyzed on polyacrylamide gel in the presence of tubulin standard samples (Figure 3.3).

For the control reaction, pellet fraction was significant, and there was some tubulin in the supernatant fraction. In the second reaction, all tubulin proteins were observed in supernatant fraction indicating that acrylamide caused total depolymerization of microtubules when it was added onto GTP-polymerized microtubules. In the third reaction, a very little protein was observed in the pellet fraction, and the great majority of tubulin was in the supernatant fraction. These results indicate that acrylamide inhibited tubulin polymerization when incubated at 37°C, even if GTP was added into reaction mixture later on. In the 4. reaction, very little protein was observed in the pellet fraction, and the great majority of tubulin was in the supernatant fraction. Although GTP was present in the reaction mixture, acrylamide effected tubulin and inhibited polymerization into microtubules. In the 5. reaction, all tubulin proteins were observed in the supernatant fraction indicating that
Acrylamide caused total inhibition of polymerization of microtubules when GTP was incubated with acrylamide and then tubulin was added. The results of 4. and 5. reactions raised a question “Whether or not acrylamide has an effect on GTP?”. Therefore, we examined the effect of acrylamide on GTP by incubating GTP (100 mM) with acrylamide (100 mM) at 37°C, then tubulin was added to the reaction mixtures and incubated at 37°C. This time acrylamide-GTP mixture was diluted (1 mM final acrylamide and GTP concentration) before addition of tubulin to provide minimum or no effect of acrylamide on tubulin (Figure 3.4).

Figure 3.4. Polyacrylamide gel of GTP-ACR interaction reactions.
1, 2, 3: Tubulin standards
4, 5: Control reaction (GTP → Tubulin)
6, 7: Reaction with acrylamide (GTP + Acrylamide → Tubulin)

A decrease in pellet formation was observed in reactions containing acrylamide compared to control reactions. The difference between the two pellet fractions was significant. These results indicated that acrylamide also had an adverse interaction with GTP. We believe that depolymerizing effect was due to acrylamide-GTP interaction rather than acrylamide-tubulin interaction. Because when tubulin was added, acrylamide concentration was too low (1mM). During preliminary experiments, acrylamide did not show any depolymerizing effect or inhibition of polymerization at such low concentrations.

Taxol-stabilized microtubules (TMTs) were also exposed to acrylamide to investigate the level of depolymerization effect of acrylamide. Tubulin proteins were incubated with GTP and taxol (a microtubule polymerizing agent) into reaction tubes to provide microtubule polymerization and stabilization of microtubules. Then, acrylamide was added into one of the reactions and incubated at 37°C. The reaction mixtures were centrifuged. Supernatant and pellet fractions were separated and analyzed on polyacrylamide gel.

TMT experiments were performed with both semi-purified tubulin and purified tubulin which was purchased from Cytoskeleton. In the experiment with pure tubulin
acrylamide showed significant depolymerization effect at 37°C, even though taxol was present (Figure 3.5).

![Figure 3.5. Polyacrylamide gel of the TMT reactions with pure tubulin purchased from Cytoskeleton. 1, 2: Control reaction. 3, 4: Reaction with acrylamide.](image)

In the experiments with semi-purified tubulin, pellet formation in reactions containing acrylamide was significantly less than control reactions indicating that acrylamide showed depolymerizing effect (Figure 3.6).

![Figure 3.6. Polyacrylamide gel of the TMT reactions with semi-purified tubulin. 1, 2: Control reaction. 3, 4: Reactions with acrylamide.](image)

These results indicated that acrylamide caused depolymerization of both purified and semi-purified microtubules. Although in control reactions all tubulin subunits did not polymerize and there were some protein in the supernatant fractions, the difference between the conditions containing acrylamide and no acrylamide was significant indicating partial depolymerization of microtubules due to acrylamide.

### 3.5. Fluorescence Spectrometer

Proteins contain three amino acid residues which may contribute to their ultraviolet fluorescence: tyrosine, tryptophan, and phenylalanine. Protein fluorescence is generally excited at the absorption maximum near 280 nm and fluorescence emission maxima range from 320 to 350 nm. However, emission from phenylalanine is rarely observed because of small yield of phenylalanine in proteins. Therefore, the absorption of proteins at 280 nm is due to both tyrosine and tryptophan residues. At wavelengths longer than 295 nm, the absorption is due primarily to tryptophan.
Tryptophan (intrinsic fluorophore) is the most highly fluorescent amino acid in proteins. The tryptophan residues of proteins generally account for about 90% of the total fluorescence from proteins. This natural fluorophore is highly sensitive to the polarity of its surrounding environment. Frequently, spectral shifts are observed as a result of several phenomena, such as binding of ligands, protein-protein association, and denaturation. Tryptophan fluorescence can be selectively excited at 295 to 305 nm. The emission maximum of tryptophan in water occurs at 336 nm, and is highly dependent on polarity.

