Microtubules in the ovarioles of *Notonecta* and *Oncopeltus*

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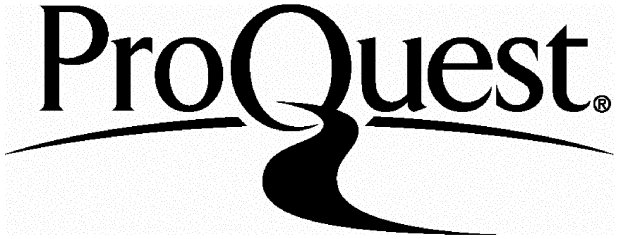

For my family, English and American

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ABSTRACT

Antibodies specific for tyrosinated, detyrosinated and acetylated tubulin were used to investigate the content and distribution of post-translational modifications of tubulin in the ovarioles of *Notonecta glauca glauca* and *Oncopeltus fasciatus*. Furthermore, antibodies were raised against tubulin purified from *Notonecta* ovarioles and characterised on a number of different cellular systems.

Using one- and two-dimensional polyacrylamide gel electrophoresis and Western blotting, ovariole extracts were probed with the isotype specific antibodies. This revealed a major and a minor α-tubulin species in *Notonecta*, both of which were detyrosinated and acetylated. In contrast, *Oncopeltus* had only one major α-tubulin species which was tyrosinated.

The *in situ* localisation of tyrosinated tubulin in the nutritive tubes of *Notonecta* ovarioles revealed a restricted distribution at the inner edge of the periphery of the functional tubes only, this distribution suggesting a novel model by which microtubules are inserted and grow in maturing tubes. Detyrosinated and acetylated tubulin were more widespread, having distributions in both the functional and redundant tubes. In *Oncopeltus*, tyrosinated tubulin could be visualised in all nutritive tubes, detyrosinated and acetylated tubulin being almost totally absent. This lack of post-translational modifications in *Oncopeltus* was shown to be due to either the lack or near total inactivation of the enzymatic machinery.

The production of monoclonal antibodies against tubulin purified from *Notonecta* ovarioles was a constantly evolving process, each change producing improved results. In the final production one cloned serum contained antibodies specific for a 114kD epitope, suggesting the presence of a kinesin specific antibody, while 4 sera recognised a 54kD epitope, suggesting the presence of α-tubulin specific antibodies. Further characterisation of the sera produced confusing results which suggested the presence of several different antibody producing

hybridomas in each clone and, hence, a conclusive analysis of the antibody content of the sera could not be reached.

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ABBREVIATIONS

1-D PAGE	one-dimensional polyacrylamide gel electrophoresis
2-D PAGE	two-dimensional polyacrylamide gel electrophoresis
β/IC1	β-heavy chain intermediate
AMP. PNP	5' adenylymido diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BAME	Nα-benzoyl-L-arginine methyl ester
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complimentary DNA
CHO-cell	Chinese hamster ovary cell
CPA	carboxypeptidase A
C-terminus	carboxyl-terminus
DAPI	4', 6-diaminodiamidino-2-phenylindole
DMEM	Dulbeco's modifcation of Eagle's medium
DMSO	dimethysulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra acetic acid
EGTA	ethyleneglycol-bis-(β aminoethyl ether) N N N' N'-tetraacetic acid
ER	endoplasmic reticulum
FCS	foetal calf serum
GDP	guanosine diphosphate
glu	detyrosinated
GTP	guanosine triphosphate
HC	heavy chain
IEF	isoelectric focussing
KLH	keyhole limpet haemocyanin
LC	light chain
MAP	microtubule-associated protein
MDCK cells	Madin-Darby kidney cells
mRNA	messenger RNA
MT	microtubule
MTOC	microtubule organising centre

NGF	nerve growth factor
N-terminus	amino-terminus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PIPES	piperazine-N, N'-bis-(2-ethanesulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
STOP	stable tubule only polypeptide
TAME	α-N-toluene *p*-sulphonyl-L-arganine methylester hydrochloride
TBS	Tris buffered saline
TEMED	N N N' N'-tetramethylethylene diamine
TPCK	L (1-tosylamido-2 phenyl)-ethyl chloromethylketone
Tris	Tris (hydroxymethyl) aminomethane
tyr	tyrosinated
UCL	University College London

PUBLICATIONS

HARRISON, A. & HYAMS, J. S. (1990). Mapping MAP-2. *J. Cell Sci.* **96**: 347-349.

HARRISON, A., STEBBINGS, H., & HYAMS, J. S. (1991). Differences in α-tubulin isotype usage in the nutritive tubes of two insects. *J. Cell Biol.* **111**: 411a.

HARRISON, A., STEBBINGS, H., & HYAMS, J. S. (1991). Different patterns of α-tubulin post-translational modification in ovarian nutritive tubes of two hemipteran insects. *J. Cell Sci.* **100**: 501-507.

1.1: THE CYTOSKELETON

The cytoskeleton is of major importance to many vital aspects of eukaryotic cell function. It provides both a structural framework around which cell morphology is maintained and altered, is largely responsible for the successful completion of mitosis and has important functions in intracellular motility. These characteristics are dependent on the cytoskeleton's ability to maintain a stable structure that, nevertheless, has the potential to undergo very rapid re-organisations. The onus for such a demanding task falls on three main, highly conserved, components: actin microfilaments, intermediate filaments and microtubules (Schliwa, 1986).

1.1I: Microtubules

Microtubules are a universal component of eukaryotic cells. They have many functions, ranging from a relatively passive structural role to their involvement in dynamic mechanisms such as intracellular transport and the movement of cilia and flagella. They also play a very important role in mitosis and meiosis as they are the major structural component of the spindle (Weber *et al*, 1975; Brinkley *et al*, 1975).

1.1Ia: Microtubule Structure

Microtubules consist of regular helical assemblies of heterodimers of two slightly elongated protein subunits, tubulins α and β, each having a molecular weight of around 50kD. The basic structure of a microtubule is that of a hollow cylinder with a diameter of 25nm comprised of parallel, longitudinally arranged protofilaments each consisting of a row of tubulin dimers. Microtubules generally contain 13 protofilaments and within the polymer adjacent dimers are slightly displaced relative to each other and thus describe a three start left handed helix with a pitch of approximately 10 degrees (Amos, 1979; Figure 1).

Proteolytic cleavage and subsequent formaldehyde treatment of the α and β sub-units revealed the nature of their relationship (Serrano and Avila, 1985). Within the dimer, the amino (N)-terminus of α-tubulin

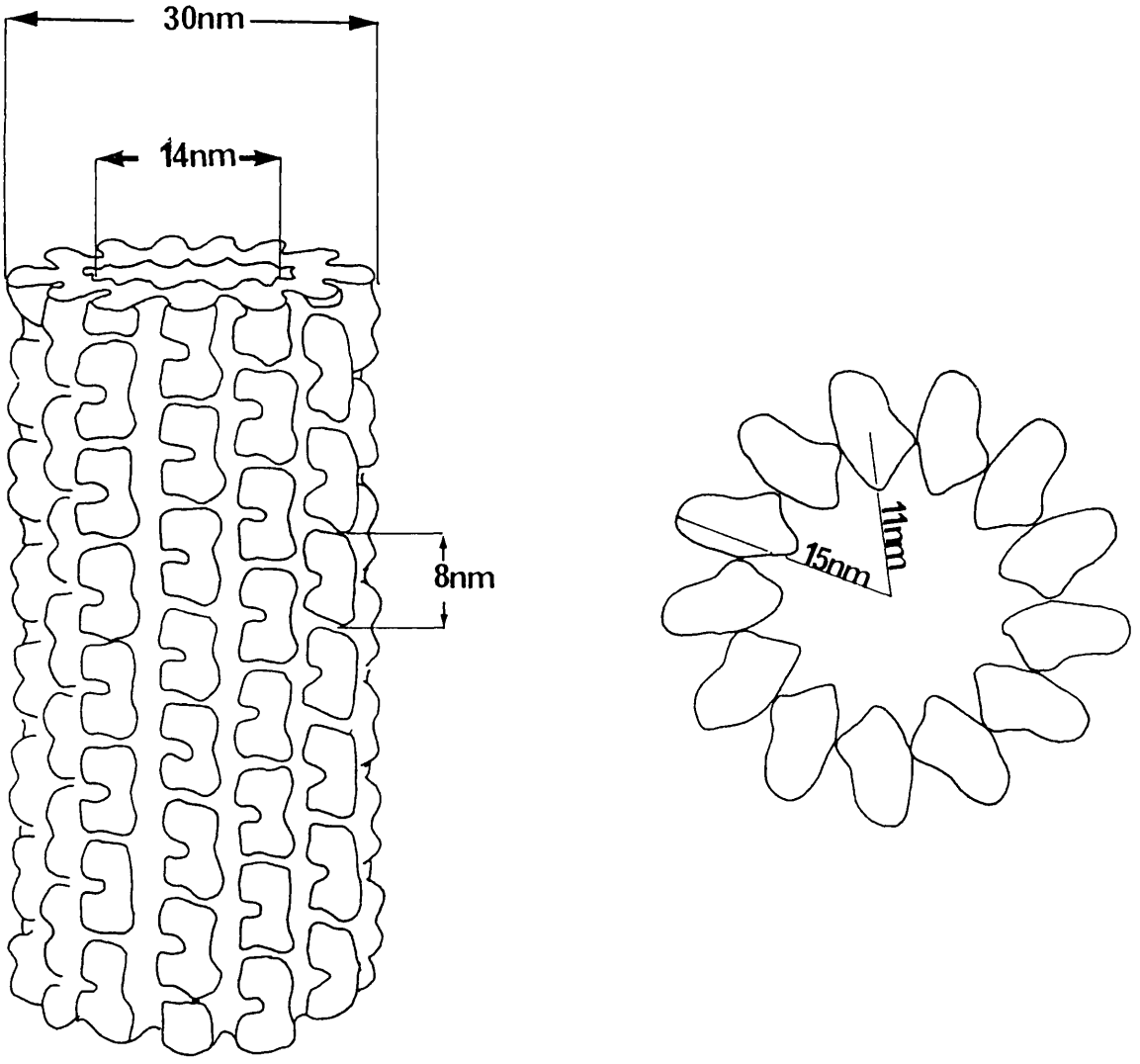


Figure 1 The Fine Structure of a Microtubule

Detailed structure of a microtubule showing its general dimensions and the interaction between α and β-tubulin dimers in the makeup of the structure (re-drawn from Amos, 1979)

binds the carboxyl (C)-terminus of β-tubulin. This contrasts with the interdimer bond in the protofilament which occurs between the C-terminus of α-tubulin and the N-terminus of β-tubulin (Kirchner and Mandelkow, 1985).

1.1Ib: Microtubule Assembly and Dynamics

Microtubules can be purified from a suitable source, such as bovine brain, relatively simply. Microtubules exposed to cold or high calcium concentrations depolymerise while at 37°C and, in the presence of a calcium chelator, GTP and magnesium, microtubule polymerisation occurs. These properties form the basis of an easy, cyclical, method of microtubule purification (Weisenberg, 1972; Olmsted and Borisy, 1975; Vallee, 1986).

Each tubulin dimer has one high affinity binding site and several low affinity sites for calcium (Solomon, 1977), the calcium acting both directly on tubulin and via the effect of calmodulin, initiating the endwise depolymerisation of microtubules (Karr *et al*, 1980). The control of calcium levels *in vivo* thus has important repercussions for microtubule distribution and reorganisation within the cell. This is particularly apparent during mitosis where sudden increases in concentration occur at the metaphase-anaphase transition, this possibly controlling chromosome movement (Poenie *et al*, 1986; Zhang *et al*, 1992). The exact effect it has on spindle depolymerisation has yet to be ascertained but it is supported by the observation that calcium sequestering vesicles co-localise with the mitotic apparatus (Harris, 1975; Silver *et al*, 1980; for a reveiw see Hepler, 1989). The levels measured are, however, too low to suggest calcium is contributing to microtubule depolymerisation (Poenie *et al*, 1986) though the effect could be modulated by calmodulin. If calmodulin is saturated with calcium, local disassembly of microtubules occurs at *in vivo* calcium levels in cultured cells (Tash *et al*, 1980; Keith *et al*, 1983), this restricted depolymerisation possibly being involved in selectively destabilising kinetochore microtubules during anaphase (Ratan and Shelanski, 1986; Berkowitz and Wolf, 1981; Harris, 1975; Silver *et al*, 1980; Zhang *et al*, 1992 for a review see Hepler, 1989). The effect of

calmodulin on the calcium depolymerisation of microtubules is also heightened by microtubule-associated proteins (MAPs). In their absence, calmodulin sequesters calcium allowing optimal microtubule polymerisation while MAPs, particularly tau, bind a calcium-calmodulin complex thus allowing depolymerisation to occur (Lee and Wolff, 1982; 1984a; 1984b). Additionally, MAP-2, which promotes microtubule assembly (see Section 1.1IIb), is prefentially digested by calcium activated proteases which could lead to a further inhibition of microtubule polymerisation (Koszka *et al*, 1985).

Microtubule assembly also has a requirement for GTP, optimal microtubule polymerisation occuring in the presence of stochiometric levels of hydrolizable GTP (Weisenberg *et al*, 1968; Gaskin *et al*, 1974; Olmsted and Borisy, 1975). Tubulin dimers associated with a non-hydrolysable GTP analogue incorporate into microtubules at a much lower rate than those associated with GTP, producing shorter, more numerous microtubules (Terry and Purich, 1980). One GTP is hydrolysed per molecule of tubulin incorporated into a microtubule, the GTP being exchangeable until after hydrolysis, the GDP subsequently being "locked into" the microtubule (Carlier and Pantaloni, 1981). This process is not, however, directly coupled to polymerisation, so that under conditions where polymerisation occurs at a faster rate than GTP hydrolysis, a stabilising 'cap' of GTP-tubulin can form at the end of the microtubule (Carlier and Pantaloni, 1981; Carlier *et al*, 1984; Chen and Hill, 1985).

A great aid to microtubule purification is the plant alkaloid taxol, which, due to a massive reduction in the critical concentration for free assembly, promotes microtubule assembly, produces stable bundles and prevents disassembly both *in vivo* and *in vitro* (Schiff *et al*, 1979; Schiff and Horowitz, 1980). This effectively prevents centrosome mediated nucleation by reducing the critical concentration of tubulin for microtubule assembly below that which can be utilised by the centrosomes (De Brabander *et al*, 1981). Taxol can also cause microtubules to spontaneously assemble into unstable asters in mitotic cells (Verde *et al*, 1991) and non-fertilised sea-urchin eggs (Schatten

et al, 1982), the former occuring in the prescence of dynein. It appears that the action of taxol is mediated by an additional ATP inhibited factor that does not recycle with tubulin but is retained in stabilised microtubules (Turner and Margolis, 1984). Treatment with a combination of cold temperature and high calcium concentration allows taxol to be released from microtubules thus reversing its effect, the recovered tubulin only reassembling under normal microtubule polymerising conditions (Collins and Vallee, 1987).

Cellular microtubules generally exist as dynamic structures. This is important as they must quickly disassemble and reassemble as part of numerous functions which include the rearrangement of the interphase cytoskeleton prior to mitosis and the microtubule mediated poleward movement of chromosomes during anaphase. An essential part of this is the need for the microtubules to have a dynamic relationship with free tubulin subunits so that the transitions can be very rapid when required. Experimental evidence has indicated the existence of two types of dynamic microtubule, the first of which is treadmilling.

The concept of treadmilling of subunits from one end of the microtubule to the other was developed as a result of the observations that a microtubule becomes progressively saturated with ^{3}H-GTP labelled tubulin, the rate of label loss during pulse chasing being dependent on the length of microtubules and the number of ends present. This hypothesis was further supported by the observation that an end blocking drug inhibited microtubule assembly but not disassembly (Margolis and Wilson, 1978). A requirement of treadmilling is that the two ends of a microtubule maintain differential critical concentrations for assembly. This seems to be dependent on the energy produced by GTP hydrolysis (Cote *et al*, 1980; Terry and Purich, 1980; Kirschner, 1980; Margolis, 1981). It may also have a structural function. GTP is bound to the β-tubulin subunit which is only exposed at one end of a microtubule, therefore GTP hydrolysis, and hence assembly, may only take place at the β-tubulin end (Carlier and Pantaloni, 1981). A mechanism for microtubule growth was suggested in which a microtubule forms by rapid polymerisation during which free exchange can occur at both the + and

the - ends, followed by a switch to treadmilling. What causes this switch is not known but it may involve a capping mechanism (Karr and Purich, 1979). In general microtubule dynamics are not well understood, as reversible equilibrium reactions, leading to assembly and disassembly, occur at both ends, increases in the free tubulin concentration slowing subunit loss from the - end (Farrell *et al*, 1979). Treadmilling has been implicated in mitotic chromosome movement as well as intercellular and slow axonal transport but in all cases the slow rate of subunit exchange is apparent (Rothwell *et al*, 1985). This may be due to differences between the *in vitro* and *in vivo* systems. For example, in interpreting these results the condition of the nucleotides must be taken into account, increasing levels of ATP speeding up the process and treadmilling not occurring in the absence of hydrolisable GTP (Margolis, 1981; Margolis and Wilson, 1979; 1981). If phosphorylated MAPs are added to microtubules *in vitro* there is an increase in the rate of treadmilling due to enhanced assembly and disassembly at the appropriate ends. Unphosphorylated MAPs, however, have no effect suggesting the importance of a MAP associated ATP-dependent microtubule protein kinase, a point overlooked in most early work (Jameson and Caplow, 1981). It is also important to make the distinction between *in vitro* treadmilling and the *in vivo* situation in which microtubules are generally anchored at one end. This should prevent treadmilling from occurring though treadmilling, coupled with GTP hydrolysis, may cause selective stabilisation of such anchored microtubules thus preventing spontaneous polymerisation of microtubules (Kirschner, 1980). The treadmilling model thus has failings so the second model of dynamic instability is favoured.

The greatest insight into microtubule dynamics was afforded by the discoveries of Mitchison and Kirschner (1984a; b). Work by Summers and Kirschner (1979) showed that opposite ends of a microtubule have differential rates of polymerisation and depolymerisation, polymerisation being higher at the + end. This led to the development of the dynamic instability model (Mitchison and Kirschner, 1984a; b). Microtubules assembled from pure tubulin and diluted below the steady-state concentration exist as two populations, some growing and some

shrinking, the ratio of the two being dependent on the tubulin concentration. There is a net loss of polymer mass, the microtubules depolymerising at random and depolymerisation, once initiated, continuing to completion. Increasing the tubulin concentration above the steady-state level produces increases in length of a constant number of microtubules leading to a net gain in polymer mass. At steady state there is a coexistance of the two phases, the majority of microtubules growing slowly being balanced by a minority shrinking quickly, the polymer concentration remaining constant. The growing microtubules have the same small critical concentration for assembly at the two ends. Subsequent work by Walker *et al* (1988) found that over a wide range of tubulin concentrations microtubules grow continuously, the transit to catastrophe, a phase of rapid shortening being a rare event that only occurs at low tubulin concentrations, while rescue, a shrinking microtubule end moving to the growing phase, can occur over a wide range of concentrations. Those microtubules which do not depolymerise to completion must be capped at some point to produce long term stability. Mitchison and Kirschner (1984b) suggested this is due to the prescence of a stable GTP cap. If a drop in free tubulin decreases the cap size the probability of GDP subunits being exposed increases. As they have a low affinity for the GDP lattice they will be rapidly lost and more GDP subunits exposed propagating further depolymerisation until the microtubule disappears or is recapped.

Dynamic instability has been demonstrated many times *in vivo* and *in vitro* (Schulze and Kirschner, 1986; 1987; Wadsworth and Salmon, 1986; Sammak *et al*, 1987; Sammak and Borisy, 1988a; b; Tao *et al*, 1988; Cassimeris *et al*, 1988) leading to a revaluation of the original model. When observed *in vitro* using dark-field microscopy the growing and shortening phase alternated frequently in a stochastic manner (Horio and Hotani, 1986). This questions the GTP cap hypothesis as the ends can interconvert frequently between the two phases without a change in the polymerisation rate. The weakness of the GTP cap model is also revealed if microtubules are transected in areas presumed to be composed entirely of GDP-tubulin, no catastrophic disassembly occuring, suggesting GTP hydrolysis is not necessary for rapid disassembly (Tao *et al*, 1988;

Belmont *et al*, 1990). Furthermore microtubule replacement has been found to be faster at the leading edge of the cell as opposed to its centre which led to the idea of tempered instability, whereby only limited disassembly occurs before rescue (Sammak *et al*, 1987; Sammak and Borisy, 1988a; b; Edson *et al*, 1993). Tempered instability may predominate as a result of plugs of aggregated tubulin in the lumens of microtubules preventing total depolymerisation by slowing the rate of disassembly and allowing the re-establishment of the GTP cap (Azhar and Murphy, 1990). In interphase and mitotic *Xenopus* oocyte extracts the rates of polymerisation and depolymerisation are similar, the frequency of catastrophe being increased in the mitotic extracts. This difference could be due to a post-translational modification in mitotic cells such as the phosphorylation of MAPs (Gard and Kirschner, 1987a; b), or an increase in microtubule capping structures like the GTP cap (Belmont *et al*, 1990). Microtubule dynamics also differ greatly between non-confluent and confluent epithelial cells, the latter's microtubules being stabilised, after the establishment of cell-cell contacts which suggests the microtubules are capped when this happens (Pepperkok *et al*, 1990; Nagasaki *et al*, 1992).

The conflict between the models of dynamic instability and treadmillimg was enhanced by the finding that with MAP free microtubules, the frequencies of rescue and catastrophe are greater at the - and + ends respectively (Walker *et al*, 1988). These results also conflict with the GTP cap model: the frequency of catastrophe is not dependent on the elongation rate and the GTP-tubulin dissociation rate is much higher than in the GTP cap model (Walker *et al*, 1988). A model was developed whereby hydrolysis only occurs when a GTP-tubulin subunit becomes buried in the polymer, catastrophe only occuring when all 13 subunits are hydrolysed. This allows the remaining GTP-tubulin subunits to dissociate without immediate cap loss and rescue occurs with the addition of a single GTP-tubulin subunit onto a rapidly shortening GDP-tubulin microtubule (Walker *et al*, 1988; Caplow *et al*, 1985). The work of Walker *et al* (1988) also went some way to resolving the conflict between the models of dynamic instability and treadmilling, the rates of association and dissociation of dynamic instability producing

treadmilling through a steady state microtubule. If GTP-tubulin subunits can dissociate during an elongation phase, the critical concentration at each end must be equal. This means that there can be two alternating phases of assembly and disassembly at each end of a microtubule, the differing frequencies of interconversion between these phases resulting in treadmilling (Walker *et al*, 1988).

The concept of dynamic instability cannot, however, be thought of purely in terms of distinct sub-populations of microtubules growing and shortening as microtubule annealing could be responsible for sudden increases in polymer length (Rothwell *et al*, 1987). The influence of various other factors in the modulation of dynamic instability should also not be overlooked. Already stable microtubules shrink at a slower rate than microtubules that have previously undergone catastrophe while microtubules show different levels of stability in different parts of a cell. Growing microtubules also tend to follow 'tracks' that were previously occupied by other microtubules and shrinking microtubules can be consistently rescued at precise points. This all indicates that associated proteins, post-translational modifications and actual physical structures in the cytoplasm modulate dynamic instability (Schulze and Kirschner, 1988). This becomes apparent when MAP-2, tau or a mixture of heat stable MAPs are added to an *in vitro* assay for dynein instability, the MAPs increasing the elongation rate and frequency rescue while reducing the shortening rate and frequency of catastrophe (Pryer *et al*, 1992). Furthermore, in *Xenopus* oocytes + end assembly is inhibited while in eggs it is specifically promoted by a MAP, XMAP, in its phosphorylated form (Gard and Kirschner, 1987a; b). It is thus a candidate for a controlling protein which would have a profound effect in modulating dynamic instability and/or treadmilling. A factor has also been identified in *Xenopus* egg extracts which severs stable microtubules, increasing the number of ends available to undergo dynamic instability. This would be particularly relevant during the major depolymerisation of interphase microtubules prior to mitosis, the increased number of ends allowing catastrophe to occur much more efficiently (Vale, 1991).

It thus seems that the primary reason why dynamic instability exists is so a cell can have a polymer with inbuilt instability. Such a polymer would grow rapidly before being stabilised by a number of yet unconfirmed mechanisms yet could also shrink rapidly when needed. This would be important when reorientating interphase microtubules during cellular locomotion or during the rearrangement of interphase microtubules at mitosis. Schulze and Kirschner (1987) also suggest that differential stability allows time for post-translational modifications (see Section 1.1Ieii) to occur which gives the stable microtubules a futher differentiated state. As the two ends of a microtubule have differing rates of assembly and disassembly a cell could also distinguish between two sets of microtubules purely by their polarity.

Although most cellular microtubules exhibit the dynamic characteristics outlined above there are subsets that have enhanced cold and drug stability (Webb and Wilson, 1980; Cassimeris *et al*, 1988; Schulze and Kirschner, 1987). It generally seems that, apart from Antarctic fish tubulin which has a modified coding sequence and no apparent stabilising factors (Detrich and Parker, 1993; Williams and Correia, 1985; Stromberg *et al*, 1986), cold stability results from factors incorporated into microtubules during assembly rather than being an inherent property of the tubulin itself (Webb and Wilson, 1980). This is apparent in the microtubules of sea urchin sperm flagella which are stabilised against cold and calcium treatment by histone H1 (Multigner, 1992). The phosphorylation state of proteins associated with cold stable microtubules is also important, a 'switch protein' being dephosphorylated in cold stable microtubules (Margolis and Rauch, 1981; Job *et al*, 1982). *In vitro*, phosphorylated MAP-1b is enriched in cold stable microtubules which suggests that the phosphorylated protein is stabilising the microtubules (Diaz-Nido, *et al*, 1988). A group of 'stable tubule only polypeptides' (STOPs), that preferentially associate with kinetochore microtubules, the most stable of the mitotic microtubules, co-purify substoichiometrically with cold stable microtubules and are found to be randomly incorporated into the polymer. If the STOPs are removed the microtubules lose their cold stability (Margolis *et al*, 1986). The other well studied group of stable

cytoplasmic microtubules are the post-translational modified microtubules which are discussed in detail in Section 1.1Ieii.

1.1Ic: Microtubule Organising Centres

Microtubule initiation occurs *in vivo* at specific structures called microtubule organising centres (MTOCs) which include mammalian centrosomes and the spindle pole bodies of fungi. Centrosomes are the major MTOCs of vertebrate cells and are composed of a pair of centrioles surrounded by diffuse, osmiophilic pericentriolar material (Peterson and Berns, 1980; McIntosh, 1983; Brinkley, 1985; Karsenti and Maro, 1986; Kalt and Schliwa, 1993). Both components efficiently nucleate microtubules, if the correct conditions prevail (McGill and Brinkley, 1975; Gould and Borisy, 1977; Roobol *et al*, 1982; Schatten *et al*, 1992; Ohta *et al*, 1993), but pericentriolar material seems to be the predominant microtubule organising region, selective destruction of PtK_2 cell centrioles having no effect on microtubule organisation or the passage through mitosis (Berns and Richardson, 1977). Supporting the dominant role of pericentriolar material in microtubule organisation is the observation that plant cells exlusively order their microtubules from pericentriolar material (Clayton *et al*, 1985). γ-tubulin has also recently been discovered closely associated with centrosomes, pericentriolar material, basal bodies and spindle poles where it is suggested to be a universal microtubule organiser (Raff *et al*, 1993; Julian *et al*, 1993; Zheng *et al*, 1991; Stearns *et al*, 1991; Horio *et al*, 1991; Joshi *et al*, 1992; Baas and Joshi, 1992; Liu *et al*, 1993; Palacios *et al*, 1993; Muresan *et al*, 1993; Gueth-Hallonet *et al*, 1993). This could be of great importance in defining microtubule polarity, its interaction with β-tubulin at the centrosome meaning that α-tubulin will be at the distal ends of the microtubule (Oakley *et al*, 1990).

As well as acting as a focus for microtubule growth the centrosome determines microtubule organisation. In tubulin-depleted cultured cells exogenous tubulin assembles into microtubules, the length, number and distribution of which is determined by the centrosome so that the number of microtubules produced reaches a plateau at high tubulin concentrations (Tucker, 1977). The multinucleating element hypothesis

suggests that there are different types of nucleating element which are individually controlled by differing combinations of tubulin and MAPs (Tucker, 1984), control mechanisms mediated by both capping proteins and the phosphorylation of MAPs being undoubtedly important (Brinkley *et al*, 1981), an increasing MAP:tubulin concentration increasing the number and length of microtubules nucleated (Bre and Karsenti, 1990). Centrosomes also act to modify microtubule dynamics and structure, the capture of one end (- end) of a microtubule allowing assembly to occur only at the distal end (+ end), so all microtubules are orientated with their assembling ends distal (Bergen *et al*, 1980; Peterson and Berns, 1979; McIntosh, 1983; Bergen and Borisy, 1980; Brinkley, 1985). Additionally brain microtubules self assembled *in vitro* have predominantly 14 protofilaments while microtubules nucleated from centrosomes only have 13 (Evans *et al*, 1985). The centrosome is thus controlling protofilament number, possibly due to the pericentriolar material attaining a level of order that allows it to both seed and control the morphology of the microtubules (Karsenti and Maro, 1986).

Despite the above properties, the full centrosomal structure is not necessary for microtubule nucleation and organisation in some systems, including melanophore arms (McNiven *et al*, 1984; McNiven and Porter, 1988) and avian erythrocytes (Swan and Solomon, 1984). The wing epidermal cells of *Drosophila* comprise one of the best characterised systems whereby large microtubule bundles assemble after the centrosomes are lost (Tucker *et al*, 1986). The bundles associate with small dense plaque-like sites at the plasma membrane, the microtubules radiating out with their + ends distal (Mogensen and Tucker, 1987; Mogensen *et al*, 1989). Interestingly, the microtubules in these bundles predominantly have 15 protofilaments, 13 protofilament microtubules forming only if the wing cells are treated with taxol before nucleation. Thus there is overiding of a non-centrosomal mediated control mechanism specifying 15 protofilaments (Mogensen and Tucker, 1990). Additionally, in axons new microtubules are nucleated by stable, post-translationally modified seeds, at their - ends, the use of existing microtubules as seeds maintaining the correct microtubule polarity (Okabe and Hirokawa, 1988; 1990; Baas and Black, 1990), while in Madin-Darby canine kidney (MDCK)

cells non-centriole associated microtubules are stable and detyrosinated (see Section 1.1Ieii). Factors in the cytoplasm could prevent the total depolymerisation of microtubules, the remaining fragments nucleating microtubules in a similar manner to centrosomes (Bre *et al*, 1987). Despite many examples of spontaneous nucleation the control mechanisms are not clear, though, *in vitro*, phosphorylated MAP-1b is enriched in polymers that occur early in the polymerisation process suggesting MAP-1b phosphorylation favours non-centrosomal microtubule nucleation *in vivo* (Diaz-Nido *et al*, 1988).

Kinetochores, specialised structures at the centromere of metaphase chromosomes, can be induced both *in vivo* and *in vitro* to nucleate microtubules at high tubulin concentrations, tightly bound tubulin possibly acting as seeds (McGill and Brinkley, 1975; Mitchison and Kirschner, 1985a; Rieder, 1982). This does not, however, seem to be the true *in vivo* mechanism, the kinetochore microtubule arrangement arising due to capture of potential spindle microtubules rather than nucleation (Euteneur and McIntosh, 1981; Mitchison and Kirschner, 1985b; Rieder *et al*, 1990; Hayden *et al*, 1990). Both + and - ends of a microtubule can be captured but the - ends dissociate at a faster rate so, at steady-state the + ends predominate (Huitorel and Kirschner, 1988). The process of capture stabilises the kinetochore microtubule from depolymerisation and allows ATP- dependent 'proximal assembly' to occur at the kinetochore which translocates away from the point of assembly due to its ATPase activity interacting with the surface of the stationary microtubule (Mitchison and Kirschner, 1985b; Cassimeris *et al*, 1990). Kinetochores have also been shown to alter the transition rates of dynamic instability, the frequency of catastrophe increasing possibly due to selective depolymerisation of kinetochore microtubules during anaphase (Hyman and Mitchison, 1990). This only occurs when the kinetochore can follow the shrinking end poleward, AMP.PNP inhibiting the rate of catastrophe, suggesting a kinetochore ATPase may modulate the transition rates, the kinetochore stabilising the microtubule until such movement can occur.

The involvement of the kinetochore in mitosis was demonstrated by

Gorbsky *et al* (1987; 1988), Nicklas (1989) and Cassimeris and Salmon (1991) who showed its ability to move along an anaphase microtubule, the microtubule disassembling as it progresses. Rieder *et al* (1990) also demonstrated this, though they could find no attendant microtubule depolymerisation. A number of kinetochore proteins have subsequently been found, one of which has the same molecular weight as the microtubule motor protein kinesin (Balczon and Brinkley, 1987). This would allow the kinetochores to move from the poles to the metaphase plate in prometaphase, the microtubule passing through a collar in the kinetochore leaving the end free to disassemble as the chromosome returns poleward (Hill, 1985; Mitchison and Kirschner, 1985b; Simerly *et al*, 1990; Huitorel and Kirschner, 1988; Rieder *et al*, 1990; Cassimeris *et al*, 1990). The collar model seems very persausive as it allows the kinetochore to maintain almost constant contact with the microtubule thus preventing dissociation (Cassimeris *et al*, 1990; Nicklas, 1989). The presence of a kinetochore ATPase is also important, anaphase A being blocked by ATP removal or by the nonhydrolysable analog 5'adenylylimido diphosphate (AMP.PNP). If ATP is replaced by ATP-γ-S which supports phosphorylation but not the reactivation of microtubule motors, anaphase A occurs as normal suggesting the requirement for ATP may relate to phosphorylation rather than the need for an energy source. Cold and calcium treatment, in the absence of ATP, also reactivates anaphase A suggesting the energy needed to move the chromosomes is stored in the spindle microtubules (Spurck and Pickett-Heaps, 1987).