There are eight tryptophan residues in a tubulin dimer. Changes in the fluorescence of tryptophan residues can be used to monitor structural transitions. When microtubules are depolymerized, tryptophan residues are exposed to the aqueous solvent. However, polymerization of tubulin dimers causes holding of tryptophan residues within the 3D structure. This structural transition causes an increase in emission spectrum. The fluorescence spectral properties of tubulin (or MTs) show differences in the presence or absence of depolymerizing agents. This illustrates the sensitivity of the spectral properties to the three-dimensional structure of the protein.

In order to monitor effects of acrylamide on microtubule polymerization, we compared fluorescence intensities of tryptophan in the presence or absence of acrylamide with or without GTP. Tryptophan gives maximum emission intensity at 336 nm when excited at 295 nm. These findings were also confirmed in our primary measurements (Figure 3.7).

![Fluorescence Spectrum](image)

**Figure 3.7.** Graphic of fluorescence spectrometer showing maximum emission intensities at 336 nm and exitation at 295 nm.
In the experiments without GTP, acrylamide caused a decrease in the emission spectrum and scattered light increased compared to control reaction. Furthermore, corrected emission values were similar in the absence and presence of acrylamide (Figure 3.8 and Figure 3.9).

**Figure 3.8.** Graphics of fluorescence spectrometer showing emission intensities versus time. In the absence of GTP A) without acrylamide and B) with acrylamide.

**Figure 3.9.** Graphics of fluorescence spectrometer for the reactions with acrylamide in the absence of GTP.
A) Emission intensities versus time.
B) Scattered light versus time.
C) Corrected emission intensities versus time.

In the absence of GTP, the presence of acrylamide caused a significant increase in the scattered light at 295 nm, and emission intensity at 336 nm decreased significantly compared to control reaction. In addition, the difference between the corrected emission intensities was very little that it could be ignored. Corrected emission intensity is obtained by division of emission intensity by scattered light.
Corrected spectra are needed for calculation of quantum yields. Since scattered light is affected by the molecules in the solution, presence of acrylamide might increase the scattered light relatively, and emission intensity might decrease due to hit of molecules with each other hence loss of the energy in the presence of acrylamide. However, when the corrected emission spectra were evaluated, approximately no change was observed due to the absence of polymerization in the absence of GTP.

In the experiments with GTP, acrylamide caused an evident increase in the scattered light, and the emission spectrum decreased significantly compared to control reaction. Furthermore, acrylamide caused a decrease in corrected emission values (Figure 3.10 and Figure 3.11).

![Graphs showing emission intensities versus time.](image)

**Figure 3.10.** Graphics of fluorescence spectrometer showing emission intensities versus time. In the presence of GTP:
A) Without acrylamide
B) With acrylamide

![Graphs showing scattered light, emission intensities, and corrected emission values versus time.](image)

**Figure 3.11.** Graphics of fluorescence spectrometer belonging the reaction with acrylamide in the presence of GTP:
A) Emission intensities versus time.
B) Scattered light versus time.
C) Corrected emission intensities versus time.
In the presence of GTP, acrylamide caused an evident increase in the scattered light which was related to the molecule amount in the solution. However, the reduction in the emission spectrum observed was due to acrylamide. Furthermore, corrected emission value reduced with acrylamide compared to control reaction. Since emission spectrum is indicative of transition into 3D structure, which indicates polymerization, we suggest that acrylamide inhibited the polymerization of tubulin subunits into microtubules. The results without GTP also support this idea, since no polymerization is expected in the absence of GTP.

3.6. Malachite Green Procedure

Highly sensitive methods for phosphate measurement are based on the change in the absorbption spectra of basic dyes upon complex formation with phosphomolybdic heteropolyacid. Malachite green is the most frequently used basic dye, and its reaction with phosphomolybdate results in the appearance of an intense absorbance band at 620-650 nm. Numerous procedures for phosphate measurement with malachite green have been proposed. The most versatile version involve the addition of one color reagent that combines all analytical components. They are, however, less sensitive because of extensive dilution of sample with the color reagent. Much higher sensitivity has been reported for the procedures which involve several pipetting steps because they permit a sample-to-reagent ratio greater than unity. A common drawback of the majority of the published procedures is that they require a filtration step during color reagent preparation because of the low solubility of malachite green.

In 1988, Baykov et al. described a modified malachite green method combining all necessary reagents in one concentrated solution for phosphate determination. In addition to this, in 1991, Geladopoulos et al. modified this method one more time by adding a deproteinization step. We preferred this second modified method because it is highly sensitive, requires only one pipetting step, eliminates the problem of dye solubility and does not cause acid hydrolysis of phosphate groups by using protein stabilization with sodium dodecyl sulfate instead of stabilization with acid solutions. The procedure is based on the finding that the dye is easily soluble and stable in the presence of 6 N acid. The addition of Tween-20 is required to stabilize the dye-phosphomolybdate complex at phosphate concentrations above 10 μM. Proteins
must be removed or stabilized prior to P<sub>i</sub> determination by sodium dodecyl sulfate. The time of color development at 25°C is about 3 minutes.