1.1Id: Autoregulation of Tubulin Synthesis

Tubulin gene expression *in vivo* is controlled by a novel autoregulation mechanism whereby the free tubulin concentration feeds back to alter mRNA levels. If the cellular concentration of free tubulin is increased by microinjection, colchicine treatment or transformation with additional tubulin genes then tubulin synthesis ceases (Cleveland *et al*, 1983; Ben-Ze'ev *et al*, 1979; Caron *et al*, 1985; May *et al*, 1990). Conversely, if vinblastine, taxol or MAPs such as tau which sequester free tubulin are used, there is a corresponding increase in tubulin synthesis (Ben-Ze'ev *et al*, 1979; Kanai *et al*, 1989). These changes in

tubulin synthesis are related to changes in the tubulin's mRNA level which is very sensitive to the tubulin pool (Cleveland *et al*, 1983). If inhibitors are used to block protein synthesis by removing the mRNA from polyribosomes or β-tubulin mRNA is truncated so it cannot be ribosomally associated, the tubulin subunit concentration has no effect on the level of synthesis. Conversely if cyclohexamide, which traps mRNAs onto stalled polyribosomes, is used the autoregulation mechanism persists. In conjunction with this there is an exclusive reduction in the tubulin-mRNA associated with the polyribosomes after colchicine treatment. The polyribosome bound mRNA is thus important for the autoregulatory mechanism (Pachter *et al*, 1987). Transfection with truncated β-tubulin genes showed that the minimum sequence needed for autoregulation to occur is encoded in the N-terminal 4 amino acids (Yen *et al*, 1988a). The importance of this sequence is apparent as it is conserved throughout evolution, confering autoregulation in every instance and, if disrupted autoregulation is lost. The RNA thus needs to be intact for autoregulation to occur but only to the point that it produces the β-tubulin with the correct recognition sequence present (Yen *et al*, 1988b). Autoregulation thus seems to occur through the free tubulin subunits acting on the nascent polypeptide as it emerges from the polyribosome. Possibly this activates a cellular RNase or stalls the ribosome, leaving the RNA exposed to non-specific RNases. This would be particularly pertinent as tubulin-mRNA has a relatively short half life compared to other cellular mRNAs (Ben-Ze'ev, 1979).

1.1Ie: Tubulin heterogeneity

Although in simple eukaryotics all cellular microtubules can be assembled from a single tubulin species (eg yeast has a single β-tubulin, Neff *et al*, 1983), as a rule higher organisms possess multiple tubulin genes. A single microtubule can thus contain a number of distinct tubulin isotypes. This genetic diversity is further compounded by post-translational modifications of the tubulin (Sullivan and Cleveland, 1986).

1.1Iei: The genetic diversity of tubulin

Vertebrates possess, on average, 6 functional α- and 6 functional β-tubulin genes (Cleveland, 1987) while a seemingly ubiquitous third member of the tubulin superfamily, γ-tubulin, has recently been found in a wide range of organisms ranging from fission yeast to *Homo sapiens* (Oakley and Oakley, 1989; Zheng *et al*, 1991; Stearns *et al*, 1991; Horio *et al*, 1991). Mouse has multiple and distinct α and β-tubulin genes which are expressed at different points in development suggesting functional specificity is occurring (Natzle and McCarthy, 1984; Bialojan *et al*, 1984; Lewis *et al*, 1985; Villasante *et al*, 1986). Similarly, in rat PC-12 cells and embryonic developing neurites multiple β-tubulin isotypes are expressed but only a restricted number are used to assemble neurite microtubules during neurite outgrowth suggesting there may be a specific requirement for a particular isotype for neurite extension (Miller *et al*, 1987; Joshi and Cleveland, 1989). In *Drosphila*, a divergent β-tubulin isotype only expressed in a subset of non-germline cells during development can assemble into just one class of microtubule when expressed in the testes in the place of the normal isoform (Hoyle and Raff, 1990).

In contrast, multiple tubulins may be functionally equivalent. This multifunctionality oocurs in the spermatocytes of developing *Drosophila* and in the ciliated protzoan *Tetrahymena* where several classes of microtubule are constructed from β-tubulins coded for by a single gene (Kemphues *et al*, 1982; Gaertig *et al*, 1993) while in cultured cells multiple functional β-tubulin genes, though differing in their C-termini, were found to assemble as co-polymers into microtubules (Lewis *et al*, 1987; Lopata and Cleveland, 1987; Gu *et al*, 1988). The functional equivalence of tubulin is also shown in *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* and *Aspergillus nidulans*, the life cycle proceeding as normal after the disruption of an α-tubulin gene due to the increased synthesis of other α-tubulin gene products (Adachi *et al*, 1986; Schatz *et al*, 1986; May, 1989). Transfection of 3T3 cells and *Aspergillus* with chimeric β-tubulin genes and mixing purified tubulin from chicken brain and erythrocytes with distinct assembly properties produces totally functional co-polymer microtubules (Bond *et al*, 1986;

May, 1989; Baker *et al*, 1990). A β-tubulin isotype in mouse and chicken which has a divergent amino acid sequence is only expressed at low levels in haematopoietic tissues and the marginal band of erythrocytes (Wang *et al*, 1986; Joshi *et al*, 1987). Despite this restricted distribution and gross difference in sequence, it can form a co-polymer with other β-tubulin isotypes in the both the interphase and spindle microtubules of transfected foetal nucleated erythrocytes and HeLa cells (Lewis *et al*, 1987; Joshi *et al*, 1987).

As there is such persuasive evidence for both points of view it seems that both systems must, in part, exist. Whitfield *et al* (1986) transformed a wild type Chinese hamster ovary (CHO) cell with a β-tubulin gene from a colcemid-resistant CHO cell. The gene was expressed but the transformant became dependent on colcemid for growth. It thus took on characteristics confered by the transforming tubulin gene. Free mingling of seemingly divergent isotypes thus suggests multiple isotypes are not involved in specifying the functional differences between different subsets of microtubules while the conservation of isotype specific sequence differences which are differentially expressed spatially and developmentally, suggests functional differences between the isotypes. The two mechanisms may thus coexist *in vivo*, the multiple genes providing an alternative means of tubulin expression during divergent pathways of cell differentiation. It also does not preclude the possibility that although isotypes freely mingle, they may interact differently with other cellular components, functional differentiation of isotypes reflecting their differing MAP associations or susceptibility to post-translational mechanisms (Gard and Kirschner, 1985; Edde *et al*, 1981).

A model was proposed by Lewis and Cowan (1988) which suggests that through evolution the isotypes have evolved along with their associated MAPs and only specifically interact with the MAPs of a particular cell type. This does not, however, interfere with the assembly properties of the isotypes and mixed populations of microtubules are still produced. The differential MAP association is thus a selective pressure maintaining isotypes throughout evolution. Evidence for this comes from

mouse testes where two β-tubulins exactly co-distribute with MAP-1a and MAP3 (Cowan *et al*, 1988). This theory is also supported by the fact that amino acid differences between the isotypes tend to occur within the same carboxyl-terminal domain that binds MAPs *in vitro* (Sullivan and Cleveland, 1986; Villasante *et al*, 1986; Wehland *et al*, 1983; Draber *et al*, 1989; Breitling and Little, 1986; Serrano *et al*, 1984a; b).

1.1Ieii: Post-translational modifications of tubulin

A further level of microtubule complexity is produced by post-translational modifications. The best defined is detyrosination whereby a tyrosine is reversibly removed by a carboxypeptidase from the C-terminus of α-tubulin leaving a new terminus of glutamic acid. Tubulin can subsequently be retyrosinated by tubulin tyrosine ligase (Barra *et al*, 1974; Ersfeld *et al*, 1993), the enzymes involved having an absolute specificity for tubulin (Arce *et al*, 1975; 1978; Kumar and Flavin, 1981; Arce and Barra, 1983; Raybin and Flavin, 1975; 1977a; b). Due to the slower reappearance of detyrosinated (glu) tubulin containing microtubules after depolymerisation by drug treatment a model was proposed in which glu-microtubules are produced by the detyrosination of tyrosinated (tyr) microtubules, monomers re-entering the tubulin pool being efficiently retyrosinated. The amount of glu-tubulin in the monomer pool has been measured as <2%. This creates a tyrosination/detyrosination cycle, detyrosination being the slower, rate-limiting step (Arce *et al*, 1978; Arce and Barra, 1985; Wehland and Weber, 1987; Gundersen *et al*, 1987; Webster *et al*, 1987; Figure 2).

In mammalian cells the distribution of tyr and glu-microtubules *in vivo* is markedly different. Tyr tubulin is found throughout the interphase and spindle microtubules while glu-tubulin is limited to a subset of sinuous, interphase microtubules plus some kinetochore and polar microtubules of the mitotic spindle, the centrioles and in the proximity of the Golgi apparatus (Gundersen and Bulinski, 1986a; b; Gundersen *et al*, 1984; Skouifas *et al*, 1990). The majority of microtubules seem to consist of variable levels of both isotypes (Geuens *et al*, 1986) though glu-microtubules are totally absent in some proliferating cultured cells such as CHO and N115 neuroblastoma

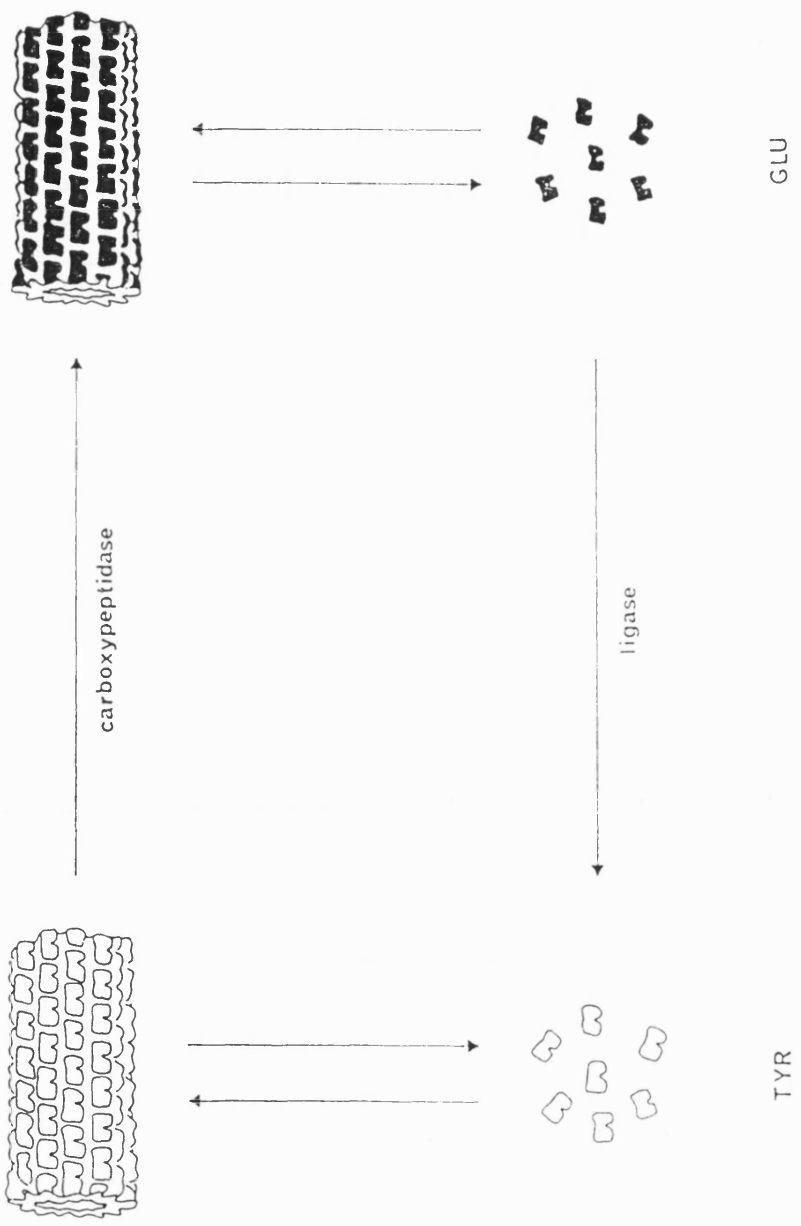


Figure 2 The Tyrosination Cycle of Tubulin
Re-drawn from Gundersen *et al* (1987)

cells and MDCK epithelial cells, only appearing in processes after differentiation has occured suggesting that at confluency there is an increase in microtubule stability which could lead to post-translational modifications taking place (Wehland and Weber, 1987; Gundersen *et al*, 1989; Pepperkok *et al*, 1990). In conjunction with this, fibroblasts migrating into experimental wounds have a leading edge of glu-microtubules suggesting these microtubules are more stable and thus defining the polarity of the movement (Gundersen and Bulinski, 1988; Nagasaki *et al*, 1992). On the formation of cell-cell contacts, however, the glu-tubulin is redistributed to a perinucleur location suggesting the stable microtubules are also having an influence on the morphology on fibroblasts in a monolayer (Nagasaki *et al*, 1992). A similar situation exists in the oocytes of *Drosophila* and in *Artemia* embryos, post-translational modifications not being detected until the onset of tissue differentiation (Warn *et al*, 1990; MacRae *et al*, 1991). Thus generally, prior to differentiation, the dynamic microtubule cytoskeleton will allow a plastic cell environment beneficial to morphogenesis to exist, stabilisation of a subset of microtubules creating an assymetric, morphological change that leads to differentiation. This suggests differentiation is activating the carboxypeptidase, the glu-microtubules indicating the prescence of stable microtubules (Wehland and Weber, 1987; Gundersen *et al*, 1989).

Glu-microtubules are very resistant to microtubule depolymerising drugs and have very low rates of tubulin incorporation so seem to be more stable than tyr-rich microtubules (Kreis, 1987; Schulze *et al*, 1987; Gundersen *et al*, 1987). This property of stability is, however, controversial, glu-microtubules being depolymerised by cold (Khawaja *et al*, 1988). There is other supportive evidence for this abscence of stability in glu-microtubules. Glu-microtubules that have been created from tyr-microtubules using endogenous carboxypeptidase A have neither increased stability nor the characteristic glu-microtubule appearance (Webster *et al*, 1990) while increasing microtubule stability with taxol subsequently produces heightened levels of glu-tubulin (Gundersen *et al*, 1987). As the action of microtubule drugs is end-dependent, this suggests some form of capping mechanism in glu-microtubules is

preventing depolymerisation from occuring, the microtubules subsequently being detyrosinated, glu-rich microtubules being a consequence not a cause of microtubule stability. This could explain why glu-microtubules appear in the spindle: kinetochore capping could stabilise a subset of microtubules despite their fast turnover, allowing detyrosination to occur (Mitchison and Kirschner, 1985b). Detyrosination may then target microtubules for further long term stabilisation mechanisms (Gundersen and Bulinski, 1986a).

Detyrosination may serve to define distinct microtubule subpopulations within a cell, for example, detyrosination may increase the affinity of microtubules for stabilising MAPs. In the brain of the Atlantic cod, glu-microtubules are highly stable, their stability being dependent on an interaction between the posttranslationally modified tubulin and MAPs (Billger, 1991). Additionally glu-microtubules assembled *in vitro* in the presence of phosphorylated MAP-2 incorporate higher amounts of MAP-2 then tyr-microtubules (Kumar and Flavin, 1982). Pertinent to this is the suggestion that the Golgi apparatus interacts primarly with glu-microtubules via glu specific MAPs (Skoufias *et al*, 1990). In all cases, however, the microtubules are stabilised by MAPs interacting with a particular subset of microtubules that are defined by some other mechanism, the microtubules being post-translationally modified subsequently (Takemura *et al*, 1992; Lee and Rook, 1992).

The other known α-tubulin post-translational modification arises by acetylation of the ε-amino group of lysine residue 40 (L'Hernault and Rosenbaum, 1985a; LeDizet and Piperno, 1987). First identified in the microtubules of the *Chlamydomonas* flagellum (McKeithan *et al*, 1983; L'Hernault and Rosenbaum, 1983; 1985a; b), it has since been found in a variety of axonemes (LeDizet and Piperno, 1986; Piperno and Fuller, 1985), in the slime mould *Physarum* (Schneider *et al*, 1987) and a wide range of mammalian cell types (Piperno *et al*, 1987; Bulinski *et al*, 1988).

The timing of acetylation seems to differ from that of detyrosination. Acetylation tends to occur at the time of microtubule assembly and not later like detyrosination. Acetylated microtubules therefore appear before glu during recovery from drug induced depolymerisation, after oocyte fertilisation and during the very early stages of mouse embryo development (Bulinski *et al*, 1988; De Pennart *et al*, 1988; Wolf *et al*, 1988; Warn *et al*, 1990; Maro *et al*, 1988). Conversely, in differentiating myoblasts acetylation occurs later and initially has a distinct, segmented distribution whereas detyrosination appears in a more uniform fashion. Acetylation, however, eventually becomes replaced by a distribution indistinguishable from that of glu-tubulin (Gundersen *et al*, 1989).

When acetylated and detyrosinated microtubules occur together, their distributions are generally similar, suggesting that, like detyrosination, acetylation preferentially occurs on stable microtubules (Schulze *et al*, 1987; Bulinski *et al*, 1988; LeDizet and Piperno, 1986; Piperno *et al*, 1987; Maro *et al*, 1988). This is supported by the observation that, in axons, the un-modified form predominates in the soluble tubulin pool (Denoulet *et al*, 1989). Acetylated tubulin has also been found in the microtubules of mitotic and meiotic spindles but, as with glu-tubulin, it is mainly associated with stable kinetochore microtubules (Piperno *et al*, 1987; Sasse and Gull, 1988; De Pennart *et al*, 1988). Conversely, in *Drosophila* embryos only interphase microtubules are acetylated possibly reflecting the differing lengths of mitosis and meiosis in these different systems (Wolf *et al*, 1988).

There are, however, anomalies in the relationship between stable microtubules and both glu and acetylated tubulin. Tyrosination does not occur in all cells investigated. In fission yeast, glu-tubulin can be found neither naturally nor after microtubule stabilisation, suggesting this simple eukaryote lacks the necessary enzymatic machinery (Alfa and Hyams, 1990). Cultured cells that do not possess glu-microtubules can also have drug resistant microtubules while there is evidence of axons having a stable population of tyr-microtubules. Conversely, the rapidly turning over spindle of *Trypanosoma brucei* is composed exclusively of

glu-tubulin (Sasse and Gull, 1988). PtK_2 cells have a subset of stable microtubules that do not have post-translational modifications of any kind while in chick embryo fibroblasts there is a large amount of post-translational modification and no stable microtubules. This suggests that post-translational modifications and stability are not permanantly linked, factors stabilising microtubules (see Section 1.1Ib), the enzymatic rate of modification then having profound effects on which microtubules become modified (Schulze *et al*, 1987; Gard and Kirschner, 1985).

A third post-translational tubulin modification is phosphorylation: purified β-tubulin having almost stoichiometric levels of bound phosphate and the ability to incorporate endogenous phosphate. This was later localised to a serine residue at the C-terminus of β-tubulin (Eipper, 1972; 1974). A protein kinase activity, which uses casein as a substrate, was found associated with tubulin, the activity of which fluctuated throughout the cell cycle of HeLa cells, being maximal during mitosis (Eipper, 1974; Piras and Piras, 1975). The kinase was subsequently identified, *in vitro*, as casein kinase II (Serrano *et al*, 1987), the activity of which could be important in modulating microtubule assembly and disassembly as well as the interaction of microtubules with membranes (Serrano *et al*, 1987; Hargreaves *et al*, 1986). Furthermore, tyrosine is phosphorylated in membrane associated α and β-tubulins at postsynaptic junctions and nerve growth cones where it is thought to modulate tubulin's interactions with the membranes, microtubule dynamics and the transport of vesicles to the leading edge (Matten *et al*, 1990). Phosphorylation also occurs to a specific β-tubulin isotype from chick neurone, the number of isotypes increasing as the system ages, indicating again its association with stable microtubules (Lee *et al*, 1990; Gard and Kirschner, 1985). This latter post-translational modification is also significant as it increases the C-terminus' negative charge and can thus alter the tubulin's interaction with MAPs (Lee *et al*, 1990).

1.1II: Microtubule-associated proteins

Microtubules are assembled from tubulin subunits with high degrees of conservation yet are components of a large variety of structures and functions. This implies that, in addition to the diversity of tubulin isotypes, there are other factors determining microtubule function. The best known are microtubule-associated proteins (MAPs) which co-purify with tubulin. Brain MAPs are comprised of a group primarily consisting of the high molecular weight MAPs, MAP-1 and MAP-2 and the smaller tau protein as well as MAP-3 and MAP-4, which are less abundant (Huber *et al*, 1985; Parysek *et al*, 1984). In addition the microtubule-associated motor proteins dynein and kinesin are present.

1.1IIa: MAP-1

MAP-1 exists as a spectrum of isotypes, classically divided into three structurally, developmentally and immunologically distinguishable co-migrating species, MAP-1a, MAP-1b and MAP-1c, with molecular weights around 350kD (Murphy *et al*, 1977; Bloom *et al*, 1984a). MAP-1a and MAP-1b are complexed with approximately equal stochiometry to three light chains (LC-1, LC-2, LC-3), MAP-1a and b having a common light chain, LC-1, while MAP-1a binds additionally to LC-2 (Vallee and Davis, 1983; Vallee and Luca, 1985; Kuznetsov *et al*, 1986; Kuznetsov and Gelfand, 1987). The precise binding of LC-3 is not known. The light chains also promote microtubule assembly and co-sediment with microtubules though a preparation lacking light chains will still bind microtubules, indicating that they are implicated in, but not essential for, the microtubule binding function (Vallee and Davis, 1983; Vallee and Luca, 1985). When MAP-1b was cloned and sequenced it was found to have two unusual repeated sequences one of which is involved in microtubule binding (Noble *et al*, 1989). Binding is, however, weak, the sequence cycling inefficiently with microtubules *in vitro*. Consequently it and MAP-1b are generally under-represented in microtubule preparations (Noble *et al*, 1989; Bloom *et al*, 1985a; Matus, 1988).

MAP-1 was found in all mammalian species studied, but was absent from invertebrates. A gene was subsequently isolated from mouse brain and was found to be expressed, albeit at much lower levels, in liver, kidney,

spleen, thymus and stomach. (Lewis *et al*, 1986a). The distribution of MAP-1 in brain is developmently regulated, such that the levels of MAP-1a in white matter increases, and that of MAP-1b decreases, as development proceeds post-natally. This leads to a low level of MAP-1b in mature brain white matter (Vallee and Luca, 1985). MAP-1a is also widely distributed throughout the nervous system of rats and while MAP-1a and MAP-1b are present in dendritic and axonal processes. MAP-1a co-localises with MAP-2 in neurons while MAP-1b is predominantly found in axons (Vallee, 1982; Bloom *et al*, 1984a; 1985a).

MAP-1 specific antibodies stain interphase and spindle microtubules in a wide range of cultured cells producing punctate patterns which suggest MAP-1 binds at discrete regions along microtubules (Bloom *et al*, 1984b; 1985b; Luca *et al*, 1984). Electron micrographs of pure, MAP free, microtubules to which MAP-1 has been added show projecting arms suggesting that MAP-1 may have a microtubule cross-bridging function (Vallee and Davis, 1983). Sequencing of a cDNA clone revealed MAP-1b has 30 potential caesin kinase II phosphorylatable sites consisting of serines or threonines (Noble *et al*, 1989). Treatment of rat PC12 cells with nerve growth factor (NGF; Aletta *et al*, 1988) and MAP-1b *in vitro* by casein kinase II (Diaz-Nido *et al*, 1988) produces an increased level of MAP-1 phosphorylation which promotes microtubule assembly. This has important implications for neuronal differentiation as the phosphorylation and synthesis of MAP-1b both increase during neurite outgrowth. The preferential association of MAP-1b with axonal microtubules thus could be involved in axon formation (Diaz-Nido *et al*, 1988; Drubin *et al*, 1988), phosphorylation possibly playing a role in the control of MAP-1 functioning.

MAP-1c isolated from brain was found to have microtubule-activated ATPase activity and an *in vitro* retrograde microtubule-translocating activity, both of which were inhibited by several known dynein inhibitors. This suggests that MAP-1c is the soluble cytoplasmic analog of axonemal dynein as well as the retrograde motor for axoplasmic transport (Paschal *et al*, 1987a; Paschal and Vallee, 1987; Shpetner *et al*, 1988; see Section 1.1IId). The similarity with axonemal dynein

stretches to the morphology of the MAP-1c molecule which consists of two globular domains that correspond to two heavy chain sub-units (Vallee *et al*, 1988). It is, however, isolated on axonal organelles that are transported in both the anterograde and retrograde direction, suggesting there is a specific mechanism which only activates the MAP-1c molecules when they are positioned to operate in the retrograde direction (Hirokawa *et al*, 1990). There are also differences in the intermediate and light chain composition which could specify the structures the molecule associates with, whether it be another microtubule, a membrane, a kinetochore or another organelle (Vallee *et al*, 1988) and the nucleotides hydrolysed which could have important repercussions for its functioning (Shpetner *et al*, 1988).

1.1IIb: MAP-2

MAP-2 consists of three heat-stable subunits, MAP-2a, MAP-2b (250 to 300kD) and the smaller MAP-2c (70kD), which all have a prodigious microtubule polymerisation promoting activity (Herzog and Weber, 1978; Kim *et al*, 1979; Garner *et al*, 1988a). All three species are encoded by the same gene, from which two distinct mRNAs are produced (Lewis *et al*, 1986b; Garner and Matus, 1988; Papandrikopoulou *et al*, 1989). There is, therefore, high homology between all three species at both the carboxyl and amino termini, the sequence for the arm portion of high molecular weight MAP-2 species being absent from MAP-2c (Papandrikopoulou *et al*, 1989).

By immunofluorescence microscopy, using monoclonal antibodies to the high molecular weight species, and *in situ* cDNA hybridisation, MAP-2 was found to be specific to the dendritic processes of neurons and the pituitary and was absent from all other tissues (Vallee, 1982; Bernhardt and Matus, 1984; Miller *et al*, 1982; Bloom *et al*, 1985b; Lewis *et al*, 1986b; Garner *et al*, 1988b; Okabe and Hirokawa, 1989). The dendritic distribution is a result of a lack of association of MAP-2 with axonal microtubules, therefore, it is preferentially turned over in the axon and selectively lost (Okabe and Hirokawa, 1989). In contrast to the distribution of MAP-2a and b, MAP-2c is abundant in embryonic and neonatal tissue and shows a far less restricted distribution (Riederer

and Matus, 1985; Garner and Matus, 1988; Garner *et al*, 1988a). Once maturation has occurred, MAP-2c is replaced by high molecular weight MAP-2. An exception to this is the adult rat retina. The cells are unique among adult neurones as they undergo constant development and contain MAP-2c (Tucker *et al*, 1988). The association of MAP-2c with embryonic tissues and its absence in adult tissues may reflect the involvement of the missing arm domain in creating a less flexible, more structured cytoskeleton.

Investigations into its tubulin binding site revealed that MAP-2 specifically interacts with a seven amino acid sequence at the C-terminus of β tubulin (Maccioni *et al*, 1988) and four amino acids at the C-terminus of α-tubulin (Paschal *et al*, 1989). If MAP-2 is chymotryptically digested the small fragments produced bind to, and promote the assembly of, microtubules, while a much larger fragment has no interaction and probably represents an arm domain that projects from the microtubule surface and interacts with other cell components or microtubules (Vallee and Borisy, 1977; Herzog and Weber, 1978; Kim *et al*, 1979; Voter and Erickson, 1982; Gottlieb and Murphy, 1985; Chen *et al*, 1992; Lopez and Sheetz, 1993). Lewis *et al* (1988) determined the complete amino acid sequence of high molecular weight mouse MAP-2 and found three imperfect repeats of 18 amino acids separated by 13 or 14 amino acids at the carboxyl terminus. These were deduced to be the microtubule binding region from the following experiments. Deletion of the entire carboxyl region destroyed the ability of MAP-2 to bind microtubules although binding still occurred if only one or two of the repeats were removed, albeit not as strongly as before (Lewis *et al*, 1989; Lewis and Cowan, 1990). Just the second repeat is sufficient to promote microtubule nucleation and elongation, but this is only the case at much higher concentrations of MAP-2 than that found *in vivo*. This suggets that the other 2 repeats act to potentiate the effect of the second repeat (Joly *et al*, 1989). Removal of the amino terminal arm portion of the molecule had no effect on either microtubule bundling or binding, indicating that both functions are associated with the carboxyl terminus (Lewis *et al*, 1989; Lewis and Cowan, 1990).

One unambiguous role for the MAP-2 arm is its possession of a binding site for a type II cAMP-dependent protein kinase, MAP-2 having up to 13 cAMP-dependent phosphorylation sites and being the major substrate for endogenous cAMP-dependent phosphorylation in brain tissue (Vallee, 1980a; b; Vallee *et al*, 1981; Miller *et al*, 1982; Theurkauf and Vallee, 1983; Sloboda *et al*, 1975). Bovine heart R_{II}, the regulatory subunit of this kinase also binds to all three MAP-2 subunits, chymotryptic digestion localising the binding site to a single conserved region of the arm. Phosphorylation could have important repercussions for the interaction of MAP-2 with microtubules as MAP phosphorylation inhibits the rate and extent of microtubule assembly without affecting the critical tubulin concentration needed for polymerisation. This suggests that the altered MAP-tubulin interaction inhibits the nucleation of microtubules (Jameson *et al*, 1980; Jameson and Caplow, 1981) though the subject is controversial as one report suggested that cAMP stimulated phosphorylation increases microtubule nucleation and growth through its effect on the calcium-calmodulin complex (Tash *et al*, 1980) while casein kinase II phosphorylation has no effect on microtubule polymerisation (Serrano *et al*, 1987).

1.1IIc: Tau

Tau, another heat stable brain MAP, exists as a heterogeneous family of at least 4 proteins, with molecular weights between 50 and 70kD, that are virtually indistinguishable by peptide mapping. These co-purify stoichiometrically with brain tubulin and promote microtubule nucleation, elongation and stabilisation *in vitro* (Weingarten *et al*, 1975; Solomon *et al*, 1979; Cleveland *et al*, 1977a; b; Drubin and Kirschner, 1986; Kanai *et al*, 1989), while *in vivo* they are almost entirely axonal though recently a specific non microtubule-associated nuclear tau has been identified (Binder *et al*, 1985; Wang *et al*, 1993). Heterogeneity is developmentally regulated, a doublet peaking at birth before being replaced by higher molecular weight species (Drubin *et al*, 1984; Couchie and Nunez, 1985). Tau is important as it is involved in the differentiation of neurites, NGF treatment of PC12 cells increases the level of cellular tau, which in turn promotes microtubule polymerisation prior to neurite extension (Drubin *et al*, 1988) while

transfection of Sf9 cells with tau induce microtubule bundling and leads to the formation of axon like processes (Knops *et al*, 1991; Baas *et al*, 1991). In conjunction with this, if the expression of tau is prevented *in vivo*, early neurite formation occurs but axon formation is prevented (Caceres and Kosik, 1990). Tau is also important as it is a major component of paired helical filaments in brain tissue of patients suffering from Alzheimer's disease (Kosik *et al*, 1986; Ksiezak-Reding *et al*, 1988; Goedert *et al*, 1989; Steiner *et al*, 1990).

The mouse brain tau sequence suggests that tau has a low interior volume being extended and hydrophilic with no extensive α-helices or β-pleated sheets (Lee *et al*, 1988). Electron microscopy shows tau to have a rod morphology consisting of a tubulin binding domain and a projecting arm domain, the arm cross-linking microtubules both *in vivo* and *in vitro* leading to the production of bundles (Hirokawa *et al*, 1988; Kanai *et al*, 1989; Chen *et al*, 1992; Lee and Rook, 1992). The sequence of mouse brain tau reveals three 18 residue microtubule binding repeats at the C-termini that are common with MAP-2, indeed the two proteins compete for tubulin binding sites *in vitro* (De La Torre *et al*, 1986; Lewis *et al*, 1988; Ennulat *et al*, 1989). It thus seems that due to the compartmentalisation of tau and MAP-2 in brain, binding is specified *in vivo* by compartment-specific tubulins, tau having an additional binding site at the N-terminus of α-tubulin (Littauer *et al*, 1986). Cloning of post-natal human brain tau also reveals the presence of an isoform which has an extra repeat, both the 4 repeat and the immature 3 repeat forms occuring in Alzheimer's disease patients (Kosik *et al*, 1989; Goedert *et al*, 1989). The distribution of the 4 repeat form is different from that of the 3-repeat tau which suggests that its altered binding characteristics may be conferring different characteristics on axons and dendrites (Goedert *et al*, 1989). It is also thought that the 4 repeat form of tau is the only one that can bundle microtubules *in vivo*, additional sequences either side of the repeats also being important (Lee and Rook, 1992).