We used the colorimetric P<sub>i</sub> microarray to determine GTPase activity in microtubule polymerization reactions. Since GTP is hydrolyzed and P<sub>i</sub> is released during microtubule polymerization, we investigated whether there is a difference in released P<sub>i</sub> amount due to the presence of acrylamide.

Firstly, color reagent containing malachite green, sulfuric acid, Tween-20 and ammonium molybdate was prepared. Two reaction mixtures were prepared: one containing GTP, and one containing GTP and acrylamide. Reactions were incubated at 37°C. Then, reaction mixtures were diluted and tubulin was added, then incubated at 37°C. Following deproteinization step with perchloric acid, color reagent was added into tubes and absorbances were measured at 630 nm.

![Phosphate determination by malachite green](image)

**Figure 3.12.** Graphic of phosphate determination using malachite green. First column indicates the absorbance of the control reaction containing GTP and tubulin. Second column indicates the absorbance of the reaction containing GTP, tubulin and acrylamide.

We observed a significant decrease in the absorbance of the reaction containing acrylamide compared to the control reaction (Figure 3.12). Since GTP is hydrolyzed into GDP and P<sub>i</sub> during microtubule polymerization, and the produced P<sub>i</sub> is well-proportioned to the number of tubulin subunits which join to the microtubule structure. These results indicated inhibition of tubulin polymerization into microtubules due to acrylamide. Acrylamide may also have adverse effects on GTP in addition to effects on tubulin. However, further analysis is needed with other GTP-related reactions.
3.7. Electron Microscopy

Electron microscopes have provided a great knowledge about detailed structures of microtubules. To examine the structures that pelleted in the sedimentation assay and fluorescence spectrometer, tubulin samples were observed by Transmission Electron Microscopy (TEM). In this technique, an electron beam passes through the specimen and results in different light/dark patterns on the specimen. The darker areas represent denser electron regions whereas the lighter areas represent the regions with fewer electron density.

In these experiments, 10 μM tubulin or 10 μM TMTs were incubated with 5 mM GTP and 100 mM acrylamide at 37°C for 1 hour. Control reactions were incubated without acrylamide. In electron microscopy experiments, GTP concentration was increased to 5 mM to stabilize microtubules against the harsh steps of the method. Then, reactions were centrifuged and pellet fractions were subjected to gluteraldehyde for 15 minutes. The samples were adsorbed to pioloform coated cupper grids and negatively stained with uranyl acetate.

In the primary experiments without taxol, microtubule structures were hard to observe (perhaps due to harsh conditions). For this reason, we chose to stabilize the microtubule structures with taxol. In the control reactions containing tubulin, GTP and taxol, an abundance of intact microtubules was observed in addition to free tubulin subunits (Figure 3.13).

![Figure 3.13. Transmission electron micrographs of control reaction1; Tubulin + GTP + Taxol. A) 50,000x and B) 100,000x](image)

In the reactions containing taxol-stabilized microtubules and acrylamide, acrylamide changed the microtubule structure. Instead of intact microtubules, relaxed,
disintegrated structures which started to depolymerize at one end were observed (Figure 3.14).

**Figure 3.14.** Transmission electron micrographs of reaction 1a; Tubulin + GTP + Acrylamide + Taxol. A) 25,000x, B) 50,000x, C) 25,000x and D) 50,000x

Taxol binds to tubulin subunits and stabilizes the microtubule structure. Therefore, intact microtubules were observed in the control reaction containing taxol. Furthermore, the presence of taxol resulted in microtubule assembly, and addition of acrylamide modified these microtubule structures such that in their natural structure. Instead, relaxed, disintegrated (dissolved) and curved structures were seen, and, microtubules started to depolymerize at one end (Figure 3.14). These results suggest that acrylamide is a strong depolymerizing agent which can affect even the taxol-stabilized microtubules.
4. CONCLUSION

Since acrylamide is a neurotoxicant disrupting axonal transport in neurons, and microtubules are one of the major elements of axonal transport, in this study, we investigated the effects of acrylamide on microtubule dynamics. Our results showed that acrylamide causes depolymerization of microtubules. Also acrylamide inhibits polymerization of tubulin dimers into microtubules. In addition, our results demonstrated that acrylamide is a strong depolymerizing agent which can depolymerize even the taxol-stabilized microtubules. Acrylamide may also have effects on microtubule polymerization through GTP. However, further analysis is needed with other GTP-related reactions. As a conclusion: one important mechanism of acrylamide’s in exhibiting its neurotoxic effects is by disrupting microtubule dynamics.
REFERENCES


RESUME

Ceren EKE KOYUNCU was born in Kadıköy in 1980. After getting her high school diploma from Pendik High School in 1998, she continued her undergraduate degree at Istanbul University, Cerrahpasa Faculty of Medicine, Department of Biomedical Sciences in 1998. She had her Bachelor’s degree in 2002. She continued to her graduate studies at Advanced Technologies in Molecular Biology – Genetics and Biotechnology program. She has been also working as a research assistant in Department of Molecular Biology and Genetics. She is still pursuing her studies in the same department.