Tau can be purified in both a phosphorylated and a dephosphorylated state (Cleveland *et al*, 1977b; Lindwall and Cole, 1984), part of the

electrophoretic complexity of tau stemming from such post-translational phosphorylation, fibroblasts transformed with tau cDNA losing one of the tau bands upon phosphorylation (Kanai *et al*, 1989). Tau is also highly elastic, paracrystals will stretch, contract and bend greatly, the stretching being correlated with the degree of phosphorylation, dephosphorylated tau being shorter and more elastic. This may be important as it allows a normally stable microtubule crosslinker to become flexible by a simple chemical change (Lichtenberg *et al*, 1988; Hagestedt *et al*, 1989). The phosphorylation site for all kinases used lies within the C-terminus of tau but only Ca^{2+}-calmodulin dependent kinase can induce an electrophoretic shift, the site of action of this kinase being localised to a single serine outside the region of microtubule-binding repeats (Baudier and Cole, 1987; Steiner *et al*, 1990). The serine phosphorylation site could also correspond to the abnormally phosphorylated residue found in Alzheimer brain tau (Baudier and Cole, 1987; Kosik *et al*, 1989; Steiner *et al*, 1990), phosphorylation possibly affecting regulatory sequences outside the repeat region that normally prevent tau forming into the paired helical filaments (Wille *et al*, 1992).

1.1IId. Dynein

Dynein is an important component of cilia and flagella and the axonemes of sperm, interconnecting the A and the B subfibers and causing them to slide relative to one another (Gibbons and Rowe, 1965; Gibbons, 1981; Sale and Satir, 1977). The composition of dynein in each of the above systems is complex, as detailed in Table 1.

Dynein has a characteristic morphology when visualised by electron microscopy. *Tetrahymena* and *Chlamydomonas* outer arm dyneins each appear as three globular heads joined by a thin stalk (Goodenough and Heuser, 1984; Toyoshima, 1987a; Johnson and Wall, 1983) while inner arm structures can be observed as one or two heads with attached stalks (Marchese-Ragona *et al*, 1988; Toyoshima, 1987a; Goodenough *et al*, 1987). Sea urchin and bovine sperm outer arm dynein are visualised as two heads corresponding to the α and the β heavy chains (Sale *et al*, 1985; Marchese-Ragona *et al*, 1987). In *Tetrahymena*, *Chlamydomonas* and sperm

	INNER ARM		OUTER ARM	
	MOLECULAR WEIGHT	COMPOSITION	MOLECULAR WEIGHT	COMPOSITION
TETRAHYMENA	330[2]-460[3]kD	1,2 heavy chains,2,3 several small chains	490[3]kD (228[3])	3 heavy chain several small chains[3]
CHLAMYDOMONAS	Minor component-1000kD (218[7]) Major component-1400-1800?kD (118[7])	Combination of heavy and intermediate chains and actin	α-340[5]kD β-340[5]kD γ-415[4]kD (128[5])	Combination of α, β and γ heavy chains plus several intermediate and small chains[4,5,6]
SPERM TAIL AXONEME	Bull-311[8]kD (128[8])[9]		Bull-311[8]kD (128[8]) 1600[8]kD (198[8]) } 188[5]	
		Chicken-Complex of 3 dyneins with 1, 2 and 3 heads respectively[9]		Combination of α and β heavy chains plus several intermediate and light chains[10,11,12]
			Trout-α-430[10]kD β-415[10]kD } 198[10]	
			Sea urchin-218[11]	Sea urchin-2 heavy chains (α, β) 3 intermediate chains 6 light chains[13,14,15]
PARAMECIUM	128[16]	Heavy chain[16]	α,β-228[16,18] γ-12-148[16]	3 heavy chains[16] Several intermediate chains[17]

1-Warner *et al*, 1985
2-Marchese-Ragona *et al*, 1988
3-Toyoshima, 1987a
4-King and Whitman, 1984
5-Pfister and Whitman, 1984
6-Goodenough *et al*, 1987
7-Piperno *et al*, 1990
8-Marchese-Ragona *et al*, 1987
9-Burgess *et al*, 1991
10-King *et al*, 1990
11-Bell and Gibbons, 1982
12-Tang *et al*, 1982
13-Moss *et al*, 1992(a)
14-Pascal *et al* , 1987(b)
15-Sale and Satir, 1977
16-Beckwith and Asai, 1993
17-Walczak *et al*, 1993
18-Larsen *et al*, 1991

Table 1 The Composition of Dynein in a Number of Different Systems

tail axonemes of sea urchin and trout all heavy chains have ATPase activity (Toyoshima, 1987a; b; King and Whitman, 1986; Pfister and Whitman, 1984; Pfister *et al*, 1982; 1985; Piperno *et al*, 1990; Tang *et al*, 1982; Bell and Gibbons, 1982; King *et al*, 1990) but in *Tetrahymena* chymotryptic digestion reveals that only two 22S heavy chains have an ATP sensitive microtubule binding ability (Toyoshima, 1987a; b; Vale and Toyoshima, 1989) while the 14S dynein does not seem to have an ATPase activity (Marchese-Ragona *et al*, 1988; Porter and Johnson, 1983a).

Both *Tetrahymena* 14S and 22S dynein can translocate microtubules in an *in vitro* assay, the latter having a higher velocity but a restricted nucleotide specificity range (Vale and Toyoshima, 1988; 1989;; Shimizu *et al*, 1991). If, however, 22S dynein is cleaved then only two headed particles can produce movement though the single headed particle still binds microtubules and has an ATPase activity (Toyoshima, 1988a; b). Similarly outer arm flagella sea urchin dynein adsorbed to a glass slide, translocates microtubules (Paschal *et al*, 1987b). The translocating subunit was subsequently identified as the β-heavy chain-intermediate chain 1 (β/IC1) subunit, the α-heavy chain and other intermediate chains having no motile activity. The velocity induced by the β/IC1 is, however, much faster than that of the intact outer arm subunit and, on ATP depletion, it detaches from the microtubule as it does not form a rigor bond. This suggests that the accessory chains have a regulating function, this function in part being due to the α heavy chain which does form a rigor bond on ATP depletion (Sale and Fox, 1988; Moss *et al*, 1992a; b)

In *Chlamydomonas* axonemes there are 13 outer dynein arm and 10 inner dynein arm components which appear to be essential for movement, though they have differing levels of involvement. This complexity suggests there are numerous fine controls for the functioning of the axoneme (Piperno and Luck, 1979; Huang *et al*, 1979; Kamiya and Okamoto, 1985; Brokaw and Kamiya, 1987; Kamiya, 1988; Piperno, 1988; Kamiya *et al*, 1991; Kagami and Kamiya, 1992; Smith and Sale, 1992; Piperno *et al*, 1992; Kurimoto and Kamiya, 1991). The sperm axoneme dynein causes the microtubules of adjacent doublets to slide apart but sliding does not

occur between random pairs of individual doublets, only adjoining doublets move and only half the doublets are displaced and therefore are active (Spungin *et al*, 1987; Satir *et al*, 1981; Satir, 1988). Possibly the binding of a dynein arm to the subfibre enhances the binding of the adjacent dynein this leading to a wave of crossbridging along the length of the microtubule (Haimo and Fenton, 1984). A further insight into the mechanics of dynein movement occured when the action of sea urchin sperm dynein was inhibited using vanadate, the dynein restricting the microtubules to oscillations along their longitudinal axes. The oscillations were, however, greater than the length of the dynein molecule indicating the microtubules were not tightly bound. Possibly weak electrostatic forces are involved or the dynein heads are continuously associating and dissociating from the microtubule. In either case the interaction could be displaced to the next tubulin monomer by a thermal fluctuation, ATP hydrolysis allowing the dynein to reassociate thus producing a unidirectional ratchet system. The weak binding would also allow dynein heads not immediately involved in force production to stay within close proximity of the microtubule until they are needed (Vale *et al*, 1989). *Tetrahymena* dynein bound to microtubules was also found to dissociate very quickly on the addition of ATP, suggesting the rigor state is short so does not inhibit dynein mediated movement (Porter and Johnson, 1983a; b).

1.1IIe: Cytoplasmic dynein

Dynein was first isolated outside the axoneme in sea urchin eggs where it was found associated with the mitotic apparatus, crosslinking microtubules (Scholey *et al*, 1984; Porter *et al*, 1988; Piperno, 1984; Hirokawa *et al*, 1985) and recently it has been shown to produce retrograde movement in an *in vitro* system (Grissom *et al*, 1992; Gilksman and Salmon, 1993). The localisation was narrowed to metaphase and anaphase polar, interzone and kinetochore microtubules where it could have important functions in chromosome movement and/or spindle elongation (Hirokawa *et al*, 1985; Hisanaga *et al*, 1987). Subsequently two isoforms of dynein were isolated, one, a ciliary dynein precursor, the other, a microtubule associated protein with different heavy chains and a low ATPase activity (Porter *et al*, 1988; Grissom *et al*, 1992). The

discovery of MAP-1C as a cytoplasmic analog of axonemal dynein (Paschall and Vallee, 1987, see Section 1.1IIa) led to the characterisation of additional cytoplasmic dyneins in pig brain (Koszka *et al*, 1987), squid optic lobes and axoplasm (Schnapp and Reese, 1989), *Paramecium* (Schroeder *et al*, 1990), chick embryo brain (Steuer *et al*, 1988), cultured chick fibroblasts (Schroer *et al*, 1989) and rat liver and testis (Collins and Vallee, 1989). Cytoplasmic dynein was also isolated from homogenates of *Dictyostelium* where it co-localised with the MTOCs in the nucleus and stained punctate cytoplasmic structures suggesting it is organelle bound. The nucleur localisation, though suggesting dynein is involved in both the transport of material to the MTOC and the anchoring of the MTOC at the nucleus was, however, poor evidence for a mitotic spindle localization (Koonce and McIntosh, 1990). Cytoplasmic dynein may, however, be involved in chromosome movement during metaphase, due to its putative association with the corona on the surface of the kinetochores, the centrosomes and spindle fibres (Pfarr *et al*, 1990; Steuer *et al*, 1990). In *Xenopus* oocytes extract cytoplasmic dynein was found to be involved in the formation of taxol initiated asters, suggesting centrosomal material is being carried to the centre of a developing aster, dynein increasing the concentration of centrosomal material above a critical concentration thus initiating centriole assembly (Verde *et al*, 1991). Cytoplasmic dynein has also been implicated in the maintenance of the pericentriolar localisation of the Golgi complex in interphase cells, vesicles being transported to the - ends of microtubules at the end of telophase (Corthesay-Theulaz *et al*, 1992).

Attempts to identify the cytoplasmic dynein binding region of tubulin produced conflicting results. Paschal *et al* (1989) found that subtilisin digestion of the C-terminus of tubulin subunits prevented binding while Rodionov *et al* (1990) found it had no effect suggesting the binding site for cytoplasmic dynein on microtubules was outside this region. Even though dynein and other MAPs may not have common binding sites it is possible that other MAPs sterically hinder the binding of dynein in a way that disrupts the cooperative binding (Haimo and Rosenbaum, 1981). This paradox has yet to be resolved though the observation that MAP-2

affects cytoplasmic dynein function tends to favour the existance of a common binding site at the C-terminus (Paschal *et al*, 1989).

There are a number of similarities between cytoplasmic and axoneme dynein. Dynein-like particles isolated from rat testes has two heads, each head corresponding to an identical heavy chain subunit. Each molecule is thus a dimer of a heavy chain, an intermediate chain and two light chains. Cytoplasmic dyneins are ATP dependent and, as with axonemal dynein their ATPase activity is microtubule stimulated (Paschal *et al*, 1987a; Shpetner *et al*, 1988). The extent of this stimulation is, however, much greater suggesting the multiple heads of axonemal dynein increase dynein-microtubule tethering and thus reduce the dynein dependence on microtubules for activation (Paschal *et al*, 1987a; Shpetner *et al*, 1988; Warner and McIlvain, 1986). Cytoplasmic dynein can also hydrolyse other nucleotides but this does not generally produce movement, this hydrolysis not being connected to force production (Shpetner *et al*, 1988). Purified cytoplasmic dynein from squid optic lobe also does not produce movement suggesting the requirement of other co-factors (Schnapp and Reese, 1989). *Dictyostelium* and rat brain cytoplasmic dynein have C-terminal homology with β heavy chain dynein from sea urchin flagella and the intermediate chain of *Chlamydomonas* flagella outer arm dynein respectively (Koonce *et al*, 1992; Paschal *et al*, 1992). This suggests that these similarities display the essential structural and mechanochemical domains of dynein molecules maybe with regard to microtubule or accessory chain binding. The lack of N-terminal homolgy, along with the simpler structure of cytoplasmic dynein, suggests the sequence and level of complexity of dynein may reflect its functioning, cytoplasmic dynein only being required for translocation of organelles along microtubules and changes in cell shape (Koonce *et al*, 1992; Paschal *et al*, 1992; Neely *et al*, 1990).

1.1IIf: Kinesin

Kinesin, an ATPase protein complex consisting of two heavy chains and two light chains (110-120kD and 60-70kD respectively), was initially identified due to its role in fast axonal transport. Both extruded and purified MAP free microtubules from squid giant axon transport

organelles in the presence of axoplasmic supernatant and ATP (Vale *et al*, 1985a; b; c; Schnapp *et al*, 1985; Allen *et al*, 1985; Schroer *et al*, 1988). Later work has, however, found that other nucleotides can support kinesin driven motility with lesser efficiency (Cohn *et al*, 1989; Shimizu *et al*, 1991) while kinesin bound to organelles can induce movement without the inclusion of axoplasmic supernatant (Schnapp *et al*, 1992). Purified microtubules can also translocate across glass and transport carboxylated glass beads, the whole translocation system being unidirectional and inhibited by AMP-PNP and high concentrations of vanadate (Vale *et al*, 1985b). In the case of classic kinesin translocation is anterograde (ie away from the cell body and towards the + end of the microtubule) and the ATPase activity is linked to a microtubule gliding activity and is greatly stimulated by the presence of microtubules. The enzymatic function is thus intimately tied to the translocation function (Kuznetsov and Gelfand, 1986; Cohn *et al*, 1987; Ingold *et al*, 1988), ATP hydrolysis promoting the release of kinesin from microtubules while AMP-PNP creates stable, non-motile, complexes between vesicles and microtubules by inhibiting an ATP binding site on the transport machinery (Harrison *et al*, 1993; Romberg and Vale, 1993; Lasek and Brady; 1985).

Kinesin has subsequently been isolated from chick and bovine brain, squid optic lobe, *Drosophila* and sea urchin eggs and embryos (Brady, 1985; Vale *et al*, 1985c; d; Johnson *et al*, 1990; Saxton *et al*, 1988; Buster and Scholey, 1991). Bovine brain kinesin is found to exist as a jointed rod with two globular domains at one end and a small fan shaped structure at the other. The globular domains consist of the heavy chains and contain the microtubule binding region, while the light chains are localised to the fanned structure which has the organelle binding region (Amos, 1987; Hirokawa *et al*, 1989). Analysis of the amino acid sequence of *Drosophila* heavy chain kinesin and the use of electron microscopy reveals a putative organelle binding region associated with the light chains at the C-terminus. More central to this there is an α-helical coiled coil with a bend near its middle and at the N-terminus there are an ATP and an ATP dependent microtubule binding site (Yang *et al*, 1989; Scholey *et al*, 1989; de Cuevas *et al*, 1992).

Kinesin has been localised by imunofluorescence microscopy to sea-urchin egg spindles (Scholey *et al*, 1985), spindle poles, pericentriolar material and primary cilia of cultured cells. The polar localisation is possibly important in anchoring and bundling spindle microtubules and reeling in kinetochore microtubules during metaphase. It is also very faintly localised to the interphase cytoplasm and the nucleoli, the general absence of interphase microtubule staining suggesting kinesin is localised to membrane bound organelles (Neighbors *et al*, 1988). This putative organelle distribution is, however, concentrated in areas where microtubules are present and if soluble proteins are extracted kinesin becomes localised to microtubules. This suggests that, during interphase, kinesin predominantly binds to organelles interacting transiently with microtubules (Pfister *et al*, 1989; Neighbors *et al*, 1988; Hollenbeck, 1989). This can be observed with kinesin's association with the endoplasmic reticulum (ER; Henson *et al*, 1992), the formation of which is both microtubule and kinesin dependent, kinesin pulling membraneous tubules along tracks layed out by microtubules until the characteristic orthogonal shape of the ER is formed (Dabora and Sheetz, 1988a; b). Kinesin has also been implicated in the microtubule dependent construction of tubular lysosomes in mouse macrophages, kinesin being anchored to the organelle surface as it translocates along the microtubules (Hollenbeck and Swanson, 1990). This is supported by the subsequent isolation of kinectin, a kinesin binding protein that is associated with the ER in chick embryo fibroblasts (Toyoshima *et al*, 1992). Furthermore, a kinesin heavy chain mutation in *Drosophila* causes loss of neuromuscular function, suggesting that it is involved in axonal transport, which is supported by the finding that kinesin associates with anterograde moving organelles in murine axons (Saxton *et al*, 1991; Hirokawa *et al*, 1991). The kinesin heavy chain mutation does not appear to have any effect on progress through mitosis, however, and is absent from both mitotic spindles and chromosomes (Saxton *et al*, 1991).

The velocity of kinesin mediated movement of latex beads along microtubules is not constant, abrupt changes in, and cessation of, movement in 4nm units suggesting kinesin specifically interacts with a domain on the periodically spaced monomers though recent work has

suggested that one kinesin motor domain binds per tubulin heterodimer (Harrison *et al*, 1993). No large lateral movements of the beads occur implying that they must maintain position over one protofilament and hence avoid collisions (Gelles *et al*; 1988; Kamimura and Mandelkow, 1992; Ray *et al*, 1993). Movement is also concentration dependent, low concentrations of kinesin allowing the bead to move for short distances along a microtubule before falling off. This suggests that kinesin releases from the microtubule during each mechanochemical cycle, high concentrations keeping the period of release short enough to prevent diffusion of the bead away (Block *et al*, 1990). Kinesin was thus found to undergo a large number of power strokes per cycle of ATP hydrolysis while remaining in contact with a microtubule (Howard *et al*, 1989).

In addition to classic kinesins, a number of kinesin-related proteins have been characterised that make up an ever increasing kinesin super-family, a small number of which are described below. The KAR3 gene product from the budding yeast, *Saccharomyces cerevisiae*, has C-terminal homology with the N-terminal of *Drosophila* kinesin heavy chain. It is localised to cytoplasmic microtubules and the spindle pole body suggesting that it is involved in microtubule sliding during nuclear fusion and possibly mitosis (Meluh and Rose, 1990). The gene products of *cut7*$^{+}$ from fission yeast, *bimC4* from *Aspergillus nidulans and nod* from *Drosophila* all have N-terminal homology with the N-terminus of *Drosophila* kinesin and, if mutated, the first two block spindle formation and pole body separation while the latter causes disjunction of non-recombinant meiotic chromosomes (Hagan and Yanagida, 1990; 1992; Enos and Morris, 1990; Zhang *et al*, 1990). The central portion of bimC4 also has an α-helical domain with noticeable areas of potential bending (Enos and Morris, 1990). The C-terminus of the *non-claret disjunctional* (*ncd*) gene product from *Drosophila* is also similar to the N-terminal motor domain of *Drosophila* heavy chain kinesin in both putative structure and nucleotide dependance. Its movement, however, is (-) end directed suggesting that sequence conservation can maintain the motor function but differences, presumably outside the C-terminus, produce the reversed direction of movement. The rest of the ncd molecule is dissimilar although the central domain is again α-helical. The mutation

in the *ncd* gene produces meiotic defects and chromosome non-disjunction in mitosis, again implying a role in microtubule sliding, or translocation of chromosomes to the poles (Endow *et al*, 1990; McDonald and Goldstein, 1990; McDonald *et al*, 1990). ncd also has a bundling activity which may suggest it has a role in spindle morphogenesis and it may be further involved in defects in the maternal MTOC, a mutation in *ncd* affecting the MTOC and thus the cytoskeletal organisation possibly with respect to the motile microtubules positioning nuclei (McDonald and Goldstein, 1990; McDonald *et al*, 1990). The suggestion that ncd is involved in spindle morphogenesis and functioning is supported by its localisation in meiotic and mitotic spindles (Hatsumi and Endow, 1992). A further kinesin super-family member Eg5 has been identified in *Xenopus* egg extracts. This is an anterograde motor which is localised at the spindles, particularly the poles, which indicates it may have a role in maintaining spindle morphogenesis (Sawin *et al*, 1992a; b). A role in spindle morphology is also suggested for human mitotic kinesin-like protein 1 and human CENP-E (Nislow *et al*, 1992; Yen *et al*, 1992). Both are thought to be involved in spindle elongation, the former cross-bridging antiparallel microtubules (Nislow *et al*, 1992) while the latter is also suggested to have a role in chromosome congression (Yen *et al*, 1992). Kinesin therefore appears to exist as a family of proteins that have a common microtubule binding motor domain with different organelle binding domains that confer their individual functions.

1.2: Nutritive tubes

The nutritive tube system of hemipteran insects is ideal for the study of microtubules and microtubule-associated transport. It is a simple system that is almost exclusively microtubule based, the microtubules all have a common polarity and transport is unidirectional (Stebbings and Hunt, 1983). Most importantly the system is easy to isolate and biochemically characterise (Hyams and Stebbings, 1979a; Stebbings *et al*, 1986; Stebbings and Hunt, 1987).

1.2Ia: Ovarian structure

A mature female of the backswimmer *Notonecta glauca glauca* has two, large paired ovaries each formed from seven ovarioles. An ovariole, in turn, is composed of an anterior syncitial tissue known as the trophic region and a developmental series of up to 25 posteriorally running, progressively maturing oocytes (Macgregor and Stebbings, 1970; Hyams and Stebbings, 1979b; Figure 3). Under polarising microscopy the nutritive tubes can be visualised as a small birefringent thread connecting the trophic core to the first oocyte. As the oocyte develops and passes posteriorally, the connection is maintained and the nutritive tube enlarges (Bennett and Stebbings, 1979; Hyams and Stebbings, 1979b). When the oocyte undergoes vitellogenesis it imports yolk proteins from the surrounding follicle cells and the nutritive tube connection is broken as it is no longer needed (Macgregor and Stebbings, 1970).

A cross-section through a *Notonecta* nutritive tube reveals a large bundle of up to 30,000 regularly spaced, stable, microtubules surrounded by large numbers of ribosomes. Each microtubule is surrounded by an electron clear zone into which ribosomes do not encroach (Macgregor and Stebbings, 1970; Stebbings and Willison, 1973; Stebbings and Hunt, 1982; Hyams and Stebbings, 1979a). The nature of the clear zone around nutritive tube microtubules has been well investigated. Colchicine treatment shows that the integrity of the clear zone is dependent on the microtubules (Stebbings and Bennett, 1976) and as some mucopolysaccharide stains produce an increase in the diameter of the microtubules visualised it seems there is some structural components within the clear zone (Stebbings and Bennett, 1975). This putative material does not, however, fill the entire zone which suggests that some other component must be involved. This may be due to electrostatic repulsion keeping the strongly anionic microtubules apart (Stebbings and Bennett, 1975; 1976), the addition of cationic compounds and the alteration of the microtubules' pH, and hence their inherent charge, changing the size of the exclusion zone (Stebbings and Hunt, 1982). The use of glycerol as a cryoprotectant causes the microtubules to associate into rows and chains suggesting the glycerol is affecting the balance of

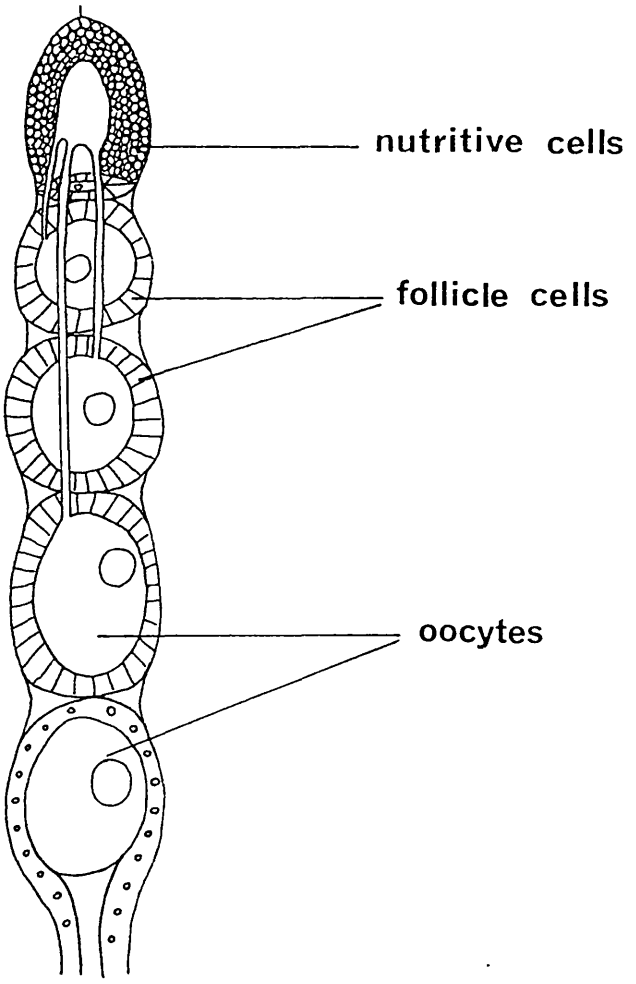


Figure 3 The Structure of an Ovariole of a Hemipteran Insect

electrostatic repulsion (Indi and Stebbings, 1985; Indi *et al*, 1985). It is also possible that MAPs occupy the clear zone and define the inter-microtubule spacing as has been suggested with cytoplasmic microtubules (Hyams and Stebbings, 1979a; b; Amos, 1979).

As nutritive tubes enlarge massively during development the microtubule-microtubule spacing remains constant. This, and low levels of tubulin synthesis within the tube, indicates that microtubules are being inserted as the tube grows, and then grow to catch up with the 'older' microtubules (Hyams and Stebbings, 1979b; Stebbings *et al*, 1985). The organisation of the new microtubules may be determined by cross bridges from the existing microtubules (Hyams and Stebbings, 1979b) because the orientation of vinblastine-induced crystals is dependent on existing microtubules (Stebbings, 1975). Conversely, when the oocyte initiates vitellogenesis the microtubules undergo a striking change. Accompanying an exclusion of transported materials, the microtubules first associate laterally into pairs and then chains before developing a very compact, 'crystalline' structure. At this point microtubule reabsorption occurs and the tubulin is probably stored for later use (Bennett and Stebbings, 1979; Hyams and Stebbings, 1979b). The initial formation of chains is interesting as it suggests that there is some kind of controlling protein, presumably a MAP, that ensures a joining microtubule is diametrically opposed to the contact of an earlier microtubule. MAPs could also be important in controlling the breakdown and reabsorption of previously stable microtubules as, during compaction, the microtubules never touch and the clear zone is still maintained. Furtheremore the compaction is not caused by constriction of the surrounding membrane as initially the tube's diameter remains constant (Bennett and Stebbings, 1979; Hyams and Stebbings, 1979b).

1.2Ib: Electrophoretic characterisation of nutritive tube components

When a homogenate of *Notonecta* nutritive tubes is run on a one-dimensional polyacrylamide gel (1-D PAGE) approximately 30 bands are resolved (Hyams and Stebbings, 1979a; Sharma and Stebbings, 1985) with tubulin comprising approximately 50-60% of total nutritive tube protein. Under two-dimensional gel electrophoresis (2-D PAGE) tubulin is

resolvable into 2 α and 2 β tubulin species, while a number of low molecular weight bands and a closely migrating high molecular weight pair are also visible. Actin and myosin are not detectable (Sharma and Stebbings, 1985; Stebbings *et al*, 1986; Hyams and Stebbings, 1979a). If the trophic region's protein content is analysed then the total number of protein bands increases while the tubulin content falls significantly, there being a lot of low molecular proteins that co-migrate with ribosomal proteins (Sharma and Stebbings, 1985). The gel staining patterns of pre- and post-vitellogenic oocytes and that of the trophic region are very similar indicating that the contents of the trophic region are probably transported to the developing oocytes, the latter synthesising very little protein themselves (Sharma and Stebbings, 1985). Two other bands can also be identified in whole ovary homogenates which have similar molecular weights to chicken brain kinesin and pig brain dynein (Anastasi *et al*, 1990).

The high molecular weight, non-motor, MAPs of the nutritive tubes are of great interest as they differ in weight and have no cross reactivity to brain MAPs. They do, however, have similar properties as they are heat stable and promote microtubule assembly (Stebbings *et al*, 1986; Anastasi *et al*, 1991). Their binding is nucleotide sensitive but, despite being removed from microtubules by increasing levels of ATP and GTP, they do not posses an ATPase activity like that of motor proteins. It is possible that, like dynein, these proteins work both as crossbridges and microtubule motors. The proteins are also interesting as their molecular weights differ slightly between different hemipteran species and they are ovary specific and do not occur in neuronal tissue (Anastasi *et al*, 1991). As the latter is also a translocation system this suggests that the proteins are not motor proteins, while the absence of neurofilaments in ovaries suggests that they have no function in cross-linking microtubules to neurofilaments. The interspecies variability between the proteins is probably important due to the wide range of conditions the species live under (ranging from sub-zero to tropical conditions) necessitating differing levels of microtubule stability. There is also a difference in the microtubule-microtubule spacing of the nutritive tubes between these species which may result

from the differing MAP content (Anastasi *et al*, 1991; Stebbings *et al*, 1991).

1.2Ic: Nutritive tube-associated transport

Ovarioles continuously synthesise rRNA which is transported from the trophic region to the oocytes both *in vivo* and in an *in vitro* system (Macgregor and Stebbings, 1970; Stebbings and Ratcliffe, 1973). The movement of labelled proteins through the nutritive tubes can be followed electrophoretically, the microtubules forming a static skeleton through which other, newly synthesised, components move at differing rates. The high molecular weight proteins (see Section 1.2Ib) seem to have the most dynamic relationship with the cytoskeleton (Stebbings *et al*, 1985). As the movement of components is generally slow it suggests the existence of a passive method of transport based on the synthesis of material at the trophic region pushing material through the tubes. In this case the microtubules' sole transport function would be to form a conduit system through which this could occur (Hyams and Stebbings, 1979a). This does not, however, explain the variable speed of components noted in both *Notonecta* and *Oncopeltus* (Stebbings *et al*, 1985; Stebbings and Hunt, 1987). It is possible that the components move down an electropotential gradient, as in *Dysdercus*, although in this example the movement of mitochondria in the nutritive tubes is supposed to be microtubule mediated and the ability to produce a steep enough voltage gradient over a long nutritive tube is questioned (Munz and Dittmann, 1987).

When nutritive tubes are isolated from *Oncopeltus* the ends fray into strands composed of small bundles of microtubules along which mitochondria translocate on the addition of ATP or GTP. Movement appears to be exclusively towards the oocyte, the retrograde direction, and is smooth and faster than i*n vivo*. This difference in velocity could be due to fraying making more ATP available to the driving motors or the removal of a structural block present in the intact system. In this sense movement is characteristic of dynein but it is inhibited by AMP-PNP, a kinesin inhibitor, and not by the classic dynein inhibitors (Stebbings and Hunt, 1987). ATP sensitive MAPs isolated from *Oncopeltus*

ovarioles do, however, have molecular weights similar to kinesin and dynein (Anastasi *et al*, 1990). A mixture of these support bidirectional motility of latex beads *in vitro*, the anterograde movement being very smooth while the retrograde movement is more jerky with beads sometimes leaving the microtubule. Both movements are inhibited by AMP.PNP while only the retrograde movement is inhibited by UV-vanadate cleavage. This suggests that there are multiple translocators bound to the beads. If the ATP extract is fractionated the kinesin homolog promotes anterograde bead movement while the dynein supports neither binding nor movement. No single fraction is capable of supporting retrograde movement. A similar lack of dynein mediated movement is found in squid optic lobes (Schnapp and Reese, 1989) and it is possible that the sucrose gradient fractions produced contain a level of dynein that is not high enough to promote motility or that some essential co-factor is lost during purification. Conversely, kinesin cannot be identified in the nutritive tubes of *Oncopeltus* suggesting it has a function elsewhere in the system, possibly in the trophic region (Anastasi *et al*, 1990).

1.3: Aims of the project

The general aim of this project was to elucidate further the structure and function of hemipteran nutritive tubes and in particular the component microtubules. Whilst this is a system which is very amenable to microtubule research, the microtubule biochemistry has been less well characterised. It was therefore decided to approach this work in two ways which, ultimately, would compliment each other. Firstly, the tubulin isotype content of *Notonecta* and *Oncopletus* ovarioles were studied using already existing, well characterised, antibodies. This would take the form of both *in vitro* immunological quantification followed by a more detailed *in vivo* analysis using frozen sections of ovariole material. Secondly, monoclonal antibodies were to be raised against purified *Notonecta* tubulin. These antibodies, when characterised, could then be used to further explore the structure and function of nutritive tubes.

2.1 MATERIALS AND METHODS

2.1I: Insects

Notonecta glauca glauca were collected from ponds in the locality of the University of Exeter and used immediately. *Oncopeltus fasciatus* were taken from laboratory stocks maintained at a temperature of 37°C and fed on a diet of dried sunflower seeds.

2.1II: Chemicals

EDTA (Ethylenediamine tetra acetic acid), EGTA (Ethyleneglycol-bis-(β aminoethyl ether) N N N' N'-tetraacetic acid) and TEMED (N N N' N'-tetramethylethylene diamine) were obtained from BDH Chemicals Ltd. Dulbeco's modification of Eagle's medium (DMEM); L-glutamine; Penicillin; Streptomycin; foetal calf serum (FCS) and Grace's medium were from Flow Laboratories. Polyethylene glycol (PEG) was obtained from Boehringer. Ampholines were from LKB Quality Chemicals and OCT compound was obtained from BDH Chemicals Ltd. All other chemicals were obtained from Sigma Chemical Company.

2.1III: Antibodies

The antibodies used are listed in Table 2.

2.1IV: Analytical procedures

2.1IVa : One-dimensional polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS) 1D-polyacrylamide gel electrophoresis (PAGE) was carried out in a Bethesda Research Laboratories model V16 Vertical Electrophoresis System or a Mighty Small II SE 250 mini gel system using 7.5% or 5 to 15% gradient separating gels according to Laemmli (1970). In both cases the 7.5% separating gels were 1.5mm thick and were composed of 375mM Tris (Tris [hydroxymethyl] aminomethane), pH 8.8; 0.1% (w/v) SDS; 7.5% or 5 to 15% (w/v) acrylamide; 0.2% (w/v) bis-acrylamide; 0.03% (w/v) ammonium persulphate; 0.1% (v/v) TEMED. The stacking gels were 2 cm and 0.75 cm deep respectively and were composed of 125mM Tris, pH 6.8; 0.1% (w/v) SDS; 3% (w/v) acrylamide; 0.8% (w/v) bisacrylamide; 0.09% ammonium persulphate; 0.2% TEMED. Gradient gels were poured by placing half a volume of 15% acrylamide mixture; 11.5%

ANTIBODY	MONOCLONAL/POLYCLONAL	ORGANISM IMMUNOGEN DERIVED FROM	IMMUNOGEN ANTIBODY RAISED AGAINST	REFERENCES
YOL1/34	MONOCLONAL	YEAST	PURIFIED YEAST TUBULIN	KILMARTIN ET AL., 1982
YL1/2	MONOCLONAL	YEAST	PURIFIED YEAST TUBULIN	KILMARTIN ET AL., 1982
aT12	POLYCLONAL	PIG	SYNTHESIZED-GLU-TUBULIN	KREIS, 1987
aT1A	POLYCLONAL	PIG	SYNTHESIZED-TYR-TUBULIN	KREIS, 1987
TA2	MONOCLONAL	PIG	SYNTHESIZED-TYR-TUBULIN	KREIS, 1987
TD5	MONOCLONAL	PIG	SYNTHESIZED-GLU-TUBULIN	KREIS, 1987
KMX1	MONOCLONAL	PHYSARUM	PURIFIED PHYSARUM TUBULIN	BIRKETT ET AL., 1985
GLU-B-1	MONOCLONAL	SEA URCHIN	PURIFIED SPERM TUBULIN	PIPERNO AND FULLER, 1985
DM1A	MONOCLONAL	CHICKEN	PURIFIED BRAIN TUBULIN	BLOSE ET AL., 1984
ANTI-GLU	POLYCLONAL	PIG	SYNTHESIZED-GLU-TUBULIN	GUNDERSEN ET AL., 1987
ANTI-TYR	POLYCLONAL	PIG	SYNTHESIZED-TYR-TUBULIN	GUNDERSEN ET AL., 1987
MAP2.2	MONOCLONAL	COW	PURIFIED MAP-2 ARM DOMAIN FROM BRAIN	OBAR ET AL., 1987
MAP2.3	MONOCLONAL	COW	PURIFIED MAP-2 ARM DOMAIN FROM BRAIN	OBAR ET AL., 1987
MAP2.4	MONOCLONAL	COW	PURIFIED MAP-2 BINDING DOMAIN FROM BRAIN	DINGUS ET AL., 1991

Table 2 The Antibodies Used in This Work

(w/v) sucrose; 0.03% (w/v) ammonium persulphate; 0.1% (v/v) TEMED in the front chamber and half a volume of 5% acrylamide gel mixture in the rear chamber of a gradient gel maker. The volumes were used so the final gel mixture would exactly equal the total separating gel volume needed. The contents of the rear chamber were allowed to run into the front chamber, which was rapidly mixed, and the resulting solution poured from the front chamber into the gel apparatus. Samples were boiled for five min. in Laemlli sample buffer [30mM Tris, pH 6.8; 5% (w/v) SDS; 10% (v/v) glycerol; 5% (v/v) 2-mercaptoethanol; 0.1% (w/v) bromophenol blue] prior to running. The running buffer contained 100mM Tris pH 8.2-8.6; 77mM glycine; 0.05% (w/v) SDS and gels were run at a constant current of 50mA using a LKB 2301-Macrodrive 1 power supply.

Gels were calibrated using Sigma high molecular weight markers consisting of: myosin, 205KD; β-galactosidase, 116KD; phosphorylase B, 97.4 KD; bovine albumin, 66KD; egg albumin, 45KD; carbonic anhydrase, 29KD which were loaded at 10µg and 30µg per lane for mini gels and full size gels respectively. Gels were either stained with a solution of 40% (v/v) isoproponol; 5% (v/v) glacial acetic acid; 0.05% Coomassie brilliant blue R-250 for 16 h and destained in 40% isoproponol; 5% glacial acetic acid or were silver stained (Section 2.1IVc) or used for immunoblot analysis (see Section 2.1IVd). Densitometry measurememts of gel bands were carried out on a positive photographic image using a Joyce Loebl Chromoscan 3.

2.1IVb: Two dimensional polyacrylamide gel electrophoresis

Two dimensional polyacrylamide gel electrophoresis (2-D PAGE) samples were prepared by mixing 100µl of sample, at a maximum concentration of 3.5mg/ml, with 50µl of lysis buffer (20mM Tris pH 7.6, 5mM MgCl; 1% (v/v) nonidet P-40; 50µg/ml leupeptin). This was then frozen in liquid nitrogen then rapidly thawed and mixed. This process was carried out three times. 3.5µl of 2.5mg/ml protease free DNase and 2.5µl of 1.5mg/ml RNase were then added and the sample incubated on ice for 15 min. 50mg of urea and 100µl of solution A [2% (v/v) nonidet P-40; 1.2% (v/v) Ampholines pH 3-10; 0.8% (v/v) Ampholines pH 5-7; 5% (v/v) 2-mercaptoethanol; 9.5M urea] were then added. The sample was subsequently warmed to room temperature and then subjected to a further cycle of

freeze/thawing.

Isoelectric focusing gels (IEF gels) were run essentially after O'Farrel (1975) in 2.5mm internal diameter x 120mm tubes. Before use the tubes were soaked in chromic acid for 1h; rinsed in deionised water followed by 50% (v/v) EtOH, 50% (v/v) 50mM KOH in deionised water then finally rinsed in deionised water. The tubes were sealed with Parafilm and loaded to a depth of 115mm with: 94% (w/v) urea; 6.43% (w/v) acrylamide; 0.37% (w/v) bisacrylamide; 3.4% (v/v) nonidet P40; 5% (v/v) Ampholines pH 3.5-10.0; 3% (v/v) Ampholines pH 5.0-7.0; 0.03% (w/v) ammonium persulphate; 0.17% (v/v) TEMED in deionised water. After polymerisation had taken place the tubes were fixed vertically into a Shandon Disc Electrophoresis IEF tank with 4% (w/v) agarose and the upper and lower electrode buffers were added [0.08% (w/v) NaOH and 0.09% (v/v) phosphoric acid respectively]. Prior to loading, gels were prefocussed at 200V, 15min; 300V, 30min then 400V, 30min. 50µl of sample was then added to each tube and overlayed with 10µl of 33% (v/v) solution A in deionised water. Gels were run for 400V, 16.5h followed by 800V, 3h. After removal from the tubes gels were equilibrated for 25 min. in Laemlli sample buffer. They were then fixed into position on top of a 7.5% 1D-PAGE with 1% agarose in 125mM Tris, pH6.8; 0.1% (w/v) SDS in distilled water and run as in Section 2.1IVa.

IEF gels that were not run in the second dimension were either stained for 15 min. in 40% (v/v) isopropanol; 5% (v/v) glacial acetic acid; 0.05% (w/v) Coomassie Brilliant Blue R-250 and destained in 40% (v/v) isopropanol; 5% (v/v) glacial acetic acid or they were wrapped in aluminium foil and stored at -20°C. The isoelectric gradient along the IEF gels was calculated by slicing the gels into 10mm sections which were then each eluted in 5 ml deionised water. The pH of each section was then measured. Alternatively Sigma IEF markers (3.6-9.3) were used.

2.1IVc: Silver staining

Silver staining was carried out using the National Diagnostics Protostain silver stain system. Gels were initially washed for 10 min. in 50% (v/v) isopropanol; 5% (v/v) glacial acetic acid in deionised water (as were all subsequent solutions). The gels then had two 10 min. washes in 5% (v/v) isopropanol; 5% (v/v) glacial acetic acid. After

thorough rinsing in deionised water the gels were sensitised for 5 min. in 20% (w/v) *Sensitizer* then rinsed thorougly in deionised water. The gels were then soaked, under fluorescent light, for 20 min. in 20% (v/v) *Silver Stain*, washed briefly in deionised water and then developed in 2.3% (w/v) *Developer* (which was changed twice after 1 min. soaking). The reaction was stopped using 2% (v/v) glacial acetic acid after which the gels were rinsed thorougly in distilled water.

2.1IVd: Immunoblotting

Transfer to 0.45µm cellulose nitrate filters (Schleicher and Schuell) was carried out in one of two ways: i) Large gels were pre-soaked for 25min in 12.4mM Tris, pH 8.4; 98mM glycine; 0.01% (w/v) SDS; 0.07% (v/v) 2-mercaptoethanol and then transfered in fresh buffer of the same composition using a Biorad-Transblot blotting tank with a constant voltage of 40V for 24h at 4°C.

ii) Mini gels were transfered in 25mM Tris, pH8.4; 192mM glycine; 0.01% (w/v) SDS; 20% MeOH using a TE Series Transphor Electrophoresis Unit and a constant voltage of 100V for 3h.

The nitrocellulose was air dried and at this point could be stored indefinitely. Two methods of immunoblotting were used:

i) Immunoperoxidase blotting was carried out according to the following schedule. Nitrocellulose was blocked in 50mM Tris, pH7.4; 0.9% (w/v) NaCl; 0.025% (w/v) BSA; 0.01% (v/v) nonidet P-40; 0.01% (v/v) Tween-20 for 30 min and was allowed to air dry. Filters were then incubated overnight in the required primary antibody diluted in wash buffer (50mM Tris, pH7.4; 0.9% (w/v) NaCl; 0.01% (v/v) nonidet P-40; 0.01% (v/v) Tween-20). The nitrocellulose was washed four times in wash buffer before being incubated for 6 hours in secondary horseradish peroxidase-conjugated antibody (diluted 1/250 (v/v) in wash buffer; anti-mouse, Sigma A 5906; anti-rabbit, Sigma A 6154; anti-rat, Sigma A 9037). Following two washes in wash buffer and two washes in TBS (50mM Tris, pH7.4; 0.15M NaCl) the nitrocellose was developed in 0.3% (w/v) 4-chloro-1-napthol; 0.01% (v/v) hydrogen peroxide in TBS.

ii) Immunophosphatase blotting was carried out according to the following schedule. Nitrocellulose sheets were blocked for 1h in 5% Marvel dried skimmed milk powder (Premier Brands, UK) in PBS-Tween (0.15M NaCl; 0.01M NaPi; 0.5% (v/v) Tween-20). They were then washed extensively in PBS-Tween and then incubated for 16h in the required primary antibody diluted in PBS-Tween. Filters were then washed twice in PBS-Tween and were incubated for 6h in secondary phosphatase-conjugated antibody (1/100 in PBS-Tween; anti-mouse, Sigma A 1902; anti-rabbit, Sigma A 8025; anti-rat, Sigma A 9529). After two washes in PBS-Tween and two washes in 0.15M sodium barbitone, pH9.6 the nitrocellulose was developed in 133mM sodium barbitone, pH9.6; 4mM magnesium chloride; 10% (w/v) nitroblue tetrazolium; 5% (w/v) 5-bromo-4-chloro-indoylphosphate.

2.1IVe: Dot-blotting

A solution of taxol purified *Notonecta* tubulin (see Section 2.1Vb), at a concentration of 3mg/ml was incubated for 16 hours at room temperature with a square of nitrocellulose to give a final weight:area ratio of 0.6mg/cm^2. The nitrocellulose was then extensively washed in PBS before being blocked for 5 hours in 3% (w/v) BSA; 0.02% (w/v) sodium azide in PBS. Following a further extensive series of washes in PBS the filter was dried and stored, prior to use, in a plastic bag at -70°C.

Before use, the nitrocellulose was divided into 1mm^2 squares by pencilled rulings and 1µl of each tissue culture supernatant was dotted into a separate square. The nitrocellulose was then incubated at 37°C for 60 min. and then washed thoroughly in wash buffer. The appropriate secondary antibody was then added and the filter incubated at room temperature for 6h. The filter was then thoroughly washed prior to being developed as in Section 2.1IVd.

2.1IVf: Immunoblot decolourisation

Previously developed immunoblots were decolourised prior to reprobing by the following procedure. Nitrocellulose was incubated in 62.5mM Tris HCl pH6.8, 100mM β-mercaptoethanol, 2% (w/v) SDS at 70°C for 30 min.

After thorough washing in TBS, pH7.4 the nitrocellulose was blocked and probed as in Section 2.1IVd.

2.1IVg: Carboxypeptidase A treatment of nitrocellulose

After blocking the nitrocellulose, as in Part (ii) of Section 2.1IVd, it was washed thoroughly in PBS then incubated in 20µg/ml carboxypeptidase A (CPA) for 30 min. at 37°C. After further thorough washes, twice in PBS and twice in PBS-Tween, the nitrocellulose was incubated in the required primary antibody, the rest of the immunolocalisation procedure being carried out using the phosphatase blotting method described in Part (ii) of Section 2.1IVd.

2.1IVh: Protein concentration determination

Protein concentrations were determined using the method of Bradford (1976). The sample was diluted 1/10 in distilled water and 100µl added to 2 ml of Bradford's reagent (15% (v/v) ethanol; 10% (v/v) ortho-phosphoric acid; 10% (w/v) Coomassie Brilliant Blue G250). The protein concentration was then calculated by measuring the absorbance at 580nm in a Philips PU 8740 scanning spectrophotomer, zeroed with a blank of buffer diluted 1/10 in distilled water, and compared to a BSA standard curve.

2.1V: Microtubule preparation

2.1Va: Bovine brain microtubule preparation

The method used essentially followed Vallee (1986). Four calves cerebral cortexes were collected on slaughter and kept on ice for a maximum of 1h. They were then cut into small pieces and cold homogenised in PEM buffer (100mM Na-PIPES [piperazine-N,N'-bis-(2-ethanesulfonic acid), monosodium salt], pH6.6; 1mM EGTA; 1mM $MgSO_4$), at a concentration of 1g of tissue to 1.5 ml of buffer, by two low speed 5 second passes in a Waring blender followed by two high speed 30 second runs in a Silverson mixer-emulsifier. The homogenate was centrifuged at 15,000g, 4°C, 90 min. (MSE 21 High Speed Centrifuge; 6x300 rotor). GTP was added to the supernatant to a final concentration of 1mM and this was

incubated, with regular, gentle swirling, at 37°C for 25 to 30 minutes. The suspension was then spun at 15,000g, 37°C for 45 min. (MSE 21, 6x300 rotor). The pellets were resuspended in 5 vols of PEM, pH6.6; 1mM GTP and homogenised to a uniform consistency in a teflon pestle homogeniser before being incubated on ice for 30 min. The suspension was centrifuged at 30,000g, 4°C, 20 min. (MSE 21; 8x50 rotor). The resulting supernatant was removed and incubated at 37°C for 15 min. (1mM GTP was added if necessary), following which the microtubules were pelleted at 30,000g, 37°C for 20 min. (MSE 21 8x50 rotor). The pellets (H_2P) could then be frozen by immersing in liquid nitrogen and stored at -70°C.

For further work pellets were thawed and resuspended in 5 volumes of PEM, pH6.6; 1mM GTP before being taken through a cycle of cold depolymerisation, cold centrifugation, polymerisation and warm centrifugation as above.

2.1Vb: *Notonecta* microtubule preparation

Ovariole homogenisation and microtubule purification were carried out essentially after Stebbings *et al* (1986). For homogenisation ovarioles from adult females were dissected out in lysis buffer (PEM pH6.9; 0.1mM PMSF; 1mM dithiothreitol; 1µg/ml pepstatin A; 10µg/ml TPCK; 1µg/ml leupeptin; 1µg/ml aprotinin; 10µg/ml TAME; 10µg/ml BAME; 7µl of buffer for every pair of ovaries) and homogenised in a teflon pestle homogeniser. For microtubule preparations a minimum of 35 pairs of ovaries were dissected out and washed three times in lysis buffer. They were then pooled in 3 vols of lysis buffer and homogenised in a teflon pestle homogeniser. EGTA was then added to 5mM and the homogenate centrifuged at 18,000g, 4°C for 30 min. (Europa 24-M Centrifuge; 8x50 rotor). EGTA was added to 2mM to the resulting supernatant which was then left on ice for 5 min. following which taxol was added to 20µM, GTP to 2mM and the solution was warmed at 37°C for 20 min. A cushion containing 15% (w/v) sucrose in lysis buffer; 20µM taxol; 2.0mM GTP was then prepared. An equal volume of this solution was layered on top of the warmed supernatant and spun at 27,000g, 30°C, 30 min. The microtubule pellet was removed and resuspended in 4-5 volumes of lysis buffer with 2.0mM GTP; 20µM taxol and centrifuged at 18,000g, 30°C for

30 min. The pellet was then frozen in liquid nitrogen where it could be stored indefinately.

2.1Vc: Carboxypeptidase assay

A pellet of 3 times cycled, taxol assembled bovine brain microtubules was resuspended in a homogenate of 30 *Oncopeltus* ovarioles (7mg/ml of protein) to give a final bovine brain microtubule concentration of 13mg/ml. This was incubated for 30 min. at 37°C. At the same time 20µg/ml of CPA was incubated with 13mg/ml bovine brain microtubules, an ovariole extract was incubated with 20µg/ml of CPA and 13mg/ml bovine brain microtubules were incubated with PEM. Each sample was incubated for 30 min at 37°C. The samples were run in duplicate on a 1D polyacrylamide gel then immunoblotted using the appropriate primary antibodies plus phosphatase-conjugated secondary antibodies.

2.1VI: Immunofluorescence techniques

A number of different cultured cell lines and tissues were prepared for visualisation by immunofluorescence. These are outlined individually below.

2.1VIa: Tissue culture cells

3T3 mouse fibroblasts, kindly provided by N. Pringle (UCL), were grown in DMEM containing 10% (v/v) FCS; 2mM L-glutamine; 100IU/ml Penicillin and 100µg/ml Streptomycin (complete DMEM) in 5 cm tissue culture dishes (Falcon) at 37°C, 5% CO_2 in a Leec MkII CO_2 incubator. For subculturing near confluent cells were detached by two 2 min. incubations in 0.25% trypsin at 37°C and diluted 1/20 into fresh medium.

For immunofluorescence cells were grown on sterile, round, 13mm, no. 1½ coverslips to near confluency. Cells were washed in TBS, pH 7.4, 37°C and fixed using one of the two following methods:

i) Coverslips were immersed in pre-chilled methanol at -20°C for 5 min. then rinsed thoroughly in TBS at room temperature.

ii) Coverslips were washed in PBS at 37°C and fixed in 4% paraformaldehyde in PBS at room temperature for 10 min. All subsequent steps were carried out at room temperature

(unless noted otherwise). After rinsing in PBS, cells were permeabilised in methanol for 2 min. then rinsed thoroughly in PBS.

In both cases, after rinsing, each coverslip was inverted over the appropriate primary antibody on a piece of Nescofilm (usually 10µl at 1/100 in TBS). They were then incubated for 60 min, 37°C in a humid chamber. After four 4 min. washes in TBS the incubation was repeated with the appropriate rhodamine-conjugated fluorescent secondary antibody (usually 1/50 in TBS; anti-mouse, ICN Flow 61-268; anti-rat, ICN Flow 61-267-1; anti-rabbit, ICN Flow 61-266). After a further four 4 min. washes in TBS each coverslip was rinsed in distilled water and inverted into 15µl elvanol (PBS, pH8.2 [137mM NaCl; 3mM KCl; 6mM Na_2HPO_4; 1mM KH_2PO_4] plus 14% (w/v) Gelvatol (Monsanto); 28% (v/v) glycerol) containing 1µg/ml DAPI (4', 6-diaminodiamidino-2-phenylindole) and 1mg/ml para-phenylene diamine (antifade). Slides were left to harden overnight at 37°C then veiwed using a Zeiss Axiophot Photomicroscope equipped with epifluorescence optics. Images were recorded on Kodak T-MAX 400 film.

2.1VIb: Insect ovarioles

Ovaries were removed under insect Ringer and fixed for 14 hours in 10% (w/v) paraformaldehyde in 0.2M phosphate buffer. The yolky oocytes were discarded and the tissue cryoprotected in PBS, pH7.4; 10% (w/v) sucrose for 60 min. before being immersed in OCT compound and frozen in liquid nitrogen. 7µm thick transverse or longitudinal sections were cut on a Reichert-Jung Cryocut E cryostat and mounted on slides coated with 0.1% (v/v) poly-lysine. They were then permeabilised in PBS; 0.2% (v/v) Triton X-100 for 30 min. After extensive PBS washes the sections were blocked in PBS; 20% (v/v) normal goat serum; 0.1% (w/v) BSA for 60 min. and then incubated for 14 hours in the required primary antibodies (diluted in PBS; 0.1% BSA). After further thorough washes in PBS the sections were incubated for 2 hours in the appropriate rhodamine-conjugated secondary antibodies (ICN Immunobiologicals, diluted 1:60 in PBS) before being mounted in PBS; 90% (v/v) glycerol; 1mg/ml p-phenylene diamine (antifade) and viewed using a Zeiss Universal microscope, equipped with epifluorescence optics. Images were recorded on Kodak T-MAX 400 film.

2.1VIc: Insect neuronal tissue

Oncopeltus ganglia were dissected under insect Ringer from the thoracic and abdominal cavities and then fixed and processed as for ovarioles (see Section 2.1VIb).

2.1VId: *Drosophila* B_{II} cell line

Drosophila B_{II} (l[2]mbn) cells, kindly supplied by Chris Clement (University of Exeter), were grown in Schneider's medium then treated with the moulting hormone 20-hydroxyecdysone for 24h (Gateff, 1978; Clement and Dinan, 1990). They were then allowed to settle on microscope slides and were fixed for 10 min. in 4% (w/v) formaldehyde in grasshopper Ringer. After rinsing in PBS the cells were permeabilised in 0.1% (v/v) Triton in PBS for 10 min., washed and then incubated in PBS plus 20% (v/v) normal sheep serum; 0.1% (w/v) BSA for 30 min. After incubating in primary antibody diluted in PBS plus 0.1% (w/v) BSA, for 16 hours, 4°C, the slides were extensively washed and incubated for 30 min, 25°C in the appropriate secondary antibody diluted 1/60 (v/v) in PBS; 0.1% (w/v) BSA. After further extensive washes the preparations were mounted in PBS; 90% (v/v) glycerol; 1mg/ml p-phenylene diamine (antifade) and viewed using a Zeiss Universal microscope. Images were recorded on Kodak T-MAX 400 film.

2.2I: Antibody production

2.2Ia: Growth media

SP2 cells (mouse myeloma), kindly provided by Nigel Pringle (UCL), were grown in complete DMEM (see Section 2.1VIa) and were subcultured every three days by a 1/20 dilution into fresh medium. Hybridomas were grown initially in AH medium before being grown in H medium prior to testing and cloning. They were cloned and subsequently grown in cloning medium. All growth took place at 37°C, 5% CO_2 in a Leec MkII CO_2 incubator.

AH medium I:

DMEM
15% foetal calf serum
2mM L-glutamine
1µg/ml azaserine
100µM hypoxanthine
100IU/ml Penicillin
100µg/ml Streptomycin

AH medium II:

DMEM
15% foetal calf serum
2mM L-glutamine
1µg/ml azaserine
100µM hypoxanthine
100IU/ml Penicillin
100µg/ml Streptomycin
15mg/ml oxaloacetate
5mg/ml sodium pyruvate
200IU/ml bovine insulin

H medium I:

DMEM
15% foetal calf serum
2mM L-glutamine
100µM hypoxanthine
100IU/ml Penicillin
100µg/ml Streptomycin

H medium II:

DMEM
15% foetal calf serum
2mM L-glutamine
100µM hypoxanthine
100IU/ml Penicillin
100µg/ml Streptomycin
15mg/ml oxaloacetate
5mg/ml sodium pyruvate
200IU/ml bovine insulin

Cloning medium:

DMEM	
20%	foetal calf serum
2mM	L-glutamine
100IU/ml	Penicillin
100µg/ml	Streptomycin
15mg/ml	oxaloacetate
5mg/ml	sodium pyruvate
200IU/ml	bovine insulin

2.2Ib: Feeder cells

All injections, subsequent tail bleeds and sacrifices were kindly carried out by Nigel Pringle and Huw Davies (both UCL). A Balb-c mouse was killed by cervical dislocation and the abdominal skin peeled back. 5 ml of AH medium was injected into the peritoneal cavity, the mouse was briefly agitated and the medium withdrawn. This was repeated three times. The cells were pelleted at 1000rpm, 37°C for 5 min. (Beckman TJ-6 refrigerated centrifuge; as were all cell pelletings in monoclonal production), resuspended in medium and aliquoted into tissue culture trays. They were grown for three days before use to check for any possible contamination.

2.2Ic: Keyhole limpet haemocyanin coupling

An equal volume of keyhole limpet haemocyanin (KLH, at the same concentration as the immunogen) in 40mM sodium ortho-phosphate, pH7.2 was mixed with the immunogen to be injected. Half a volume of 20mM glutaraldehyde in 40mM sodium ortho-phosphate, pH7.2 was added dropwise with stirring to the KLH-immunogen mixture and then stirred at 25°C for 30 min. The mixture was dialysed (29x44 Visking tubing) for 18 h at 4°C against 2 litres of 40mM sodium ortho-phosphate, pH7.2.

Alternatively the KLH was fixed prior to coupling. 1 ml of KLH, at the final concentration used for coupling, was mixed with 1 ml of 20mM glutaraldehyde in 40mM sodium ortho-phosphate, pH7.2 and mixed at 25°C for 25 min. The mixture was dialysed for 18 h at 4°C against 2 litres 40mM sodium ortho-phosphate, pH7.2. The KLH was then reacted with the immunogen as above.

2.2Id: Mouse immunisation schedule

In all cases, after carrying out a pre-immunisation bleed, Balb-c mice were immunised with 250µl of KLH coupled immunogen homogenised with an equal volume of complete Freund's adjuvent (the concentrations are detailed in the results section). Boosts were given using Freund's incomplete adjuvent (as were all subsequent injections) at 21 days. After a further 10 days tail bleeds were taken and tested. Initially all positive mice were immunised three days later with 250µl of immunogen interperitoneally (Method I) or, in later experiments 11 days later, 100µl intravenously and 100µl interperitoneally (Method II). In both cases their spleens were removed 3 days after boosting and fused.

2.2Ie: Testing tail bleeds

Blood was taken from a nick at the end of the tail and incubated at 37°C for 1 hour before being transfered to 4°C for 16 h. The resulting clot was pelleted by centrifugation for 5 min. at full speed in a MSE microcentaur. The serum was removed and centrifuged a second time for 10 min. Sodium azide was added to 0.02% to the supernatant and the sera was tested by immunoblotting nitrocellulose strips of the immunogen (see Section 2.1IVd).

2.2If: Fusion I

SP2 cells were passaged 3 times prior to the fusion. A final volume of 150 ml of cells, approaching near confluence, and at a concentration of 10^6/ml was used for each fusion. A positively testing mouse was sacrificed and the spleen was removed aseptically and placed in 10 ml DMEM in a 5 cm petri dish. It was then finely teased apart using forceps and filtered through a 425µm metal millipore grid. Both the SP2 cells and the splenocytes were separately pelleted, resuspended in 10 ml DMEM and counted. They were then mixed in a ratio of one splenocyte to six SP2s (all the splenocytes being used) and pelleted. The pellet was placed in a 37°C sterile water bath and 1 ml. of DMEM plus 50% (w/v) polyethylene glycol (Boehringer 1500) at 37°C was slowly mixed in over a minimum period of 3 min. followed by 10 ml of DMEM at 37°C over 5 min. Cells were pelleted and resuspended in 36 ml AH selection medium I at 37°C, 0.5 ml then being aliquoted into the central 8 wells of nine 24

well tissue culture plates containing feeder cells. Cells were maintained in AH medium I until there was greater than 90% cell death. The medium was then changed by first adding 50% H medium I at 37°C, leaving for 24 h, then by carefully removing the medium and replacing totally with H medium I at 37°C.

2.2Ig: Fusion II

This was carried out exactly the same as in Fusion I (see Section 2.2If) except AH medium II and H medium II were used.

2.2Ih: Screening

When the hybridoma colonies were visible by eye they were screened. 750µl of tissue culture supernatant was removed from each cell containing well and used neat to probe by blotting nitrocellulose containing samples of the original immunogen (see Section 2.1IVd).

2.2Ii: Cloning I

Positive wells were resuspended in 3.5 ml DMEM; 20% FCS medium at 37°C which was then transfered to one well of a 6 well tissue culture plate. The cells were incubated for 16 h. After resuspending the cells 50µl of suspension was removed and diluted 1/100 in 5 ml FCS. The volume of live cells was counted and a volume containing 128 cells was added to 30 ml complete DMEM at 37°C. 100µl was pipetted into the central 60 wells of three 96 well dishes containing previously prepared feeder cells (see Section 2.2Ib). The remaining cells were transfered to 12 ml of complete DMEM, grown up and frozen. When colonies in the 96 well dishes were visible by eye they were diluted in 2.5ml of complete DMEM at 37°C and transfered to a 6 well dish. The colonies were then screened and the positives re-cloned.

2.2Ij: Cloning II

10µl of positive hybridoma cells were added to a Falcon tube containing 3ml of cloning medium. The cells were then successively diluted by removing 0.3ml of the cell dilution from the Falcon tube and adding it to a tube containing 2.7ml of cloning medium II. This was carried out a further two times to give final dilutions of 1:10, 1:100

and 1:1000. 100µl of each dilution was then added into 24 wells of a 96 well tissue culture plate, the wells already containing 50µl of feeder cells. Each plate thus has 24 wells of the 1:10 dilution, 24 wells of the 1:100 dilution and 24 wells of the 1:1000 dilution. Once the cells had grown up to be visible by eye they were tested.

2.2Ik: Cloning III

Positive wells were resuspended in cloning medium at 37°C which was then transfered to one well of a 6 well tissue culture plate. The cells were incubated for 16 hours. After resuspending the cells 50µl of suspension was removed and diluted 1/100 in 5 ml FCS. The volume of live cells was counted and a volume containing 128 cells was added to 30 ml cloning medium at 37°C. 100µl was pipetted into the central 60 wells of three 96 well dishes containing previously prepared feeder cells (see Section 2.2Ib). The remaining cells were transfered to 12 ml of cloning medium, grown up and frozen. When colonies in the 96 well dishes were visible by eye they were diluted in 2.5ml of cloning medium at 37°C and transfered to a 6 well dish. The colonies were then screened and the positives re-cloned.

2.2Il: Expanding and freezing positive clones

Cells from positive clones were transfered from the well, in 0.5 ml of cloning medium at 37°C, into a 24 well tray containing feeder cells (see Section 2.2Ib). When a high cell density was reached cells were transfered in 2 ml of cloning medium into a 6 well tray. After further growth cells were diluted in 5 ml cloning medium, at 37°C, and transfered to a 5 cm dish. When the cells had reached a concentration of 10^6/ml they were pelleted, the medium was removed and the pellet resuspended in 2 ml FCS, 5% (v/v) dimethylsulfoxide (DMSO) and divided between two 1 ml. cryotubes. The tubes were gradually cooled on ice for 10 min. and were then placed at -70°C for 24 h before being moved to liquid nitrogen for long-term storage.

2.2Im: Thawing cells

Tubes were thawed at 37°C and the contents added to 3 ml of the

required medium at 37°C. Cells were then pelleted and resuspended in 10 ml of the same medium and split between two 5 cm dishes.

3.1: RESULTS

3.1I: Post-translational modifications in 3T3 cells

The glu- and tyr-tubulin specific antibodies used in this study of tubulin isotypes of *Notonecta* and *Oncopeltus* were initially screened on 3T3 mouse fibroblasts. Figure 4a shows the characteristic pattern produced by the tyr-specific antibody 1A2 in interphase cells (Kreis, 1987). Extensive networks of microtubules are labelled. When compared with a similar cell stained with the broad spectrum, α-tubulin-specific antibody YOL1/34 (Kilmartin *et al*, 1982) no identifiable differences were apparent indicating that the tyr-specific antibodies recognise a large population of cellular microtubules (Figure 4b). Unfortunately, no mitotic cells could be identified as these round up and are lost during processing. The localisation of tyr-microtubules in the mitotic spindle could thus not be verified.

The distribution of microtubules labelled by glu-tubulin specific antibodies was quite different to that of tyr-microtubules. The glu-tubulin specific antibody ID5 (Wehland and Weber, 1987) recognised a very restricted subpopulation of interphase microtubules which had a characteristic sinous morphology (Figure 4c; Gundersen *et al*, 1984; Bulinski *et al*, 1988). This is particularly apparent in the microtubule identified by the arrow (Figure 4c). A similar distribution was obtained using an anti-acetylated tubulin monoclonal antibody (data not shown). Labelling by the glu-tubulin specific antibodies of the appropriate microtubules was further demonstrated by the increased levels of glu specific staining resulting from taxol stabilisation of the microtubules (Figure 4d; Gundersen *et al*, 1987).

3.1II: Post-translational modifications in *Notonecta* and *Oncopeltus* ovaries

3.1IIa: *Notonecta*

In initial experiments the number of α and β-tubulin isotypes in the two species was determined by Western blotting of insect protein

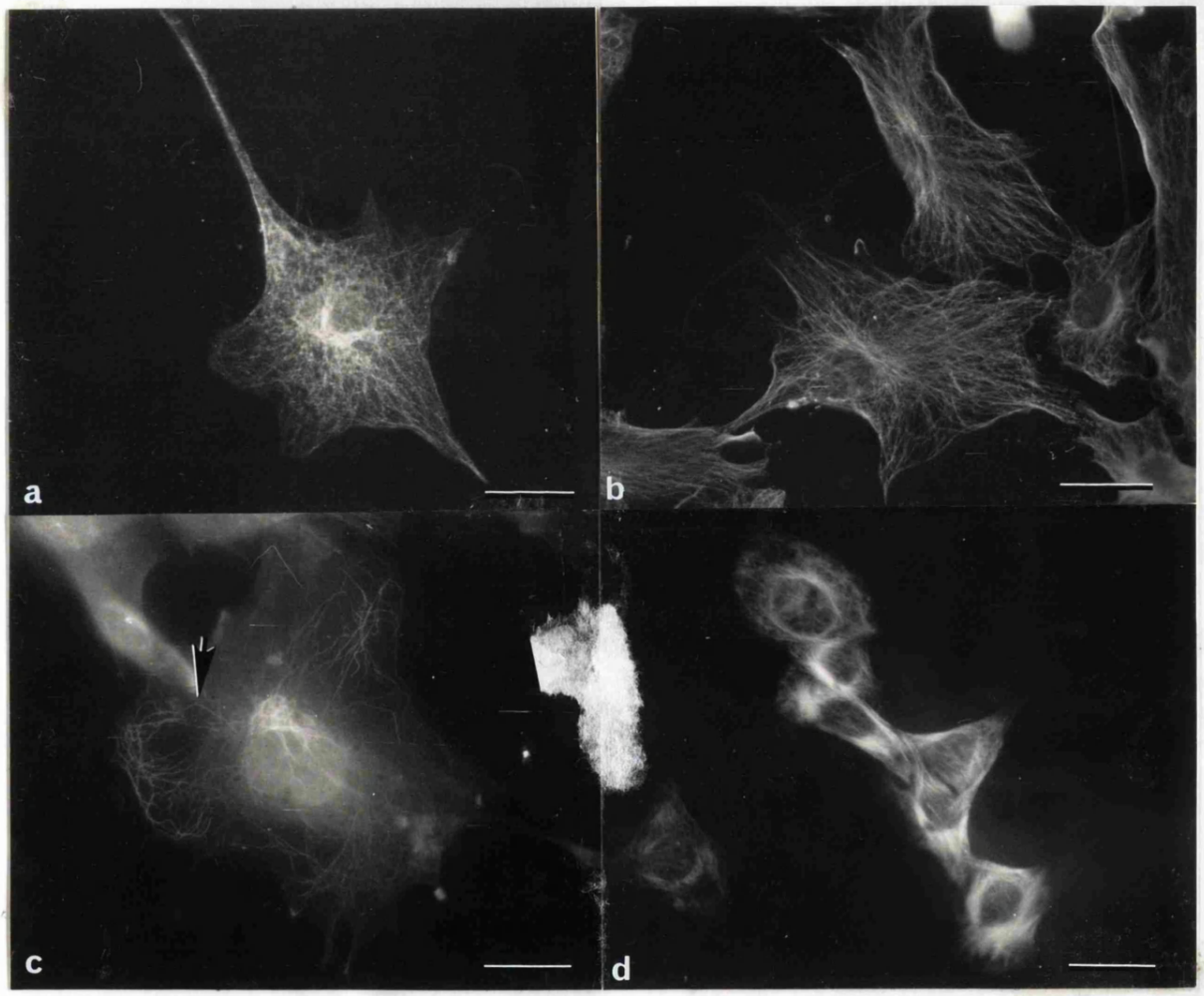


Figure 4 The Distribution of Tyr and Glu- Tubulin in 3T3 Cells
Tubulin isotype specific antibodies were used to stain methanol fixed 3T3 cells. (a) YOL1/34 (α-tubulin antibody); (b) αT13 (tyr-tubulin antibody); (c) ID5 (glu-tubulin antibody; the arrow indicates a sinous microtubule characteristic of a glu-tubulin containing microtubule); (d) Cells treated for 16 hours with 10μm taxol prior to fixing stained with ID5. YOL1/34 and αT13 were used at a dilution of 1:1000 while ID5 was used undiluted. In all cases the bar is 20μm.

separated by 1-D gel electrophoresis. Probing with an α-tubulin specific antibody produced one specific band with a molecular weight of approximately 52kD (Figure 5 Lane 1). In a further analysis, homogenates of *Notonecta* ovaries were separated by 2-D PAGE and probed with an anti-α-tubulin antibody, an elongated spot developing. This could be resolved into a major and a minor component (Figure 6a), both with a molecular weight of 52kD. The major component had an isoelectric point of 5.75, the smaller component being slightly more acidic. Probing with an anti-β-tubulin antibody revealed a single, very faint, spot with a molecular weight of 51kD and an isoelectric point of 5.70 (data not shown).

Having established that *Notonecta* ovarioles contain one major and one minor α-tubulin plus a single β-tubulin, the post-translational modifications of the α-tubulin were investigated. 1-D PAGE revealed the prescence of both tyr and glu-tubulin (Figure 5, Lanes 2 and 3). When the same antibodies were used to probe samples separated in two dimensions both the major and the minor tubulins were labelled with each antibody (Figure 6b and c). The identity of these spots was confirmed by first probing a nitrocellulose with an anti-α-tubulin antibody (Figure 7a) and then decolourising the spot that developed (Figure 7b). Subsequent reprobing with either the anti-tyr or the anti-glu antibody revealed the exact correspondence between the α-tubulin spot and the glu and tyr-tubulin spots (Figure 7c for an example). The one obvious difference occured when 2-D separated samples were probed with an antibody that recognised anti-acetylated tubulin. In this case only the minor, more acidic, α-tubulin component was recognised. There was no visible staining of the major component (Figure 6d). Consequently, following the convention for naming post-translationally modified tubulins (L'Hernault and Rosenbaum, 1983), I propose that the major component should be named α1 and the minor component α3 (Figure 6a).

3.1IIb: *Oncopeltus*

Similar experiments were carried out using *Oncopeltus* ovariole extracts. Extracts separated by 1D PAGE and probed with an anti-α-tubulin antibody again revealed the presence of only one specific band

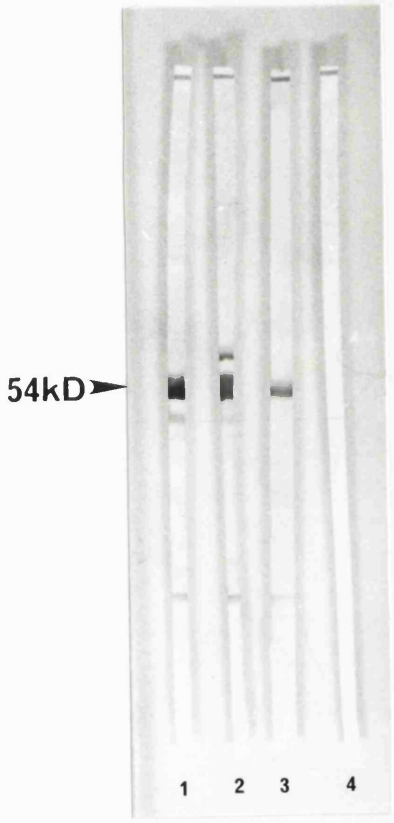


Figure 5 Tubulin Isotype Content of *Notonecta* Ovarioles (1-D)
Tubulin isotype specific antibodies were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strip 1: YOL1/34 (α-tubulin antibody); strip 2: anti-tyr (tyr-tubulin antibody); strip 3: ID5 (glu-tubulin antibody); strip 4: wash buffer used instead of a primary antibody. YOL1/34 and anti-tyr were used at a dilution of 1:1000 while ID5 was used undiluted. The amount of protein in each strip was approximately 25μg and all blots were developed using the peroxidase method. Tubulin is arrowed on the left

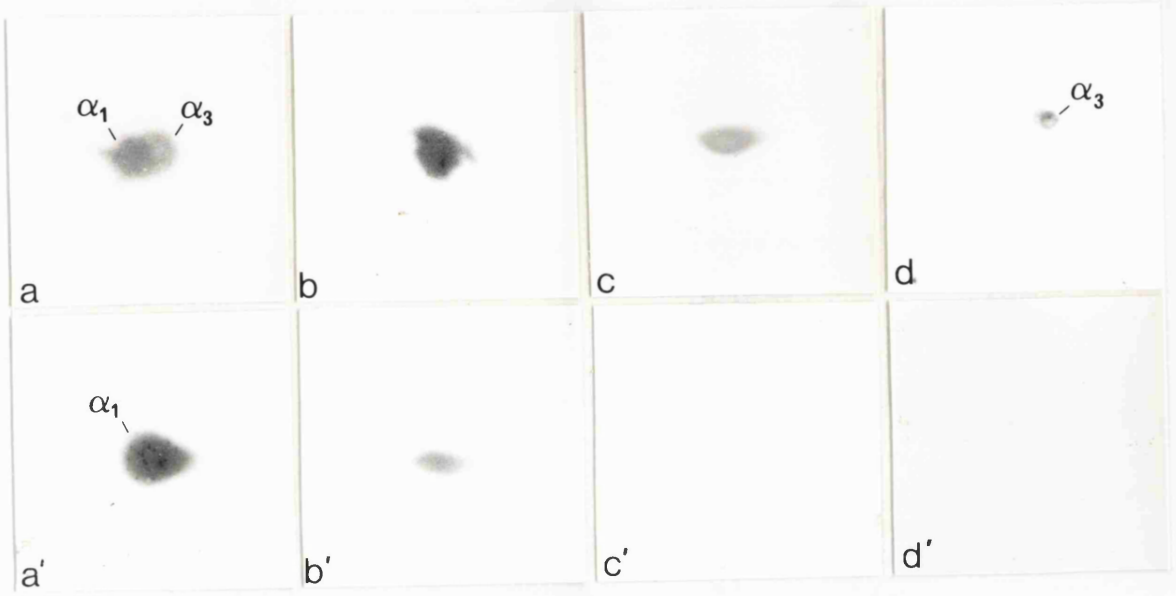


Figure 6 Tubulin Isotype Content of *Notonecta* and *Oncopeltus* Ovarioles (2-D)

Tubulin isotype specific antibodies were used to probe nitrocellulose containing taxol purified microtubule proteins from the ovaries of *Notonecta* (a to d) and *Oncopeltus* (a' to d') separated by 2-D PAGE. a and a': YOL1/34 (α-tubulin antibody); b and b': YL1/2 (tyr-tubulin antibody); c and c': ID5 (glu-tubulin antibody); d and d': 6-11B-1 (acetylated-tubulin antibody). YOL1/34 and YL1/2 were used at a dilution of 1:1000, 6-11B-1 at a dilution of 1:100 and ID5 was used undiluted. The amount of protein in each blot was 175µg and all blots were developed using the peroxidase method. The tubulin isotypes are indicated.

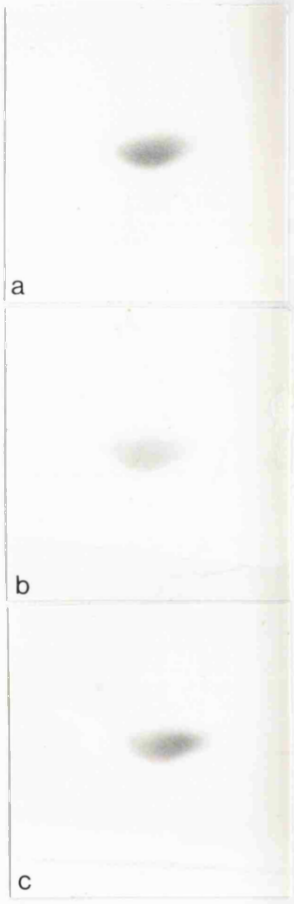


Figure 7 Nitrocellulose Decolourisation Showing the Coincidence of α-Tubulin Using Different Primary Antibodies

Nitrocellulose containing a 2-D separation of microtubule proteins from *Notonecta* homogenate was probed with YOL1/34 (α-tubulin antibody; a). After decolourisation (b) it was then reprobed with ID5 (glu-tubulin antibody; c). YOL1/34 was used at a dilution of 1:1000 while ID5 was used undiluted. The amount of protein in the blot was 175μg and it was developed using the phosphatase method.

with a molecular weight of approximately 52kD (not shown). A single α-tubulin band can, however, be observed in Figure 22. In contrast to *Notonecta*, an anti-α-tubulin probed image of a 2D PAGE also revealed only one spot with a molecular weight of 52kD and an isoelectric point of 5.9 (Figure 6a'). Subsequent probing with an antibody that recognises tyr-tubulin revealed the same spot (Figure 6b'). In contrast, antibodies to glu and acetylated tubulin produced no specific reactions (Figure 6c' and d') despite the fact that each blot gave a subsequent positive reaction with an α-tubulin antibody (data not shown). The single spot is presumably *Oncopeltus* ovariole α1. The identity between the α1 isotypes of *Notonecta* and *Oncopeltus* was shown by mixing samples of ovary homogenates from both species and probing by 2D PAGE as above. The *Notonecta* α1 spot and the *Oncopeltus* α1 spot were so closely associated that they appeared as a single, although elongated, spot (Figure 8).

Despite the fact that the glu and acetylated-tubulin specific antibodies recognised the appropriate isotypes in *Notonecta*, it was possible that they might not be able to recognise the same isotypes in *Oncopeltus*. Thus the negative reaction would not reflect a lack of these isotypes. To ascertain wether these antibodies could recognise nutritive tube tubulin in a post-translationally modified state, purified *Oncopeltus* microtubules were detyrosinated *in vitro* using CPA before being separated on a 2D gel and probed. As expected, all tyr-tubulin staining was lost, to be replaced by glu-tubulin staining (Figure 9a and b). As glu and acetylated tubulin have near identical distributions *in vivo* (Bulinski *et al*, 1988) and both glu and acetylated-tubulin specific antibodies react with *Notonecta* ovary tubulin it is assumed that the anti-acetylated-tubulin antibody would react with *Oncopeltus* acetylated-tubulin, if present.

3.1III: Distribution of α and β-tubulin in *Notonecta* and *Oncopeltus* ovarioles

Having determined the α-tubulin isotype content of both *Notonecta* and *Oncopletus* ovarioles, and the required specificity of the antibodies by immunoblotting, the distribution of the isotypes *in situ* was

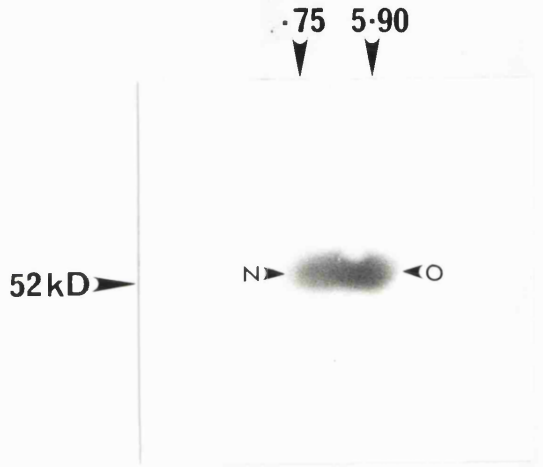


Figure 8 Co-migration of Notonecta and *Oncopeltus* Ovariole α-Tubulin on a 2-D gel

A 1:1 mixture of taxol purified microtubule proteins from *Notonecta* and *Oncopeltus* ovarioles were separated on a 2-D gel and then probed with YOL1/34 (α-tubulin antibody) at a dilution of 1:1000. The total amount of protein was 175µg and the blot was developed using the phosphatase method. The isoelectric points and molecular weights of the respective spots are indicated at the top and the left hand side of the figure respectively.

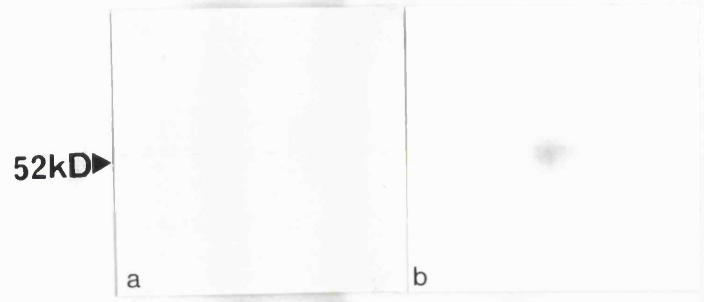


Figure 9 *In vitro* Abolition of Tyr-tubulin and Establishment of Glu-tubulin in *Oncopeltus* Ovariole Microtubules

Nitrocellulose containing taxol purified microtubule proteins from *Oncopeltus* ovarioles separated by 2-D PAGE was treated with 20µg/ml CPA for 30min. at 37°C. It was then probed initially with YL1/2 (tyr-tubulin antibody; a) followed by ID5 (glu-tubulin antibody; b). YL1/2 was used at a dilution of 1:1000 while ID5 was used undiluted. The amount of protein in the blot was 175µg and the blot was developed using the phosphatase method. The position of tubulin is indicated by arrows to the left of the figure.

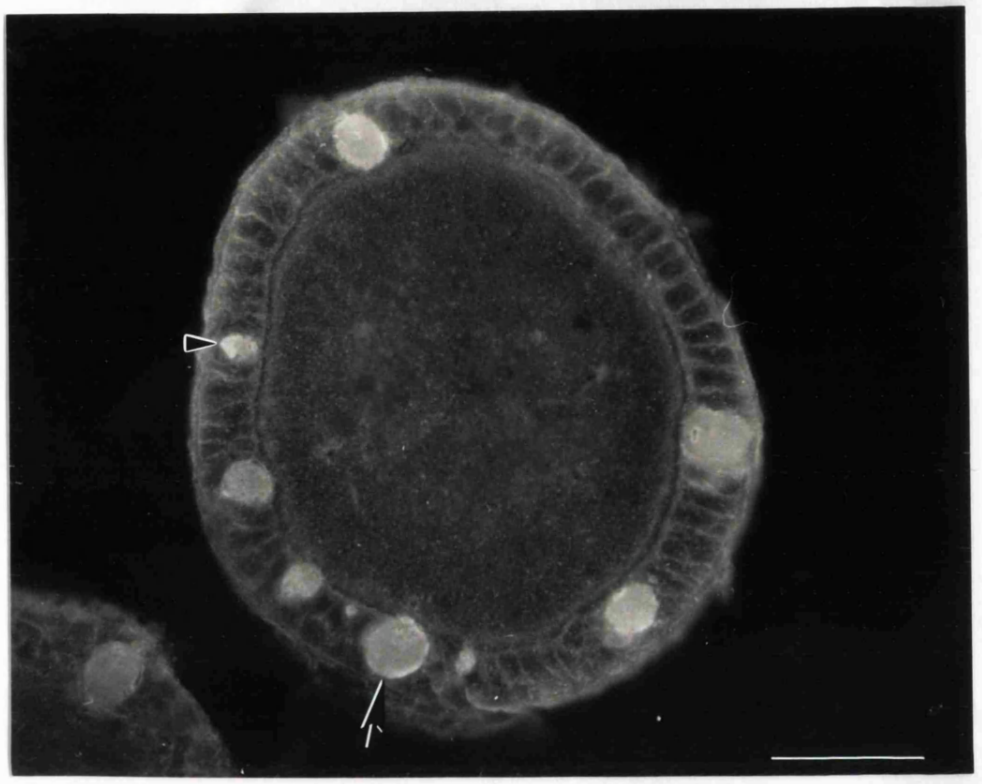


Figure 10 The Arrangement of Nutritive Tubes in the Ovariole of *Notonecta*

Transverse frozen sections of *Notonecta* ovarioles, fixed with formaldehyde, were stained with YOL1/34 (α-tubulin antibody; used at a dilution of 1:100). The position of a functional nutritive tube is indicated by an arrow while that of a redundant nutritive tube is shown by an arrow head. Bar 50μm.

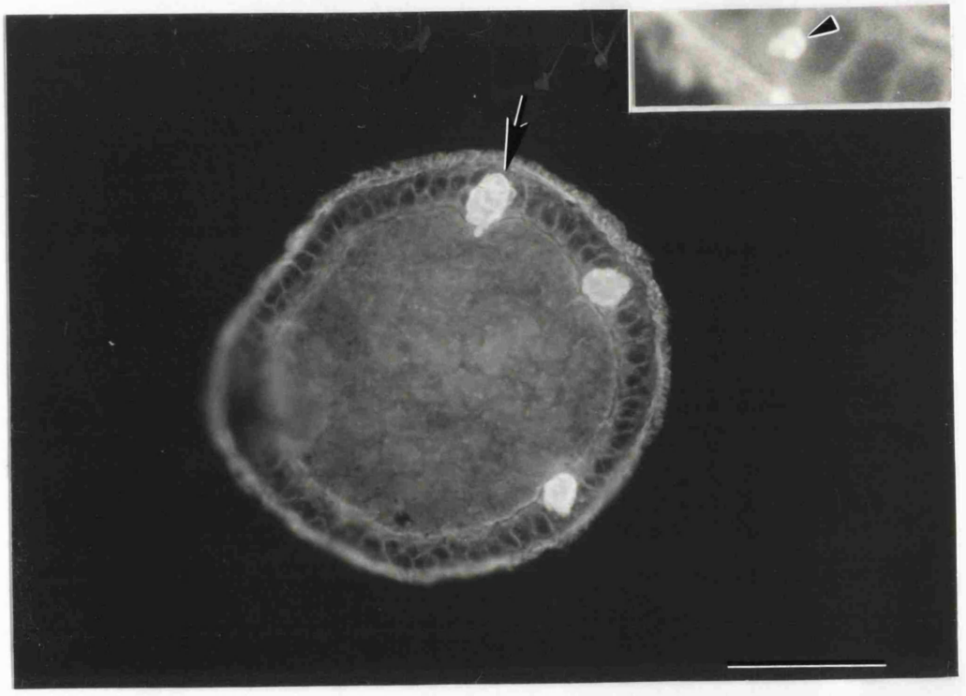


Figure 11 The Arrangement of Nutritive Tubes in the Ovariole of *Oncopeltus*

Transverse frozen sections of *Oncopeltus* ovarioles, fixed with formaldehyde, were stained with YOL1/34 (α-tubulin antibody; used at a dilution of 1:100). The position of a functional nutritive tube is indicated by an arrow. Bar 50μm.

The position of a redundant nutritive tube is more clearly visible as indicated by the arrow head on the inset.

investigated. 7μm thick transverse frozen sections were probed with the appropriate antibodies. In initial experiments, both broad spectrum α- and β-tubulin antibodies labelled all nutritive tubes (Figures 10 and 11; β-tubulin not shown). There was no discrimination between tubes on the basis of their level of redundancy, narrow diameter redundant tubes (Figure 10 and 11, arrow head) labelling in the same manner as the larger, functional tubes (Figure 10 and 11, arrow). Microtubules in the surrounding follicle cells and in the oocyte itself were also labelled (Figure 10 and 11). At this point it is also important to note that the number of oocytes, and hence nutritive tubes, present in *Oncopeltus* is significantly smaller than in *Notonecta* (five or six compared with up to 30 respectively), hence the smaller number of tube profiles in the *Oncopeltus* sections (compare Figure 10 and 11). The smaller number of tube profiles in *Oncopeltus* also means that the probability of finding redundant tubes, which are also much reduced in number in *Oncopeltus*, is very small (a redundant *Oncopeltus* nutritive tube is indicated by the arrow in the inset of Figure 11).

3.1IIIa: Distribution of α-tubulin isotypes in *Notonecta ovarioles*

Since all microtubules in all classes of nutritive tubes label equally with broad range α- and β-tubulin specific antibodies, the distribution of post-translationally modified isotypes was of interest. The distribution of tyr-tubulin containing microtubules in *Notonecta* nutritive tube sections taken at random was significantly different from that observed with α-tubulin. In the larger functional tubes, strong staining was restricted to microtubules forming a band at the inside edge, the microtubules in the central portion of the tubes being almost totally devoid of staining (Figure 12, arrow). The smaller redundant tubes lacked staining. In contrast, the microtubules of the surrounding follicle cells and of the oocyte were brightly stained (Figure 12 and inset). When similar sections were stained with either glu- or acetylaed tubulin specific antibodies, a very different microtubule arrangement was revealed (Figures 13 and 14 respectively). Unlike tyr-microtubules glu and acetylated microtubule staining occurred throughout both functional tubes (Figure 13 and 14, arrow) and redundant tubes (Figure

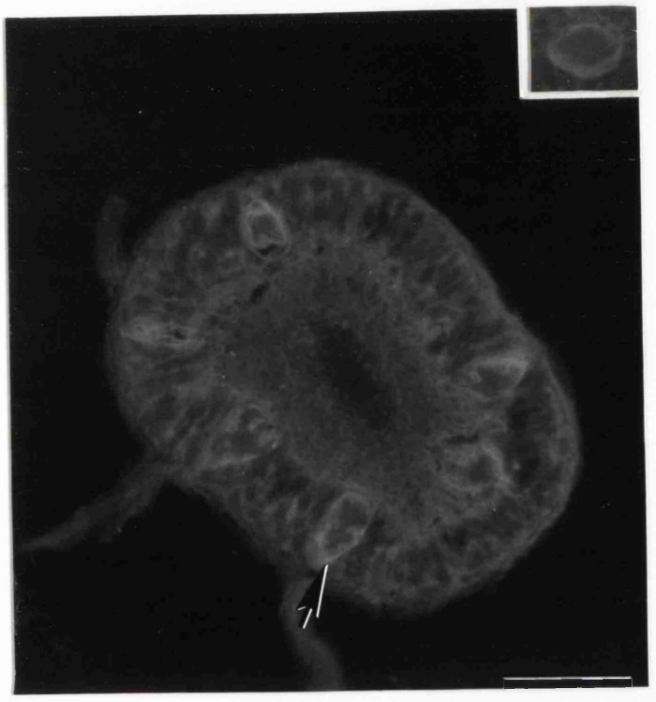


Figure 12 The Distribution of Tyr-tubulin in the Ovariole of *Notonecta*
Transverse frozen sections of *Notonecta* ovarioles, fixed with formaldehyde, were stained with 1A2 (tyr-tubulin antibody; used at a dilution of 1:100). The position of a functional nutritive tube is indicated by an arrow. Bar 50μm.

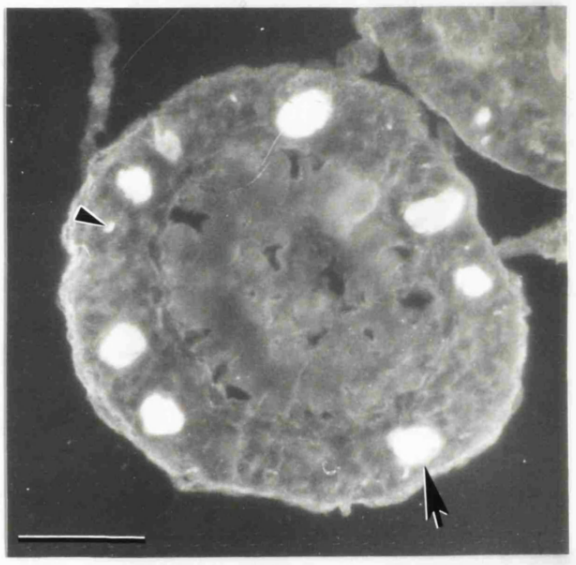


Figure 13 The Distribution of Glu-tubulin in the Ovariole of *Notonecta*
Transverse frozen sections of *Notonecta* ovarioles, fixed with formaldehyde, were stained with ID5 (glu-tubulin antibody; used at a dilution of 1:5). The position of a functional nutritive tube is indicated by an arrow while that of a redundant nutritive tube is shown by an arrow head. Bar 50µm.

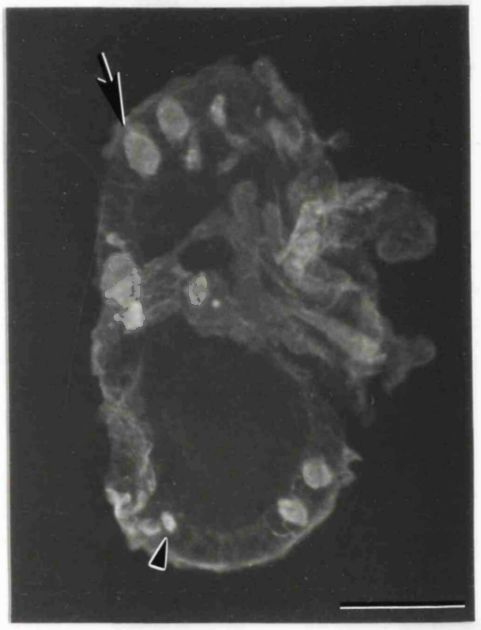


Figure 14 The Distribution of Acetylated-tubulin in the Ovariole of *Notonecta*

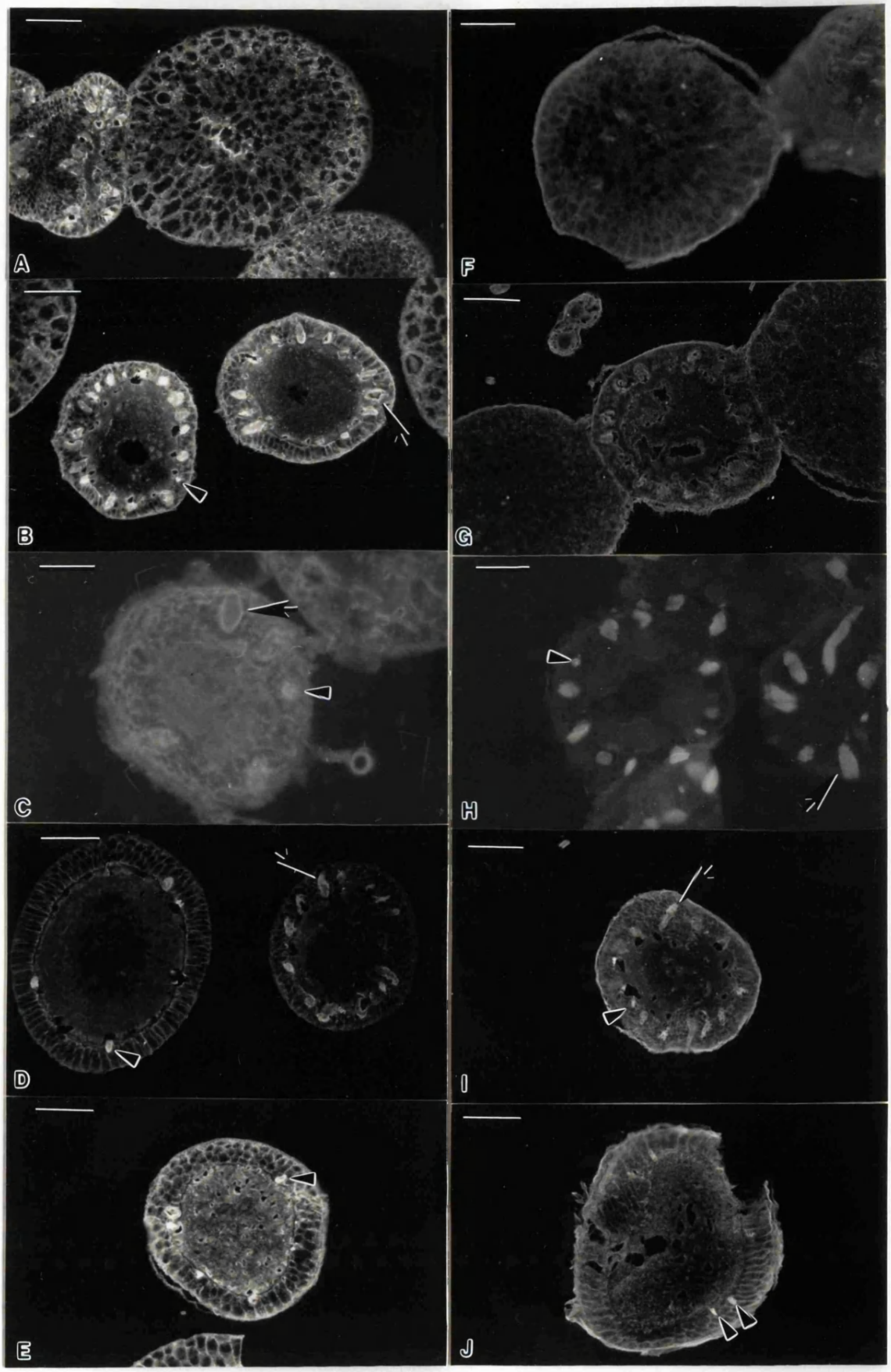
Transverse frozen sections of *Notonecta* ovarioles, fixed with formaldehyde, were stained with 6-11B-1 (acetylated-tubulin antibody; used at a dilution of 1:20). The position of a functional nutritive tube is indicated by an arrow while that of a redundant nutritive tube is shown by an arrow head. Bar 50µm.

13 and 14, arrowhead). There was, however, a complete absence of microtubule staining in the follicle cells and the oocyte (Figures 13 and 14).

When sections were cut at successive levels through the nutritive tubes, a distinct and reproducible pattern of staining, which was dependent on the distance from the trophic region, became apparent. In the mid-trophic region, strong tyr-tubulin staining was restricted to amorphous patches in the central region of the sections (Figure 15a, arrow) with faint staining occuring throughout the tissue at cell boundaries. Glu-tubulin staining had a similar, though more amorphous distribution, the strength of signal being much lower (Figure 15f). Further down the trophic region, at the neck where the nutritive tubes proper begin, tyr-tubulin staining was apparent at the periphery of the functional tubes (Figure 15b, arrow) as well as that of smaller tubes (Figure 15b, arrow head). In contrast both redundant and functional tubes were completely stained with antibodies to glu-tubulin, though the staining was again very faint (Figure 15g). Tyr-tubulin staining patterns within the nutritive tubes proper were similar to that shown in Figure 12. Antibodies again only stained the inner periphery of the functional tubes (Figure 15c, arrow), no staining being present in the redundant tubes (Figure 15c, arrow head), while both functional and redundant tubes stained equally with the glu-tubulin specific antibodies (Figure 15h, arrow and arrow head respectively). At the point where the nutritive tubes enter the oocyte region the total number of tube profiles decreases though a number of peripheral rings can still be observed around functional tubes (Figure 15d, arrow). As before, there was still an abscence of tyr-tubulin staining redundant tubes. In contrast, both the remaining functional and the increasingly redundant tubes stained faintly with glu-tubulin specific antibodies (Figure 15i, arrow and arrow head repectively). Finally in the nutritive tubes within the oocytes themselves the tyr-tubulin staining was restricted almost totally to the redundant tubes (Figure 15e, arrow head), very few functional tubes remaining (Figure 15e). A similar pattern was found for glu-tubulin, though the staining was totally confined to redundant tubes

Figure 15 The Distribution of Tyr and Glu-tubulin at Progressive Points Through a *Notonecta* Ovarioles

Transverse frozen sections, fixed with formaldehyde, were taken at progressive points through *Notonecta* ovarioles and then stained with either 1A2 (tyr-tubulin antibody; a to e) or ID5 (glu-tubulin antibody; f to j). (a) Midtrophic region: the arrow indicates amorphous staining; (b) Trophic region-nutritive tube neck: the arrow indicates peripheral staining of functional tubes while the arrow head shows redundant tubes stained throughout; (c) Nutritive tube proper: the arrow indicates peripheral staining of functional tubes while the arrow head shows all the redundant tubes staining; (d) Nutritive tube-oocyte connection: the arrow indicates peripheral staining of functional tubes while the arrow head shows redundant tubes stained throughout; (e) Oocyte; (f) Midtrophic region; (g) Trophic region-nutritive tube neck; (h) Nutritive tube proper: the arrow and arrowhead indicate both the functional and redundant tubes staining throughout; (i) Nutritive tube-oocyte connection: the arrow and arrowhead indicate both the functional and redundant tubes staining throughout; (j) Oocyte: arrow heads show brightly staining redundant tubes. 1A2 was used at a dilution of 1:100 while ID5 was used at 1:5. All bars 50μm.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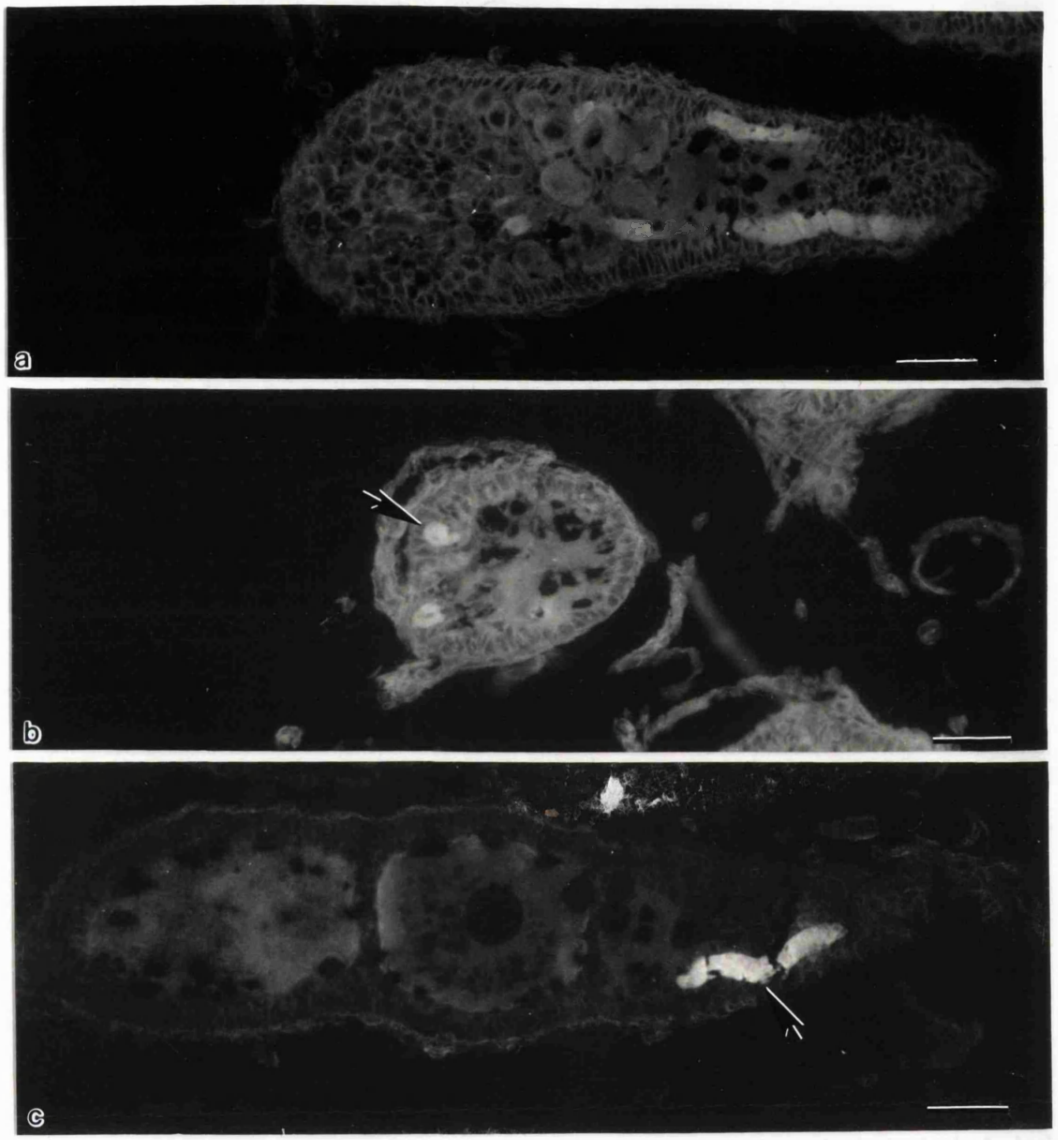

Figure 16 The Distribution of Tyr and Glu-tubulin in Juvenile Ovarioles of *Notonecta*

Longitudinal (a and c) and transverse (b) frozen sections of ovaries from juvenile *Notonecta*, fixed with formaldehyde and stained with tubulin isotype specific antibodies. (a) 1A2 (tyr-tubulin antibody): staining shows embryonic nutritive tubes; (b) 1A2: arrow shows entire tubes staining, no peripheral staining being apparent; (c) ID5 (glu-tubulin antibody): arrow shows staining is restricted to the most distal part of the tissue, with respect to the trophic region. 1A2 was used at a dilution of 1:100 while ID5 was used at 1:5. All bars 50µm.

and, compared with more anterior sections, the staining was significantly brighter (Figure 15j, arrow heads).

3.1IIIb: Distribution of α-tubulin isotypes in immature *Notonecta* ovarioles

The effect of age on the distribution of tyr and glu-tubulin in *Notonecta* ovarioles was also investigated by looking at ovarioles from juvenile animals. When probed for tyr-tubulin juvenile ovarioles revealed a wide distribution that is not just confined to the nutritive tubes. Most of the cells making up the ovarian structure stained positively for tyr-tubulin (Figure 16a and b). The nutritive tubes themselves also stained strongly and were visualised in both longiudinal and transverse sections (Figures 16a and b respectively). It is, however, interesting to note that, in transverse sections, almost the entire tubes stained (arrows), the extreme inner peripheral staining found in more mature specimens not occuring (compare Figure 16b with Figure 12). In contrast, glu-tubulin staining was much fainter (Figure 16c). Nutritive tubes did stain but they are not as numerous as when labelled for tyr-tubulin and the staining was restricted to the most distal part of the tissue, with respect to the trophic region (Figure 16c, arrow).

3.1IIIc: Distribution of α-tubulin isotypes in *Oncopeltus* ovarioles

When *Oncopeltus* sections were stained using antibodies to tyr-tubulin both functional and redundant tubes were stained. Staining occured throughout the tubes without compartmentalisation (Figure 17a). It is important to note that, due to the small number of nutritive tubes and the inceased rapidity of redundant nutritive tube breakdown in *Oncopeltus*, the number of redundant tubes and hence the number of redundant profiles picked up by immunofluorescent staining is reduced. Sections cut through the trophic region also revealed tyr-tubulin staining but it was more diffuse, being restricted to amorphous patches in the central region (Figure 17a, arrow). In contrast, when *Oncopeltus* nutritive tube sections were stained with antibodies specific for glu

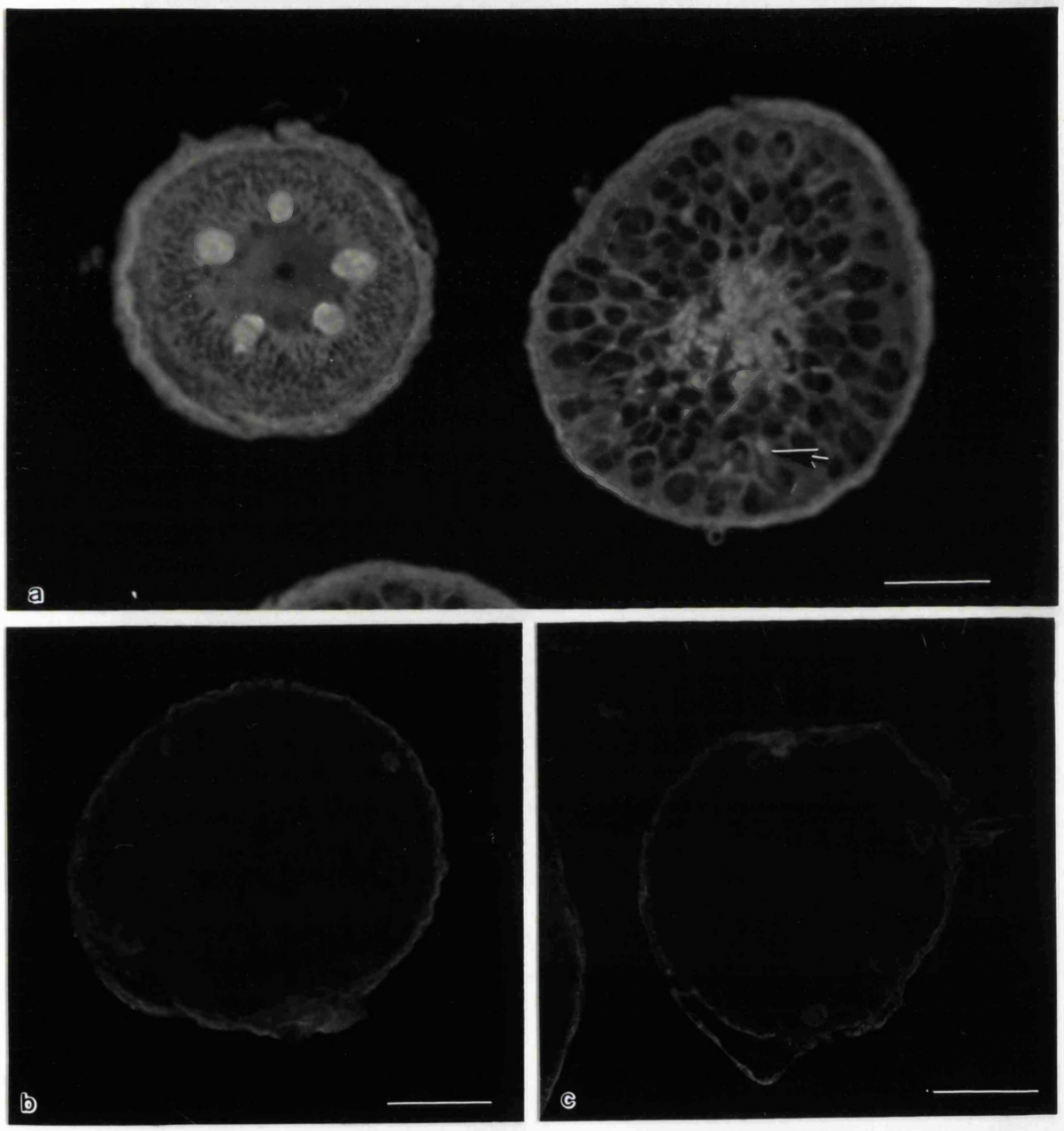

Figure 17 The Distribution of Tyr, Glu and Acetylated tubulin in the Ovarioles of *Oncopeltus*

Transverse frozen sections of *Oncopeltus* ovarioles, fixed with formaldehyde, were stained with tubulin isotype specific antibodies. (a) 1A2 (tyr-tubulin antibody) staining showing functional tubes. This also shows a section through the trophic region, the arrow indicating amorphous patches of staining. (b) ID5 (glu-tubulin antibody). (c) 6-11B-1 (acetylated-tubulin antibody). 1A2 was used at a dilution of 1:100, ID5 at a dilution of 1:5 while 6-11B-1 was used at 1:20. All bars 50μm.

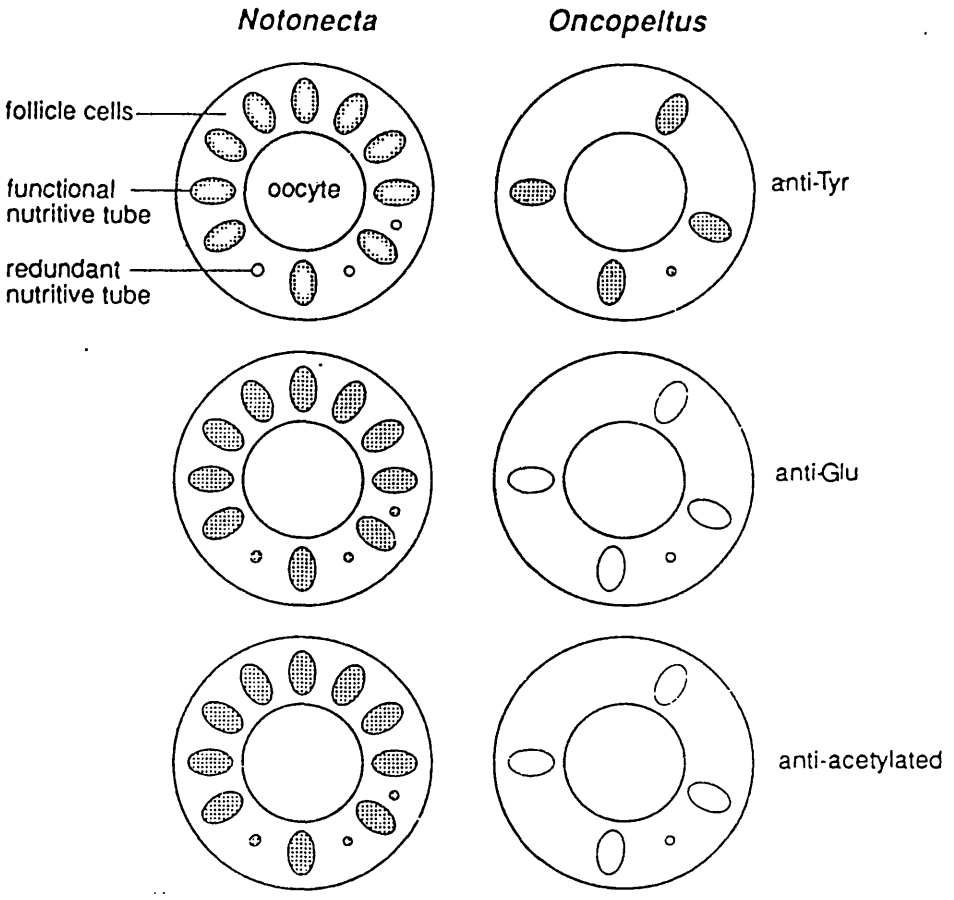

Figure 18: Diagram Summarising the Immunofluorescence Staining Patterns Obtained with *Notonecta* and *Oncopeltus*

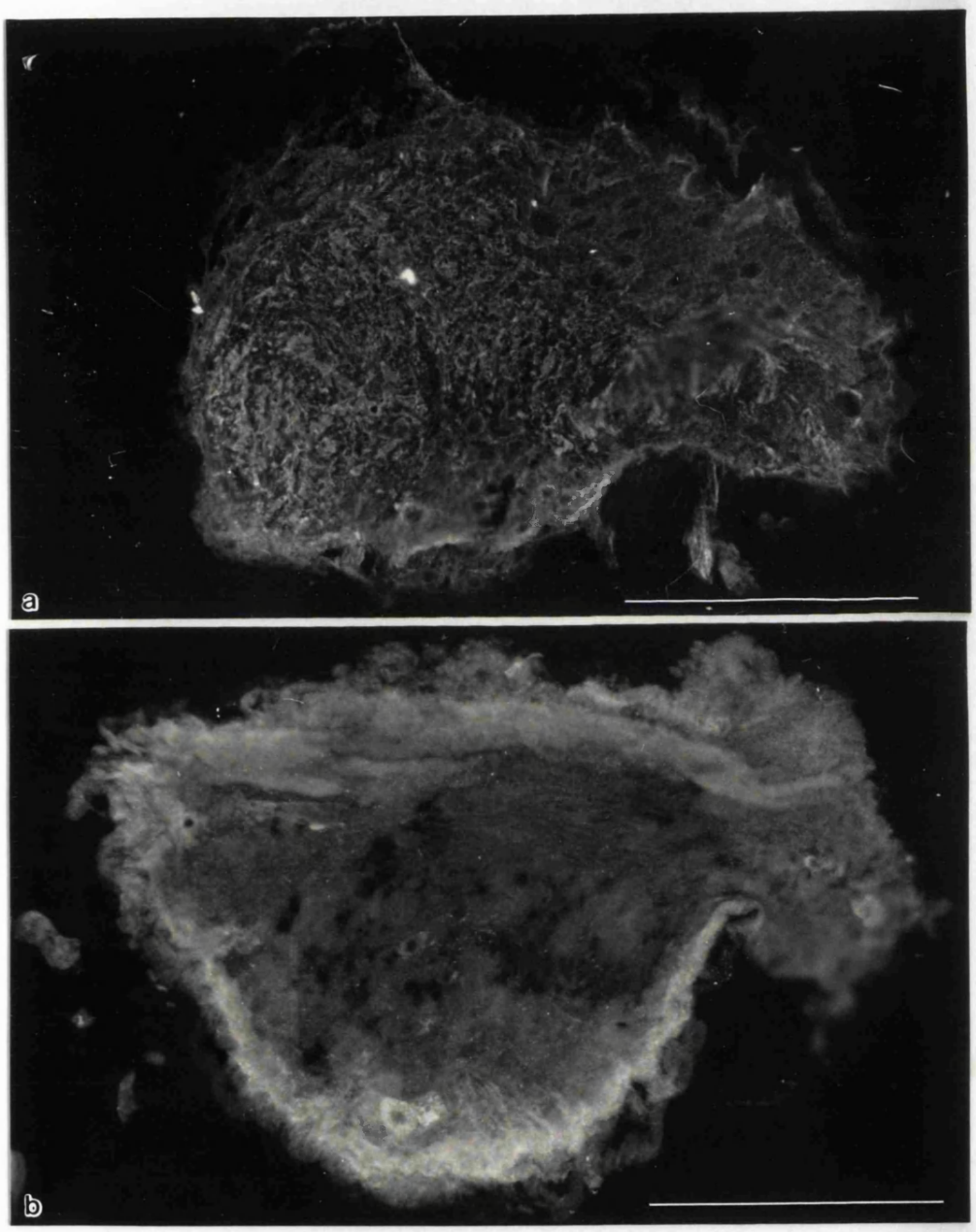

Figure 19 The Distribution of Tyr and Glu-tubulin in the Ganglion Tissue of *Oncopeltus*

Frozen sections of *Oncopeltus* ganglia from the thoracic and abdominal cavities, fixed with formaldehyde, were stained with tubulin isotype specific antibodies. (a) 1A2 (tyr-tubulin antibody) showing particulate staining at the centre of the tissue. (b) ID5 (glu-tubulin antibody) showing more amorphous staining at the periphery. Both 1A2 and ID5 were used at dilutions of 1:100. Bar 50μm.

(Figure 17b) or acetylated tubulin (Figure 17c) staining, if present at all, was very faint. This absence of staining was reproducible, even when *Notonecta* sections placed adjacently on the same slide stained with their normal intensity. Figure 18 summarises the distribution of glu and tyr-tubulin in both *Notonecta* and *Oncopeltus* nutritive tubes.

3.1IIId: Distribution of α-tubulin isotypes in *Oncopletus* ganglia

Attempts to repeat *in situ* the work in which *Oncopeltus* glu-tubulin, created by *in vivo* CPA treatment, is recognised by glu-tubulin specific antibodies (see Section 3.1IIb) was unsuccesful (data not shown). It was, therefore, decided to look for glu-tubulin in another microtubule based system from *Oncopeltus*. As glu-tubulin is predominantly associated with stable microtubules (Kreis, 1987; Schulze *et al*, 1987; Gundersen *et al*, 1987) the stable ganglion microtubules, were used. Frozen sections cut from the thoracic and abdominal cavities were probed with antibodies to both tyr and glu-tubulin, the tyr-tubulin antibodies producing particulate staining at the centre of the tissue mass (Figure 19a). By comparison, glu-tubulin staining was fainter and consisted of amorphous staining at the edges of the tissue (Figure 19b).

3.1IIIe: Investigation into the mechanism of post-translational modification in *Oncopeltus* ovarioles

Determining that *Oncopeltus* ganglia have a glu-tubulin isotype (see Section 3.1IIId) and the ovariole tubulin could be detyrosinated and subsequently recognisied by glu-tubulin specific antibodies (see Section 3.1IIb), led to the development of a preliminary method of determining whether *Oncopletus* ovarioles have the necessary detyrosinating enzymes. *Oncopletus* ovariole homogenate was mixed with bovine brain microtubules and incubated along with mixtures of bovine brain microtubules and CPA or bovine brain microtubules and PEM buffer. When probed with the appropriate antibodies it was found that the CPA treated brain microtubules had been totally detyrosinated (Figure 20, compare Lanes 2 and 6), whereas brain microtubules that had been incubated in buffer remained an equal mixture of glu and tyr-tubulin (Figure 20, compare

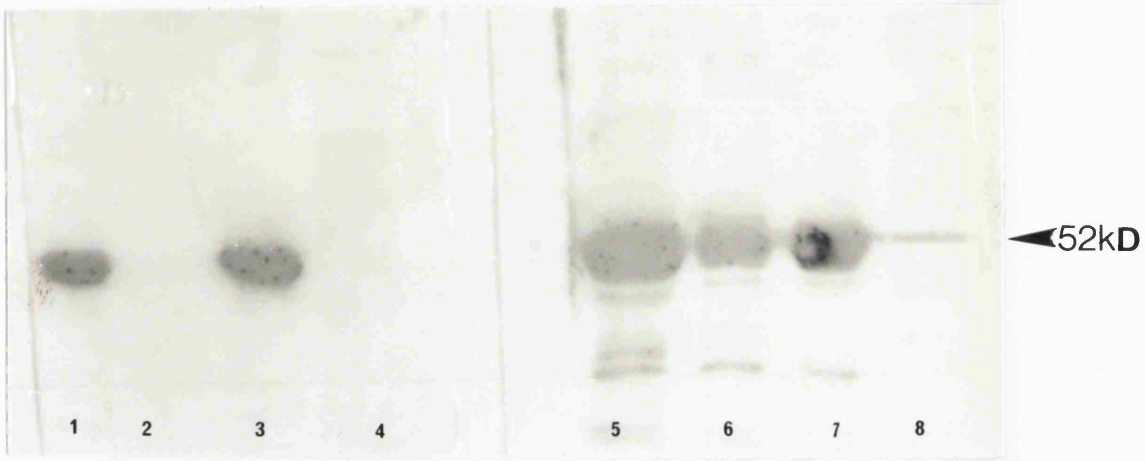

Figure 20 *In vitro* Assay for Carboxypeptidase Activity in the Ovarioles of *Oncopeltus*

Nitrocellulose containing different combinations of ovarian homogenate from *Oncopeltus*, bovine brain microtubules and commercially available CPA incubated together for 30 mins at 37°C was probed with isotype specific antibodies. Lanes 1 to 4: YL1/2 (tyr-tubulin antibody); Lanes 5 to 8: ID5 (glu-tubulin antibody). Lanes (1) and (5): ovarian homogenate plus bovine brain microtubules; Lanes (2) and (6): bovine brain microtubules plus CPA; Lanes (3) and (7): bovine brain microtubules plus PEM buffer; Lanes (4) and (8) ovarian homogenate plus CPA. YL1/2 was used at a dilution of 1:1000 while ID5 was used undiluted, both blots being developed using the phosphatase method. All sample loadings were 65µg. Note the maintainance of tyr-tubulin specific staining when bovine brain microtubules are incubated with ovarian homogenate (Lanes 1 and 5) as compared to the brain microtubules incubated with CPA (Lanes 2 and 6).

Lanes 3 and 7). An identical situation occured with the brain microtubules that had been incubated with the *Oncopeltus* ovariole homogenate. An equal mixture of glu and tyr-microtubules remained after treatment, there being no detyrosination (Figure 20, compare Lanes 1 and 5). It is interesting to again note that CPA treatment of *Oncopeltus* ovariole homogenate allows glu-tubulin specific antibodies to recognise apparently previously fully tyr-microtubules (Figure 20, compare Lanes 4 and 8).

3.2: Production of monoclonal antibodies

The production of monoclonal antibodies has followed several different, yet closely linked methods. These have, with increasing practical experience, evolved into a successful protocol, the development of which is outlined below.

3.2I: Mononclonal production-1

In all monoclonal antibody production mice were immunised with *Notonecta* ovariole microtubule protein purified by taxol stabilisation (see Section 2.1Vb; Figure 21a, Lane 3), tubulin comprising approximately 22% of the total protein concentration (Figure 21b). In the early runs, however, the method involved using the immunisation schedule outlined by Method I (see Section 2.2Id) and in all cases when the process was halted the hybridomas were frozen and saved at -70°C.

In the first attempt, 3 mice were immunised, using Method 1, with 525µg of KLH coupled microtubule protein purified from *Notonecta* ovarioles at a concentration of 3.5mg/ml. Due to the shortage of *Notonecta* material the tail bleeds were tested on nitrocellulose containing brain tubulin and *Oncopeltus* homogenate. Nevertheless, 2 sera were found to very faintly label a band with a molecular weight of 52kD (Figure 22, Lanes 6 and 7). All other tail bleed tests were negative. In an attempt to elucidate further the nature of the antigen recognised, 3T3 cells were incubated with the positive tail bleed sera. In both cases the results were ambiguous (Figure 23a and b). Structural

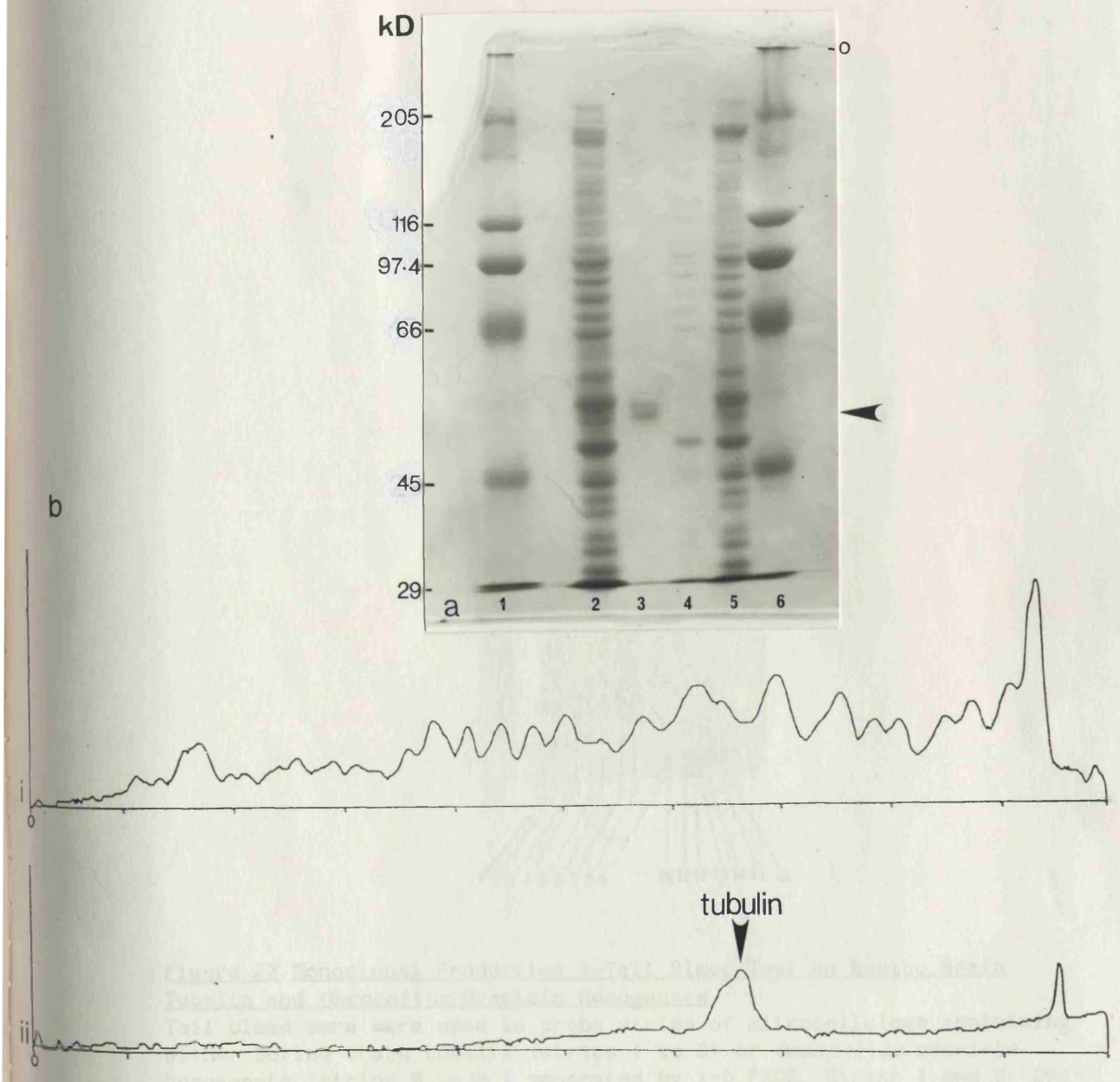

Figure 21 Purification of *Notonecta* Tubulin
(a) Coomassie stained gel of *Notonecta* ovariole protein extracts, purified by hot and cold cycling coupled with taxol stabilisation. Lane 1: molecular weight markers; Lane 2: total protein extract; Lane 3: pellet after final 37°C incubation; Lane 4: cushion supernatant; Lane 5: initial cold pellet; Lane 6: molecular weight markers. All loadings are 25µl. The position of tubulin is indicated on the right while the weights of the molecular weight markers is indicated on the left.
(b) Densitometry scans of *Notonecta* ovariole homogenate (i) and subsequently purified tubulin (ii), showing the extent of purification.

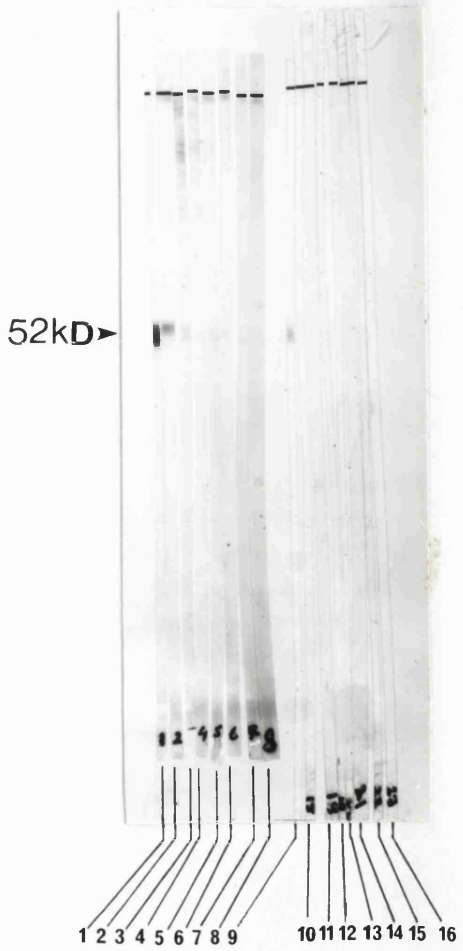

Figure 22 Monoclonal Production 1-Tail Bleed Test on Bovine Brain Tubulin and *Oncopeltus* Ovariole Homogenate

Tail bleed sera were used to probe strips of nitrocellulose containing either bovine brain tubulin (strips 1 to 8) or *Oncopeltus* ovariole homogenate (strips 9 to 16) separated by 1-D PAGE. Strips 1 and 9: DM1A (α-tubulin antibody); strips 2 and 10: 6-11B-1 (aceylated tubulin antibody); strips 3 and 11: MAP2-2 (MAP-2 arm domain antibody); strips 4 and 12: MAP2-3 (MAP-2 arm domain antibody); strips 5 and 13: MAP2-4 (MAP-2 binding domain antibody); strips 6 and 14: Left ear serum; strips 7 and 15: Right ear serum; strips 8 and 16: No ear serum. DM1A, MAP2-2, MAP2-3 and MAP2-4 were used at a dilution of 1:1000, the tail bleed sera were used at a dilution of 1:10 while 6-11B-1 was used undiluted. The amount of protein in the *Oncopeltus* strips was 22μg while the bovine-brain was 30μg and all blots were developed using the peroxidase method. Bands of interest are arrowed on the left.

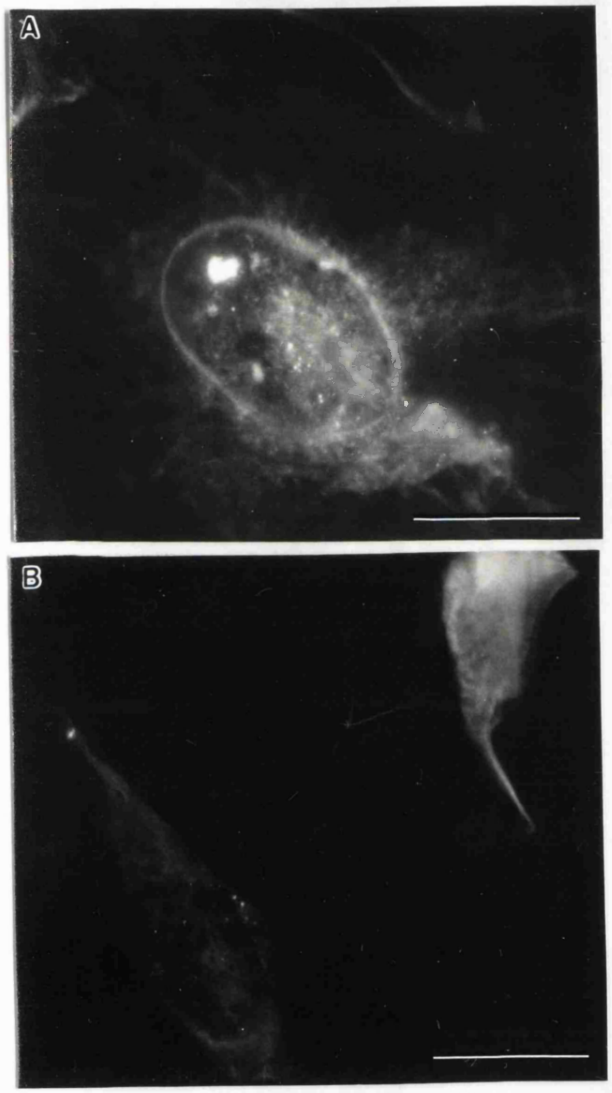

Figure 23 Monoclonal Production 1-Tail Bleed Test on 3T3 Cells
The positive tail bleed sera from Lanes 6 and 7 of Figure 22 were used to probe methanol fixed 3T3 cells. (a) Lane 6 serum: this shows ambiguous particulate components, no real structure being identified; (b) Lane 7 serum: this shows a similar pattern to (a) but the staining seems to be more concentrated around the nucleus. The serum was used at a dilution of 1:10 and the bar is 50μm.

components of the cells were recognised with both sera, particularly around the nucleus (Figure 23b) but the structures labelled could not be conclusively identified as microtubules.

Despite this ambiguity, and due to the apparent recognition of tubulin in the immunoblotting test, the mice were further boosted and the spleens removed and fused using Fusion method I (Section 2.2If). One fusion became very contaminated and was abandoned while the other had a low success rate, only 57 wells growing up, but 3 of these wells produced sera that recognised an antigen that had a molecular weight slightly less than 52kD but was recognised by the other α-tubulin controls (Figure 24, Lanes 4, 5 and 6). One of these wells was subsequently cloned using Cloning method I (see Section 2.2Ii) but of the 37 wells that grew up, there were no positives, when screened by immunoblotting or immunofluorescent staining of tissue culture cells (results not shown). The experiment was therefore terminated at this point.

3.2II: Monoclonal production-2

Mice were again immunised with 525μg of KLH coupled microtubule proteins purified from *Notonecta* ovarioles at a concentration of 3.5mg/ml following Method I, and the tail bleed sera tested on nitrocellulose containing *Notonecta* ovariole homogenate. Of the three mice tested one was negative (Figure 25, Lane 2) while the other two were positive. Of the positives one recognised 2 bands with molecular weights of 70kD and 135kD (Figure 25, Lane 3) while the other reacted faintly with a band at 135kD (Figure 25, Lane 2). The two positive mice were boosted and the spleens subsequently removed and fused using Fusion method I (see Section 2.2If). Similar testing of the sera from the fusion wells did not, however, reveal any specific, positive bands. One serum stained an antigen with a molecular weight of 125kD (Figure 26, Lane 9) while all other bands produced corresponded to proteins with molecular weights between 34 and 41kD (results not shown). As the one high molecular weight protein stained was not recognised in the tail bleed sera tests it is probably spurious and the lower molecular weight

Figure 24 Monoclonal Production 1-Fusion Sera Test on *Notonecta* Ovaria Homogenate

Fusion sera were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strip 1: YL1/2 (tyr-tubulin antibody); Strip 2: MAP2-4 (MAP-2 binding domain antibody; Strip 3: DM1A (α-tubulin antibody); Strip 4: **9B/4**; Strip 5: **11B/4**; Strp 6: **6B/4**; Strip 7: 4B/5; Strip 8: 3C/3. YL1/2, MAP2-4 and DM1A were use at a dilution of 1:1000 while the sera were used undiluted. The amount of protein in the strips used was 25µg and all blots were developed using the peroxidase method. The sera of interest are highlighted in bold and the important bands are arrowed on the right.

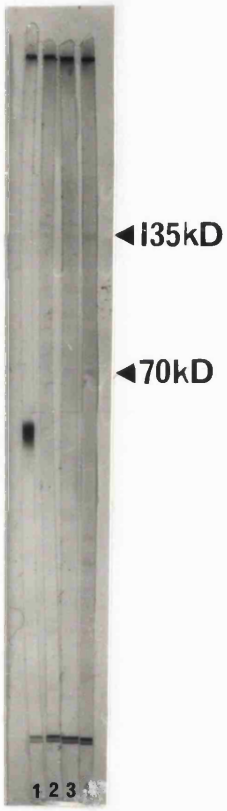

Figure 25 Monoclonal Production 2-Tail Bleed Sera Test on *Notonecta* Ovarian Homogenate

Tail bleed sera were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strip 1: DM1A (α-tubulin antibody); Strips 2, 3 and 4: Tail bleed sera. DM1A was used at a dilution of 1:1000 while the tail bleed sera were used at a dilution of 1:10. The amount of protein in the strips used was 25μg and all blots were developed using the peroxidase method. Bands of interest are arrowed on the right.

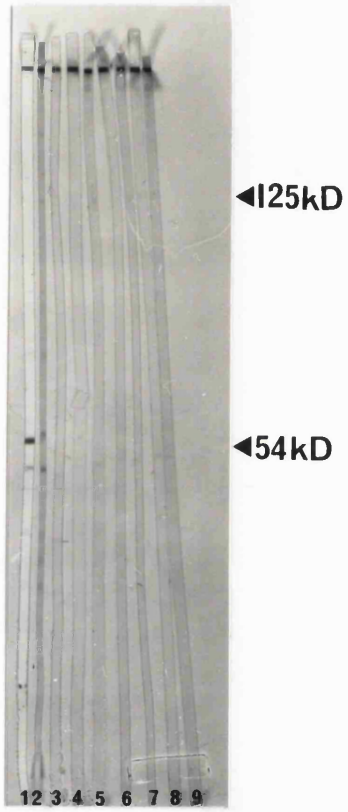

Figure 26 Monoclonal Production 2-Fusion Sera Test on *Notonecta* Ovarian Homogenate

Fusion sera were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strip 1: KMX-1 (β-tubulin antibody); Strip 2: MAP2-2 (MAP-2 arm domain antibody); Strip 3: Secondary antibody only, wash buffer used in place of the primary; Strip 4: 5B/2; Strip 5: 5B/5; Strip 6: 7B/3; Strip 7: 8B/3; Strip 8: 8C/3; Strip 9: **8C/4**. KMX-1 and MAP2-2 were used at a dilution of 1:1000 while the sera were used undiluted. The amount of protein in the strips was 25μg and all blots were developed using the peroxidase method. The sera of interest are highlighted in bold and the important bands are arrowed on the right.

bands are probably non-specific. The experiment was, therefore, halted at this point.

3.2III: Monoclonal production-3

As the first two monoclonal production runs were essentially negative the levels of protein in the immunisation samples were increased to 2.175mg at a concentration of 14.5mg/ml. The immunisation schedule again followedthat outlined in Method I. Two of the mice contracted infections which killed one and severely debilitated another, so it could not be used, but the remaining one survived. The sole survivor was bled from the tail and the serum tested on a nitrocellulose strip of ovariole homogenate. In contrast to the previous two tail bleed tests the nitrocellulose stained relatively strongly (Figure 27, Lane 3). The strip was messy with a smear of staining stretching from 60kD to the higher molecular weight edge of the nitrocellulose but a number of specific bands could be picked out. The more obvious bands included ones with molecular weights of 51, 56, 61, 115, and 175kD. The sera was then tested on 3T3 cells. The staining produced was quite faint and organelle like (Figure 28a, b), being predominantly concentrated around the nucleus. In the cell processess, however, microtubule like staining could be observed (Figure 28a, b, arrow). Due to the results of both immunoblotting and immunofluorescence the mouse was boosted but prior to the scheduled time of spleen fusion the mouse succumbed to an infection of the peritoneal cavity. After a course of antibiotics it recovered enough to complete the immunisation schedule. On sacrificing in preparation to carry out the fusion the organs within the peritoneal cavity were all found to be fused. Because of this it was impossible to extract the spleen and this, coupled with the risk that there could still be a pathogenic infection present that could contaminate and proliferate in the fusion wells, led to the experiment being halted.

3.2IV: Mononclonal production-4

Two mice were immunised with 2.175mg of microtubule proteins purified from *Notonecta* ovarioles at a concentration of 14.5mg/ml, the

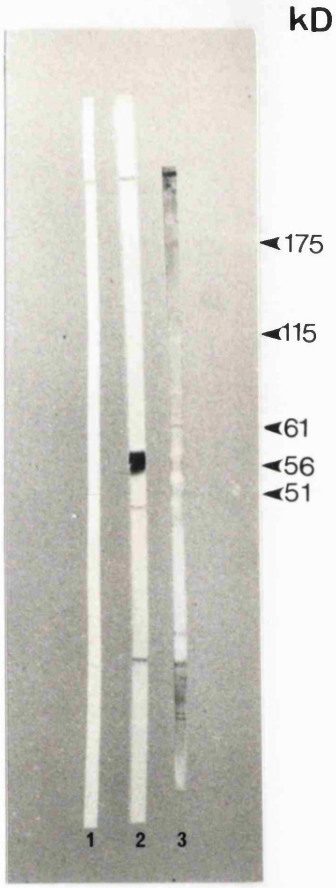

Figure 27 Monoclonal Production 3-Tail Bleed Serum Test on *Notonecta* Ovarian Homogenate

A tail bleed serum was used to probe a strip of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strip 1: Secondary antibody only, wash buffer used in place of the primary; Strip 2: YOL1/34 (α-tubulin antibody); Strip 3: Tail bleed serum. YOL1/34 was used at a dilution of 1:1000 while the serum was used at a dilution of 1:10. The amount of protein in the strips used was 25μg and all blots were developed using the peroxidase method. The bands of interest are arrowed on the right.

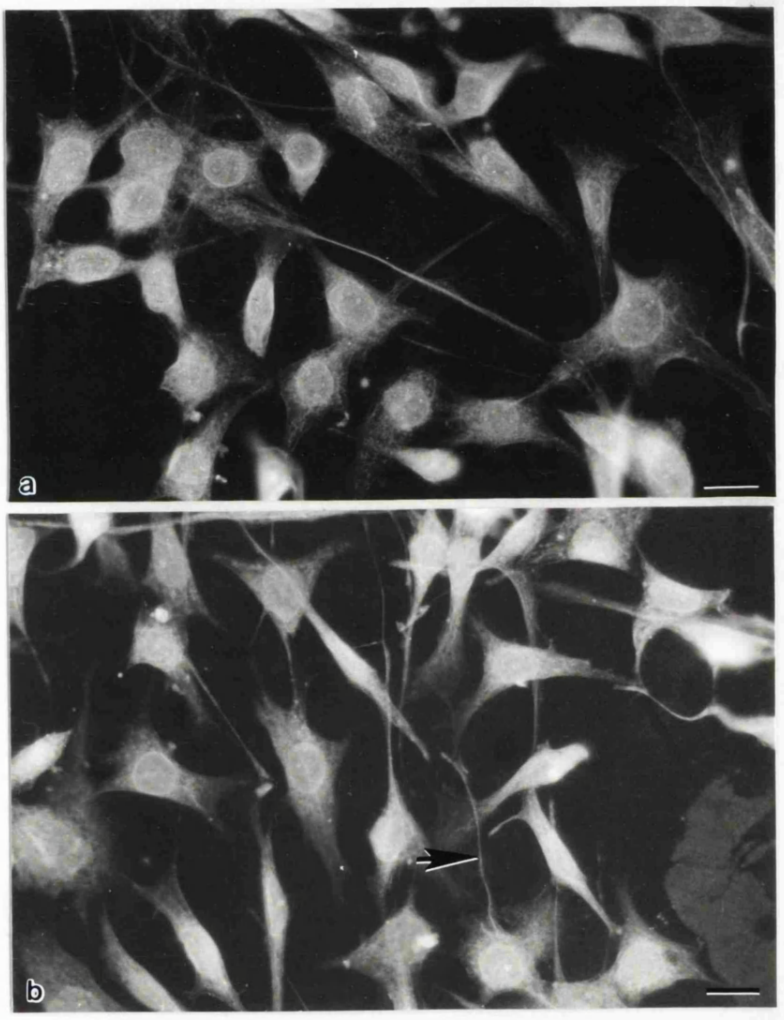

Figure 28 Monoclonal Production 3-Tail Bleed Test on 3T3 Cells
The positive tail bleed serum from Lane 3 of Figure 27 was used to probe methanol fixed 3T3 cells. (a) and (b): Both show a faint, particulate staining pattern that is particularly concentrated around the nucleus. The arrow in (b) does, however, indicate microtubule like staining in the cell processess. The serum was used at a dilution of 1:10 and the bar is 20μm.

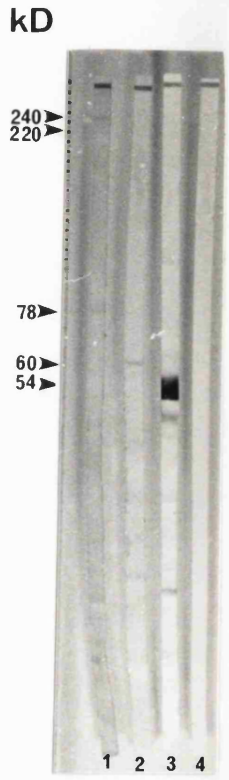

Figure 29 Monoclonal Production 4-Tail Bleed Sera Test on *Notonecta* Ovarian Homogenate

Tail bleed sera were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strips 1 and 2: Tail bleed sera; Strip 3: YOL1/34 (α-tubulin antibody); Strip 4: wash buffer. YOL1/34 was used at a dilution of 1:1000 while the tail bleed sera were used at a dilution of 1:10. The amount of protein used in the strips was 25μg and all blots were developed using the peroxidase method. Bands of interest are arrowed on the left.

immunisation schedule following Method II. Tail bleed tests showed that the sera from both mice again produced indistinct results but in both cases a number of seemingly specific, high molecular weight bands could be resolved (Figure 29, Lanes 1 and 2). The first serum recognised antigens with molecular weights of 240, 220 and 80kD (Figure 29, Lane 1) while the second recognised antigens at 78 (very faint) and 60kD (Figure 29, Lane 2). The mice were boosted and the spleens removed and fused using Fusion method II (see Section 2.2Ig). Unfortunately all subsequent fusion well sera tests on nitrocellulose strips containing *Notonecta* ovariole homogenate produced negative results despite the controls being positive (results not shown). A series of dot blots did, however, identify 5 positive wells (results not shown) so these wells were cloned using the Cold Spring Harbor cloning method (Cloning method II, Harlow and Lane, 1988). When the sera removed during the cloning method were tested on nitrocellulose strips one identified a positive band with a molecular weight of approximately 190kD (Figure 30, Lane 8). This band was not identified by the tail bleed sera so it was deduced to be non-specific and, with the concomitant lack of other positive reactions, the experiment was halted.

3.2V: Monoclonal production-5

Thoughts on the nature of the interaction of the immunogen with the KLH suggested that it may be better to pre-couple the KLH before coupling it to the immunogen to prevent the immunogen being "swamped" by the carrier. This procedure was duly carried out, and a mouse was immunised with the resulting sample. Mice were also immunised with an un-pre-coupled sample and a mixture of pre-coupled and un-pre-coupled samples, all immunisations following Method II. When the sera from the tail-bleeds were tested no specific bands developed but the nitrocellulose strips were very "dirty", the serum from the mouse immunised with the exclusively pre-coupled immunogen producing the strongest reaction (Figure 31, Lane 2). Interestingly, the positive, α-tubulin control also failed to produce a positive band (Figure 31, Lane 1) so it was decided that the fault must lie in the nitrocellulose used. Unfortunately the results could not be retested due to the shortage of

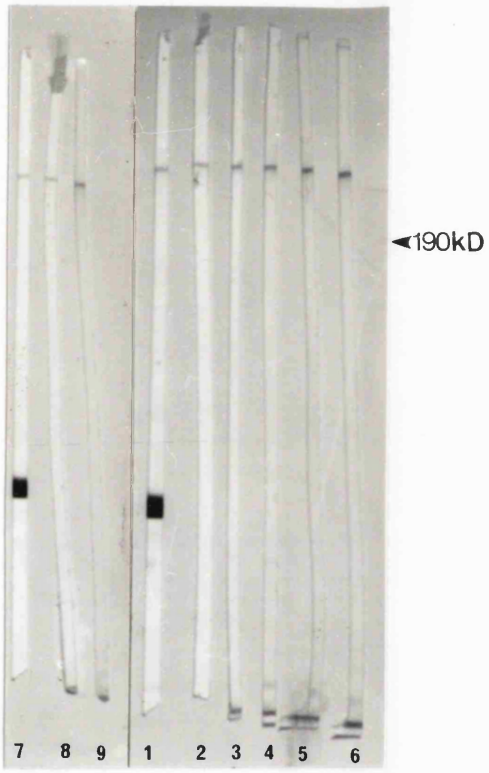

Figure 30 Monoclonal Production 4-Clone Sera Test on *Notonecta* Ovarian Homogenate

Clone sera were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strips 1 and 7: YL1/2 (tyr-tubulin antibody); Strip 2: 4C/2; Strip 3: 5C/3; Strip 4: 8B/5: Strip 5: 6B/4; Strip 6: 7B/4; Strip 8: **4C/5**; Strip 9: 8C/5. YL1/2 was used at a dilution of 1:1000 while the sera were used undiluted. The amount of protein in the strips used was 25µg and all blots were developed using the peroxidase method. The serum of interest is highlighted in bold and the important band of 190kD is arrowed on the right.

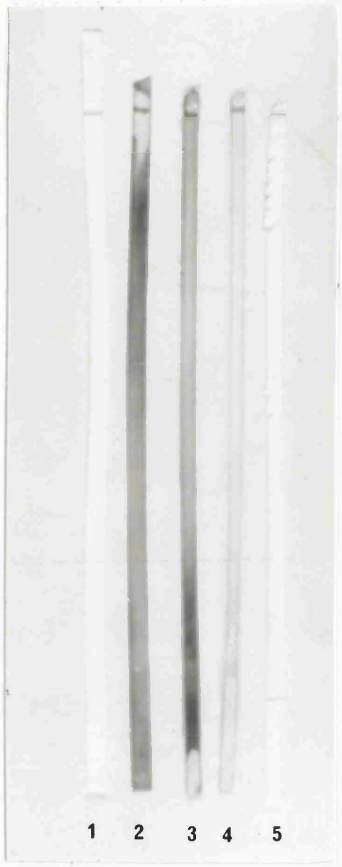

Figure 31 Monoclonal Production 5-Tail Bleed Sera Test on *Notonecta* Ovarian Homogenate Showing the Benefit of Pre-Coupling the KLH before Immunisation

Tail bleed sera were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strip 1: YOL1/34 (α-tubulin antibody); Strip 2: Tail bleed serum from mouse immunised with *Notonecta* ovarian microtubules coupled with pre-coupled KLH; Strip 3: Tail bleed serum from mouse immunised with a 1:1 mixture of *Notonecta* ovarian microtubules coupled with either precoupled KLH or non-precoupled KLH; Strip 4: Tail bleed serum from mouse immunised with *Notonecta* ovarian microtubules coupled with non-precoupled KLH; Strip 5: Secondary antibody only, wash buffer used in place of the primary. YOL1/34 was used at a dilution of 1:1000 while the tail bleed sera were used at 1:10. The amount of protein in the strips used was 20μg and all blots were developed using the phosphatase method. It is important to note the strength of reaction, albeit smeared, with strips 2 and 3.

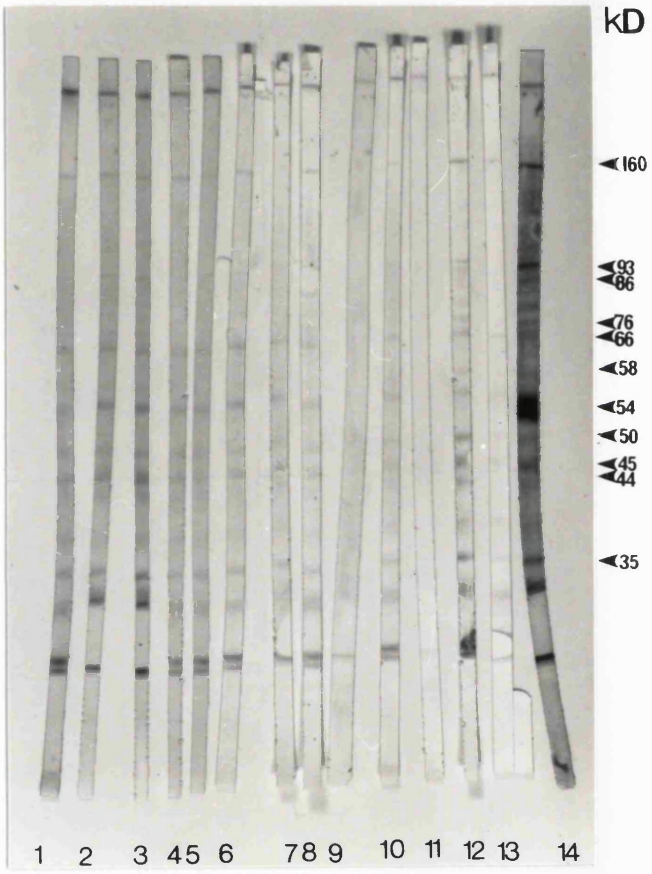

Figure 32a Monoclonal Production 5-Fusion Sera Test on *Notonecta* Ovarian Homogenate

Fusion sera were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strip 1: BC/5; Strip 2: LB/3; Strip 3: LB/5; Strip 4: KB/3; Strip 5: EC/5; Strip 6: BC/4; Strip 7: GB/5; Strip 8: CC/4; Strip 9: FC/2; Strip 10: CC/2; Strip 11: DB/2; Strip 12: GC/3; Strip 13: Secondary antibody only, wash buffer used in place of the primary; Strip 14: YOL1/34 (α-tubulin antibody). YOL1/34 was used at a dilution of 1:1000 while the sera were used undiluted. The amount of protein in the strips used was 20μg and all blots were developed using the phosphatase method. The important bands are arrowed on the right.

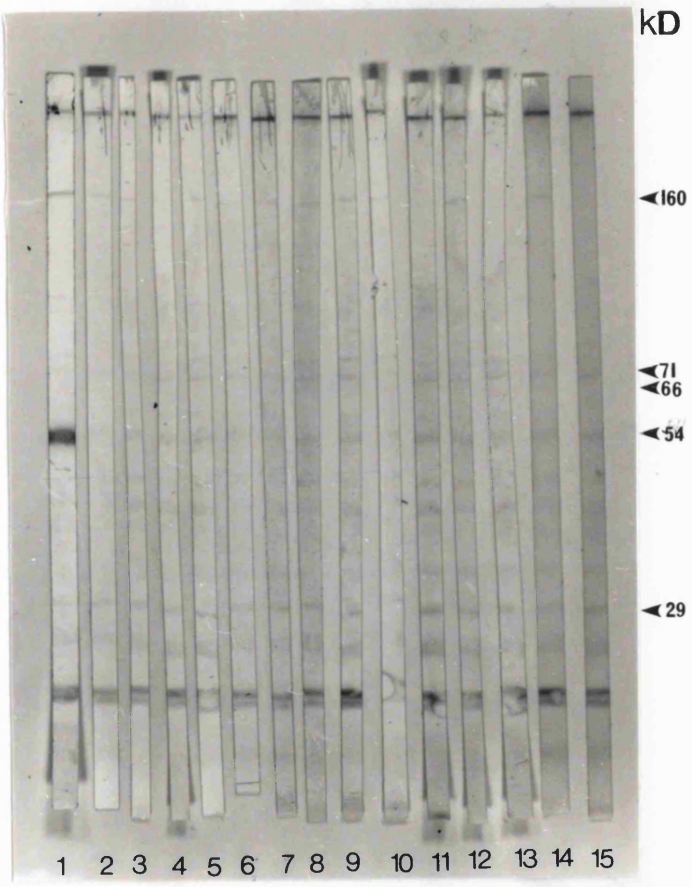

Figure 32b Monoclonal Production 5-Fusion Sera Test on *Notonecta* Ovarian Homogenate

Fusion sera were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strip 1: YOL1/34 (α-tubulin antibody); Strip 2: AB/2; Strip 3: BB/3; Strip 4: EB/5; Strip 5: CC/3; Strip 6: EC/3; Strip 7: EB/2; Strip 8: GB/4; Strip 9: LB/4; Strip 10: KC/3; Strip 11: BB/4; Strip 12: GC/5; Strip 13: FC/4; Strip 14: CB/3; Strip 15: CB/8. YOL1/34 was used at a dilution of 1:1000 while the sera were used undiluted. The amount of protein in the strips used was 20μg and all blots were developed using the phosphatase method. The important bands are arrowed on the right.

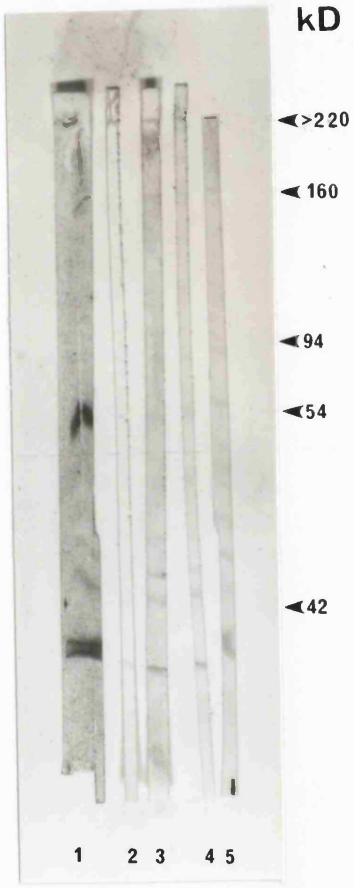

Figure 32c Monoclonal Production 5-Fusion Sera Test on *Notonecta* Ovarian Homogenate

Fusion sera were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strip 1: YOL1/34 (α-tubulin antibody); Strip 2: EC/2; Strip 3: BB/2; Strip 4: BC/2; Strip 5: BC/3. YOL1/34 was used at a dilution of 1:1000 while the sera were used undiluted. The amount of protein in the strips used was 20μg and all blots were developed using the phosphatase method. The important bands are arrowed on the right.

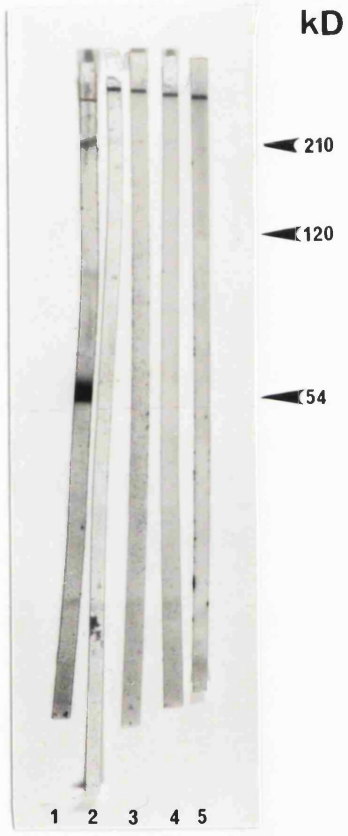

Figure 32d Monoclonal Production 5-Fusion Sera Test on *Notonecta* Ovarian Homogenate

Fusion sera were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strip 1: YOL1/34 (α-tubulin antibody); Strip 2: GC/4; Strip 3: BB/5; Strip 4: DC/4; Strip 5: GC/2. YOL1/34 was used at a dilution of 1:1000 while the sera were used undiluted. The amount of protein in the strips used was 20μg and all blots were developed using the phosphatase method. The important bands are arrowed on the right.

Notonecta ovariole homogenate available (these tests were carried out when the *Notonecta* had died off for the winter and even during the height of the season when *Notonecta* are available there are only a finite number available). As the mouse immunised with the pre-coupled immunogen produced the strongest reaction its spleen was removed and fused using Fusion method II (see Section 2.2Ig), the fusion well supernatants being tested on new nitrocellulose loaded with *Notonecta* ovariole homogenate. A number of specific bands developed, having molecular weights ranging between 29 and over 210kD (the full range of bands is shown in Figure 32 and Table 3). Because of the large number of different positive bands two wells, which produced sera that stained the majority of the relevant bands identified, were chosen for cloning (Figure 32a, Lane 12 and Figure 32c, Lane 3; Table 3), the cloning reverting back to the original schedule with the addition of an improved cloning medium (Cloning method III, see Section 2.2Ik). A number of cloning wells identified a band with a molecular weight of 54kD (Figure 33, Lanes 5 to 10 and 12) while another identified a well at 114kD (Figure 33, Lane 3). The other bands which developed were not identified by supernatant from the original fusion well so are probably non-specific.

The clones were named on the basis of the nitrocellulose strip they were tested on and the molecular weight of the band that they identified (for example the positive clone that recognised a 114kD antigen on Lane 3 of Figure 33 is named CL3/114). To further characterise the antigens recognised by the positive clone wells individual clone supernatants were used in immunofluorescence investigations on a number of different tissue culture cells, including a *Drosophila* B_{II} cell line that had been treated with moulting hormone to stimulate process extension (Clement and Dinan, 1990). Figure 34 shows such cells stained with a broad base-specific α-tubulin antibody. The arrow head indicates microtubules at the cell periphery. The clones were also tested on frozen sections of *Notonecta* ovarioles.

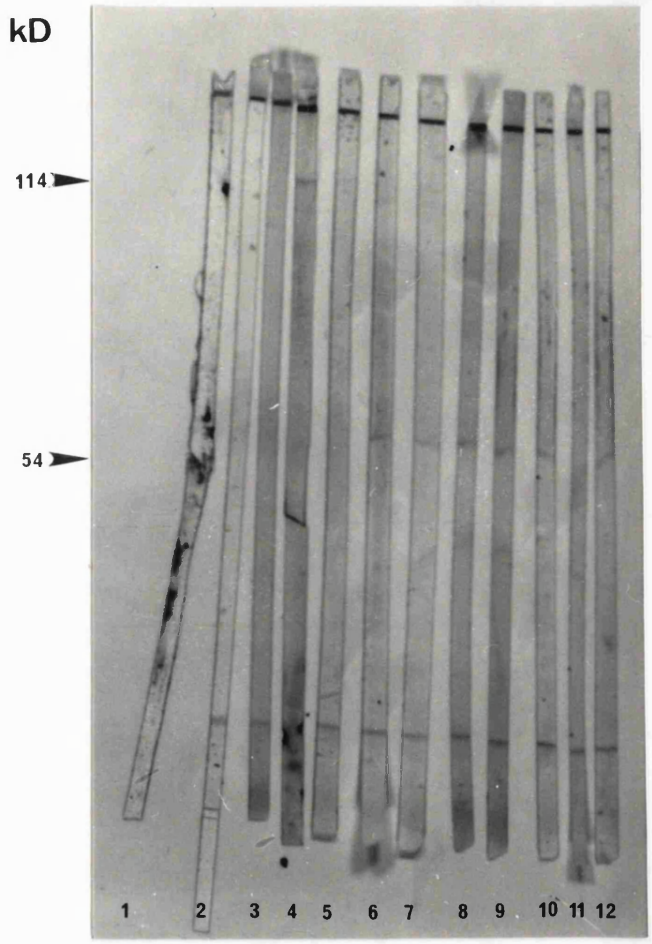

Figure 33 Monoclonal Production 5-Clone Sera Test on *Notonecta* Ovarian Homogenate

Clone sera were used to probe strips of nitrocellulose containing *Notonecta* ovariole homogenate separated by 1-D PAGE. Strip 1: YOL1/34 (α-tubulin antibody); Strip 2: BF/6; Strip 3: **BF/7**; Strip 4: CG/7; Strip 5: **CC/5**; Strip 6: **CF/7**; Strip 7: **CF/2**; Strip 8: **CG/10**; Strip 9: **CG/9**; Strip 10: **CG/11**; Strip 11: BF/3; Strip 12: **BD/6**. YOL1/34 was used at a dilution of 1:1000 while the sera were used at 1:5. The amount of protein in the strips used was 20μg and all blots were developed using the phosphatase method. The sera of interest are highlighted in bold and the important bands are arrowed on the left.

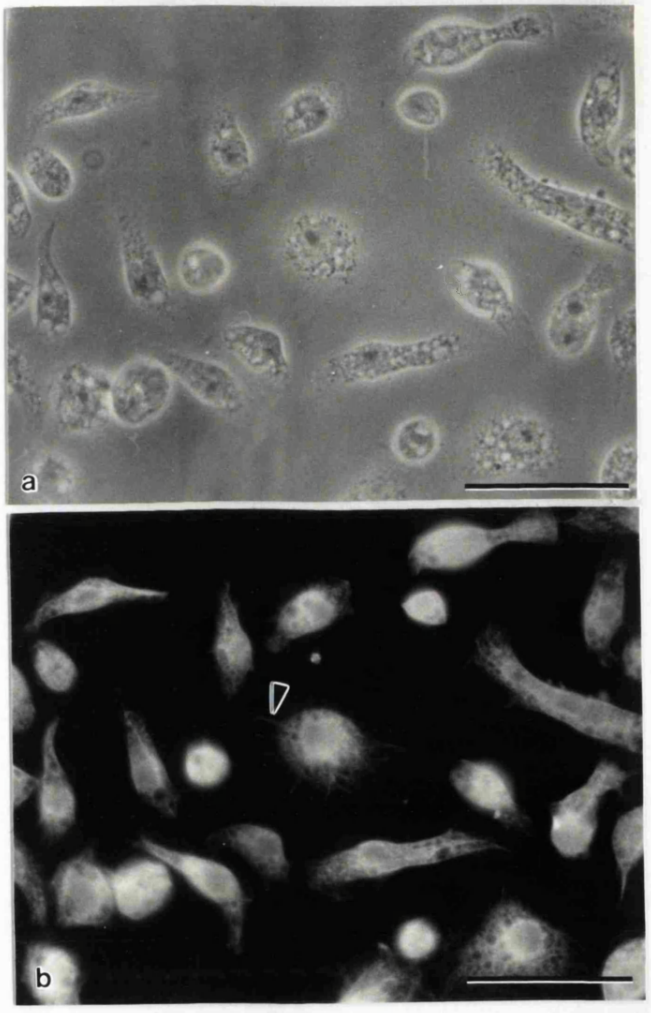

Figure 34 The Distribution of Tubulin in *Drosophila* B_{II} Cells
Drosophila B_{II} cells, treated with the moulting hormone 20-hydroxyecdyone and fixed with formaldehyde, were stained with YOL1/34 (α-tubulin antibody). (a) Phase contrast. (b) The same field stained with YOL1/34 (diluted 1:100). The arrow head shows microtubules and in both cases the bars are 30μm.

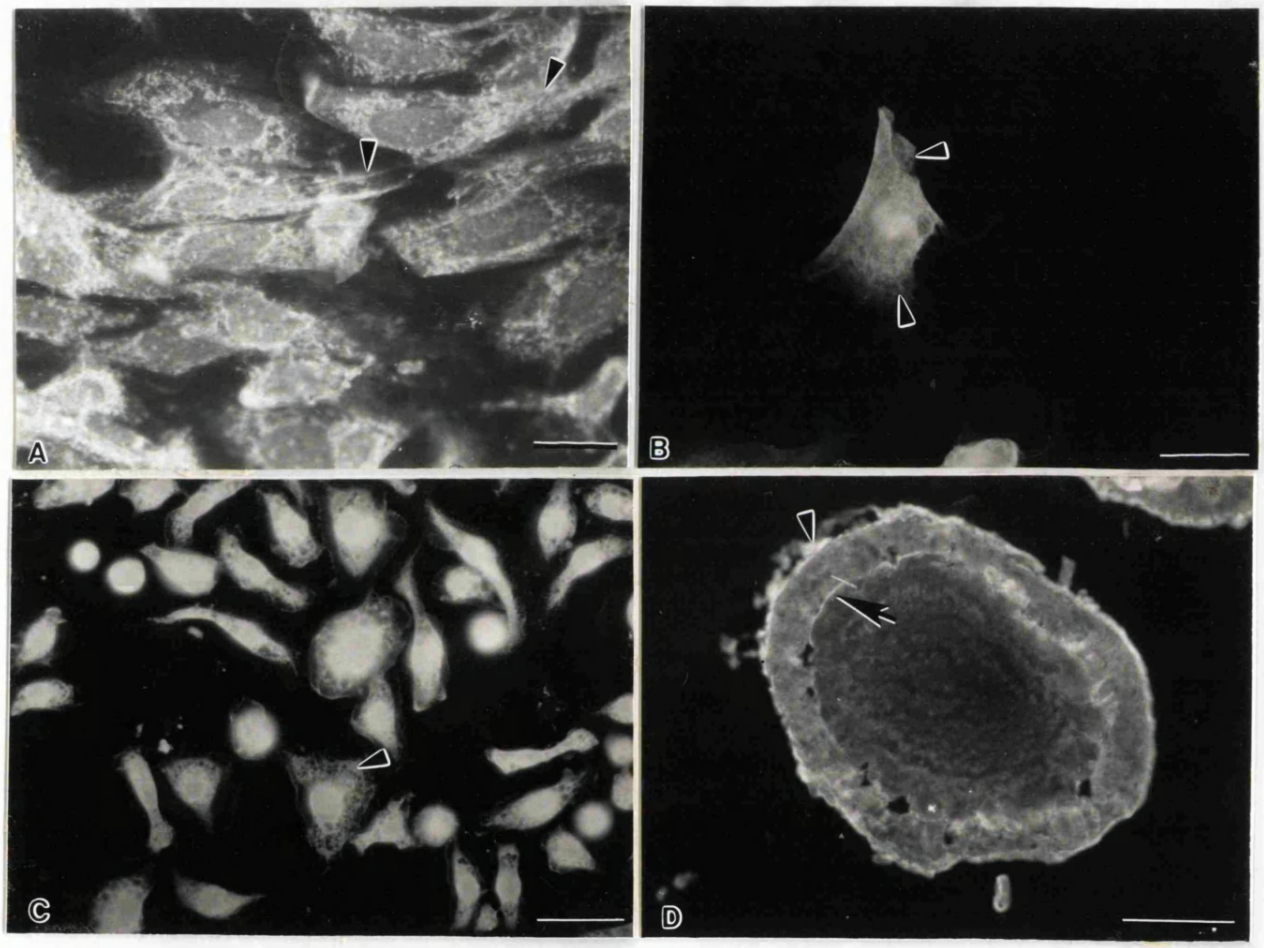

Figure 35 Monoclonal Production 5-CL3/114 Test on 3T3 and *Drosophila* B_{II} Cells and Ovarian Sections from *Notonecta*

The serum from clone CL3/114 was tested on a number of different tissue culture cells and ovarian tissue from *Notonecta*, the serum being used undiluted in each case. (a) Methanol fixed 3T3 cells. The arrow heads indicate faint microtubule like staining. The bar is 20μm. (b) Formaldehyde fixed 3T3 cells. The arrow head indicates particulate patches of staining. The bar is 20μm. (c) Formaldehyde fixed *Drosophila* B_{II} cells. The arrow head indicates particulate patches of staining. The bar is 20μm. (d) Formaldehyde fixed transverse section of a *Notonecta* ovariole. The arrow and heads respectively indicate staining on the inner and outer edges of the nutritive tube staining region. The bar is 50μm.

3.2Va: Testing CL3/114

CL3/114 was initially tested on 3T3 cells that had been fixed in methanol (Figure 35a). The staining produced was quite particulate and spread throughout the cytoplasm, although very sparse, fragmented microtubule like staining was also apparent (Figure 35a, arrow head). When CL3/114 was tested on 3T3 cells fixed using paraformaldehyde a different distribution of staining was revealed, the staining being amorphous with some particulate patches (Figure 35b, arrow) and a centre of more intense nuclear staining. Again the microtubule like staining was sparse but visible, outlining sinous microtubule like structures (Figure 35b, arrow head). The supernatant was then tested on *Drosophila* B_{II} cells. As with the 3T3 cells the staining was not exclusively centred on one single structure, the staining again being very amorphous with some particulate patches visible (Figure 35c, arrow head). The final test on frozen sections of *Notonecta* ovarioles produced staining around the inner and outer margins of the region which contains the nutritive tubes (Figure 35d, arrow head) but no staining was apparent within the tubes themselves (Figure 35d).

3.2Vb: Testing CL6/54

All subsequent clones underwent the same tests as CL3/114. CL6/54 produced a very abberant immunomicroscopic picture with methanol fixed 3T3 cells, staining occuring on a wide range of different structures. A faint microtubule like staining pattern could, however, be visualised (Figure 36a, arrow head). Only amorphous, cytoplasmic staining could be visualised in paraformaldehyde fixed 3T3 cells (Figures 36b) while on treated *Drosophila* B_{II} cells CL6/54 produced a general granular staining (Figure 36c). Staining of *Notonecta* ovariole sections produced non-specific staining throughout the sections, although peripheral staining could be resolved at both the outer edge of the ovariole (arrow head) and, more faintly, on the inner periphery of the region containing the nutritive tubes (arrow). There is also one suggestion of a nutritive tube with the inner, peripheral staining visualised with tyr-tubulin specific antibodies (asterix, Figure 36d).

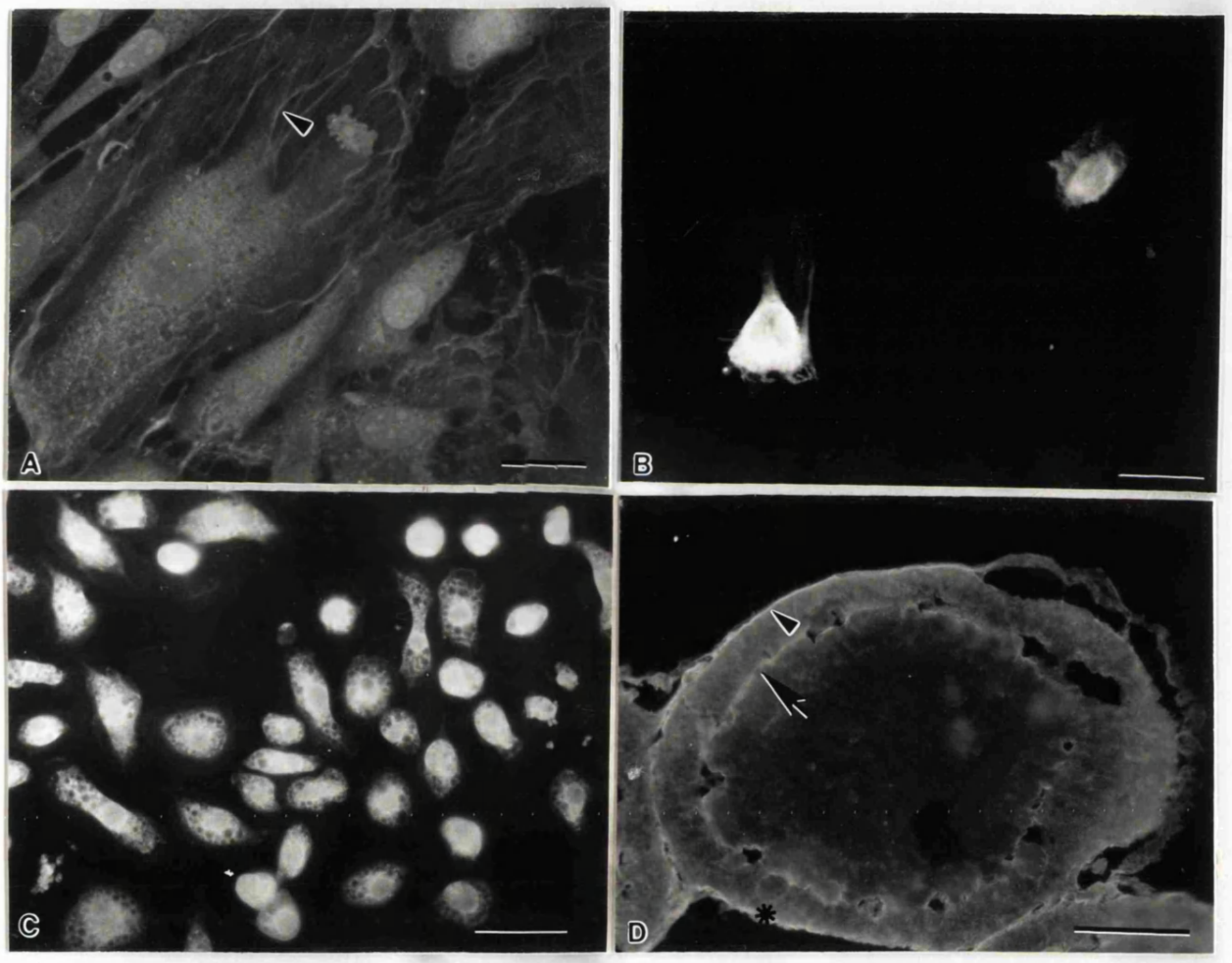

Figure 36 Monoclonal Production 5-CL6/54 Test on 3T3 and *Drosophila* B_{tr} Cells and Ovarian Sections from *Notonecta*

The serum from clone CL6/54 was tested on a number of different tissue culture cells and ovarian tissue from *Notonecta*, the serum being used undiluted in each case. (a) Methanol fixed 3T3 cells. The arrow head indicates possible microtubule like staining. The bar is 20µm. (b) Formaldehyde fixed 3T3 cells. The bar is 20µm. (c) Formaldehyde fixed *Drosophila* B_{tr} cells. The bar is 20µm. (d) Formaldehyde fixed transverse section of a *Notonecta* ovariole. The arrow and arrow heads respectively indicate staining on the inner and outer edges of the nutritive tube staining region. The bar is 50µm.

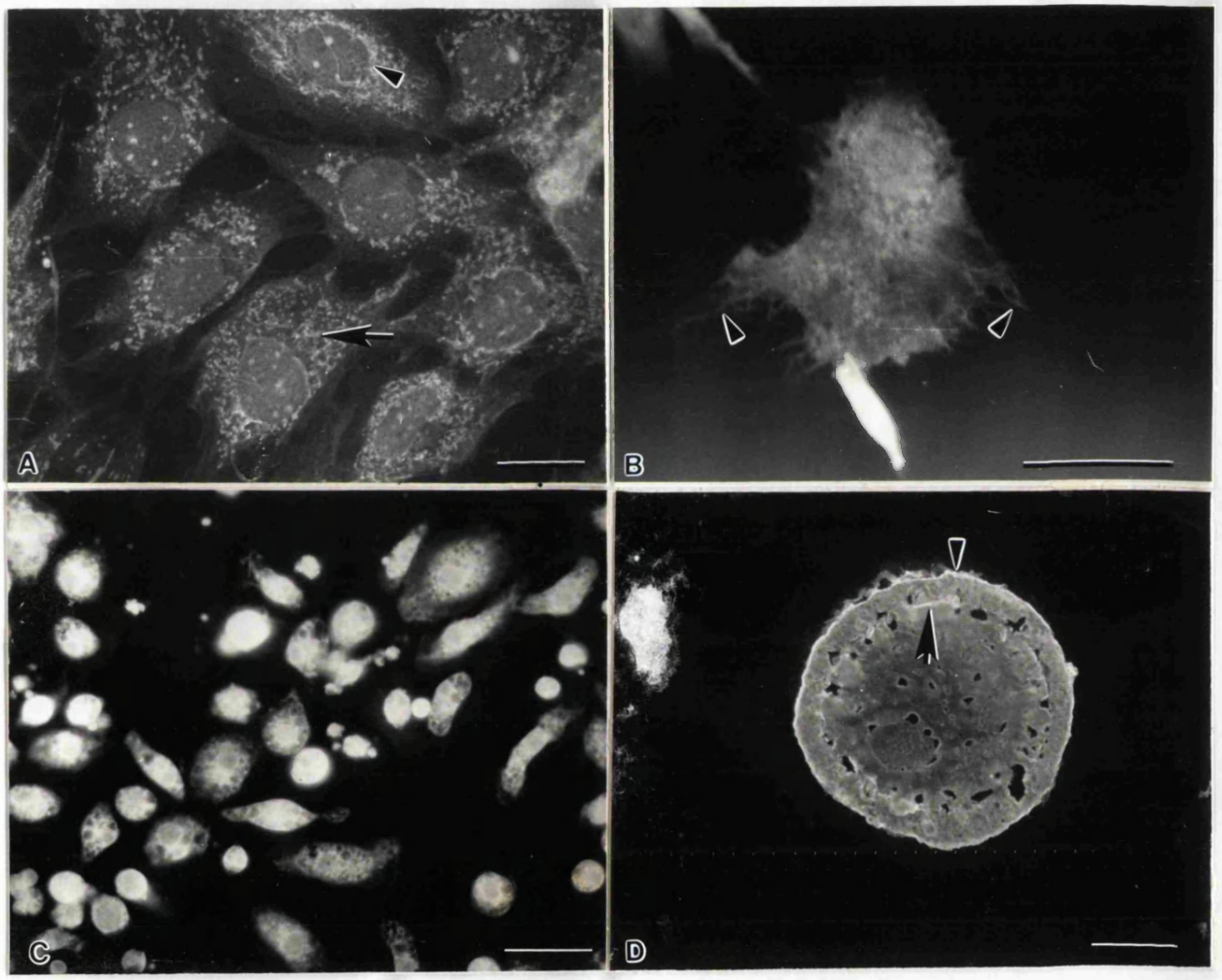

Figure 37 Monoclonal Production 5-CL7/54 Test on 3T3 and *Drosophila* B_{II} Cells and Ovarian Sections from *Notonecta*

The serum from clone CL7/54 was tested on a number of different tissue culture cells and ovarian tissue from *Notonecta*, the serum being used undiluted in each case. (a) Methanol fixed 3T3 cells. The arrow indicates particulate cytoplasmic staining while the arrowhead shows particulate staining at the nucleus. The bar is 20μm. (b) Formaldehyde fixed 3T3 cells. The arrow heads indicate wispy projections at the cell edge. The bar is 20μm. (c) Formaldehyde fixed *Drosophila* B_{II} cells. The bar is 20μm. (d) Formaldehyde fixed transverse section of a *Notonecta* ovariole. The arrow and arrowheads respectively indicate staining at the inner and outer edges of the nutritive tube containing region. The bar is 50μm.

3.2Vc: Testing CL7/54

CL7/54 staining of methanol fixed, 3T3 cells produced a particulate staining that was partly cytoplasmic (arrow) and partly concentrated around the periphery of the nucleus (arrow head, Figure 37a). The particles stained were of a roughly uniform size and, apart from the nuclear concentration there seemed to be no distinct concentrations in the cytoplasm. In paraformaldehyde fixed 3T3 cells the staining was amorphous and wholly cytoplasmic though there were "wispy" projections at the cell edge (arrow heads, Figure 37b) while in *Drosophila* B_{II} cells the clone again produced a granular staining pattern, the entire cytoplasm seeming to be stained (Figure 37c). As with the previous clone tested the staining of *Notonecta* ovariole sections produced a very faint staining around the outer edge of the ovariole (arrow) and the inner periphery of the region containing the nutritive tubes (arrow head), there being no actual staining within the tubes themselves (Figure 37d).

3.2Vd: Testing CL8/54

Methanol fixed 3T3 cells incubated with CL8/54 produced a fragmented, particulate cytoplasmic staining pattern with the suggestion that the fragments were derived from tubular structures (arrow, Figure 38a). The staining was also more concentrated at the periphery of the nucleus (arrow head, Figure 38a). In contrast, paraformaldehyde fixed 3T3 cells incubated with the clone serum exhibited a more general amorphous, cytoplasmic staining though particulate staining was visible concentrated at the periphery of the nucleus (arrow, Figure 38b). At the cell edges there were also "wispy" projections (arrow head, Figures 38b). Staining of *Drosophila* B_{II} cells, as with CL6/54, produced a suggestion of sinous tubules amongst a general background of amorphous staining (arrows, Figure 38c) but treatment of *Notonecta* sections with CL8/54 produced general tissue staining that was not concentrated specifically in nutritive tubes though the outer edge of the ovariole seemed to be faintly stained (arrow head, Figure 38d).

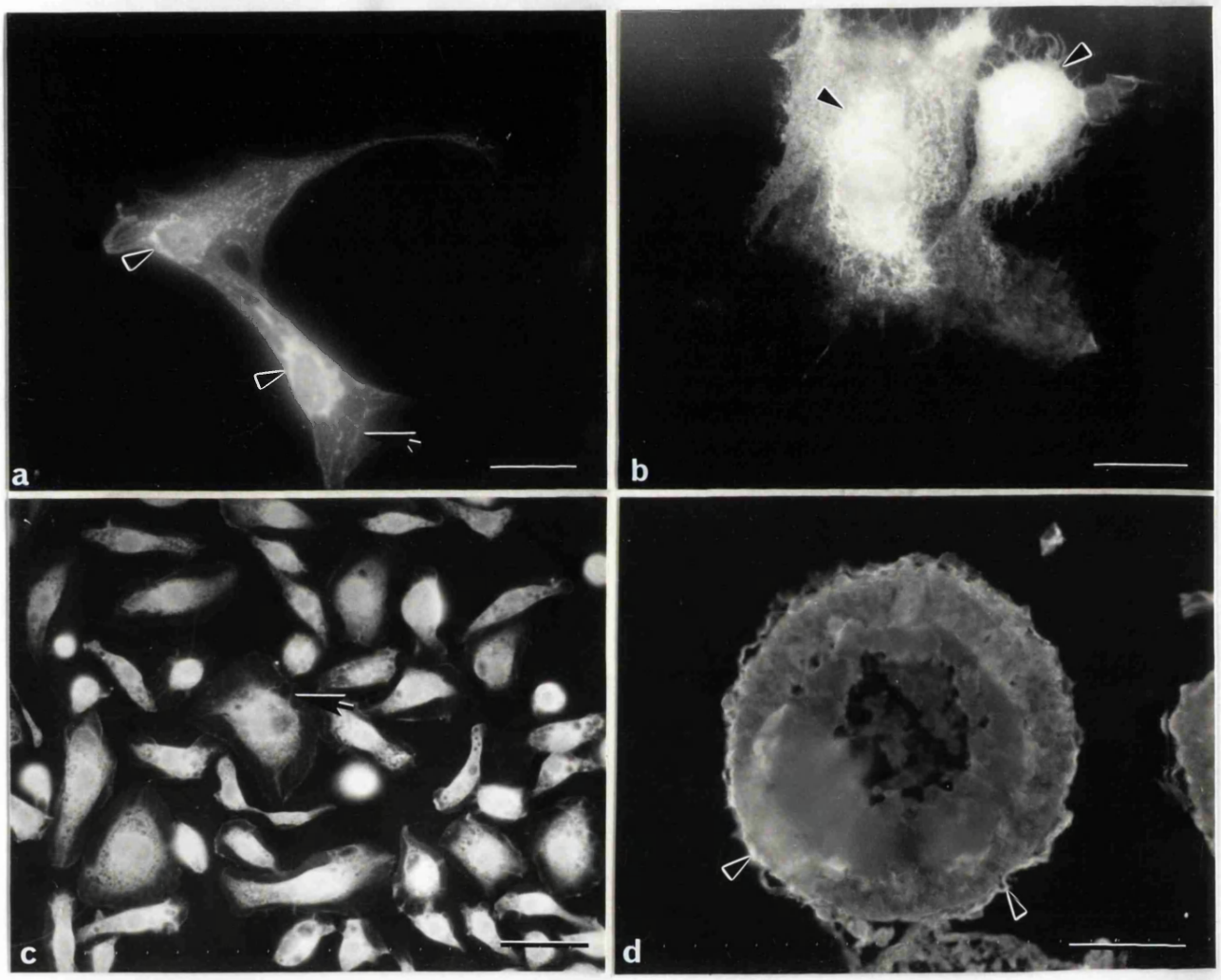

Figure 38 Monoclonal Production 5-CL8/54 Test on 3T3 and *Drosophila* B_{II} Cells and Ovarian Sections from *Notonecta*

The serum from clone CL8/54 was tested on a number of different tissue culture cells and ovarian tissue from *Notonecta*, the serum being used undiluted in each case. (a) Methanol fixed 3T3 cells. The arrow indicates structures which have possibly derived from fragmented microtubule like structures while the arrowheads show more perinuclear staining. The bar is 20μm. (b) Formaldehyde fixed 3T3 cells. The arrow heads indicate particulate, perinuclear staining. The bar is 20μm. (c) Formaldehyde fixed *Drosophila* B_{II} cells. The arrow indicates sinous tubules. The bar is 20μm. (d) Formaldehyde fixed transverse section of a *Notonecta* ovariole. The arrow heads indicate faint staining at the outer edge of the ovariole. The bar is 50μm.

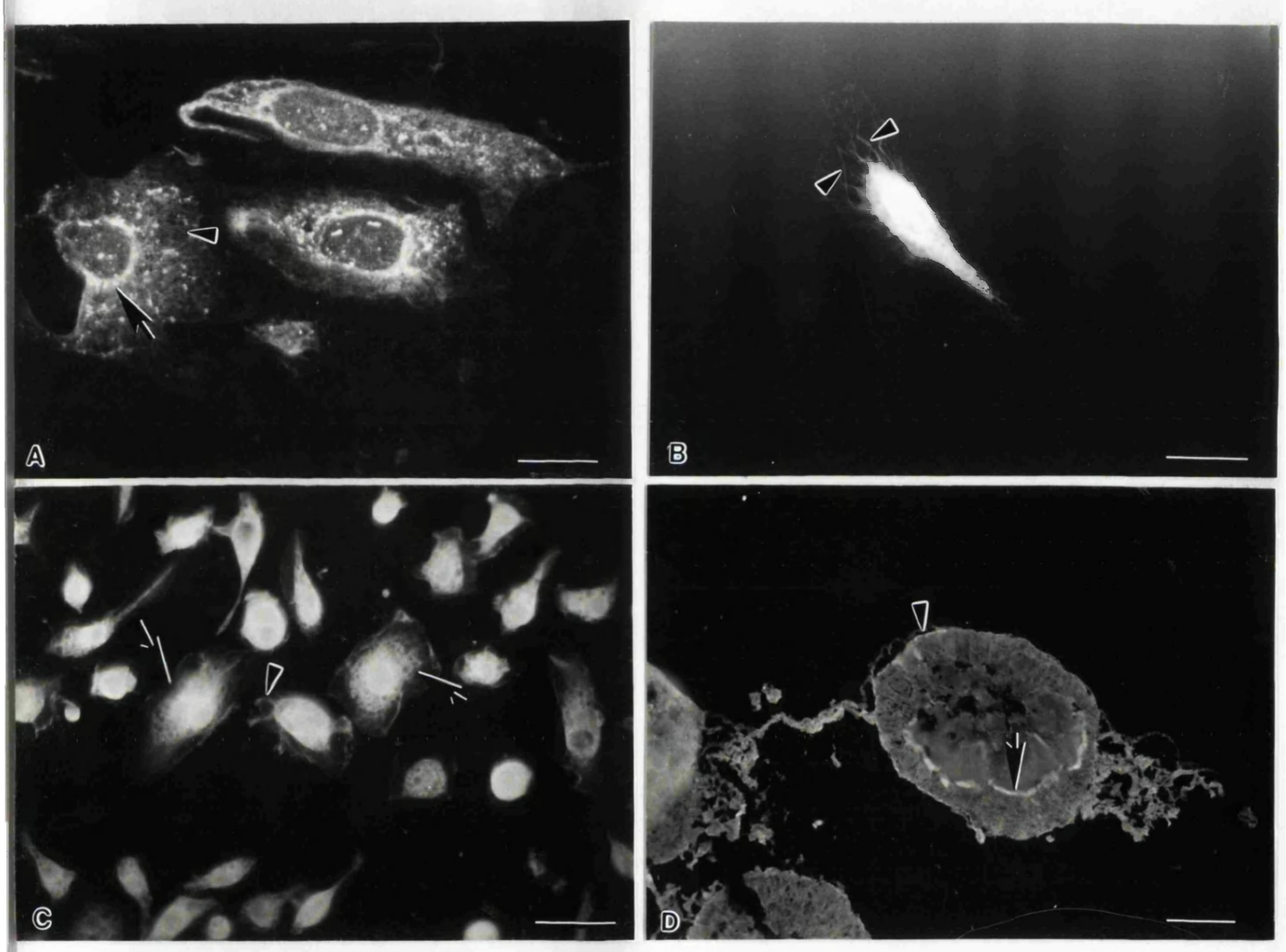

Figure 39 Monoclonal Production 5-CL9/54 Test on 3T3 and *Drosophila* B_{II} Cells and Ovarian Sections from *Notonecta*

The serum from clone CL9/54 was tested on a number of different tissue culture cells and ovarian tissue from *Notonecta*, the serum being used undiluted in each case. (a) Methanol fixed 3T3 cells. The arrow heads indicate sparse microtubule like staining while the arrow shows perinuclear staining. The bar is 20μm. (b) Formaldehyde fixed 3T3 cells. The arrow heads indicate short sinous tubules at the cell margins. The bar is 20μm. (c) Formaldehyde fixed *Drosophila* B_{II} cells. The arrow head indicates a sinous microtubule like structure while the arrows show granular staining concentrated at the nucleus. The bar is 20μm. (d) Formaldehyde fixed transverse section of a *Notonecta* ovariole. The arrow and arrow heads respectively indicate staining at the inner and outer edges of the nutritive tube containing region. The bar is 50μm.

3.2Ve: Testing CL9/54

The last clone to be tested on methanol fixed, 3T3 cells identified an intensely particulate staining pattern in which there was very sparse microtubule like staining (arrow head, Figure 39a). Again there were regions of intense staining concentrated around the nuclear periphery (arrow, Figure 39a). When the clone was used to stain paraformaldehyde fixed 3T3 cells the majority of the cytoplasm stained intensely but short, sinous, microtubule like structures could also be identified at the cell margins (arrow head, Figure 39b). Staining of *Drosophila* B_{II} cells produced a granular staining pattern particularly concentrated at the nucleus (arrow, Figure 39c) while in some cells there was a sparse amount of sinous, microtubule like staining towards the cell margins (arrow head, Figure 39c). This was not, however, a consistent result as many cells had unstained edges. In contrast, treatment of *Notonecta* ovariole sections yeilded staining of the inner periphery of the region containing the nutritive tubes (arrow, Figure 39d), the outer edge of the ovariole only having slight staining (arrow head, Figure 39d). The tubes themselves were not stained (Figure 39d).

The remaining positive clones could not be tested in the time available. They were, therefore, frozen down and stored in a liquid nitrogen fridge for subsequent evaluation.

4. 1: DISCUSSION

Though working on a common system this body of work has diversified into two main sections: the characterisation of tubulin and the analysis of the distribution of microtubules in the ovaries of both *Notonecta glauca glauca* and *Oncopeltus fasciatus* and the production of monoclonal antibodies exclusively using *Notonecta* ovarian tissue. Due to this investigative diversification, which only truly reaches common ground at the antibody testing stage, I will initially address the interpretation of the results separately before drawing some parallels in the final analysis.

4. 1II: Characterisation of nutritive tubes in *Notonecta* and *Oncopeltus*

4. 1IIa: Immunological characterisation of the nutritive tubes of *Notonecta glauca glauca*-Initial results

Using an established method of tubulin purification, tubulin and microtubule-associated proteins were purified from a homogenate of *Notonecta* ovaries. In the original homogenate tubulin accounted for approximately 8% of the total soluble protein while in the purified microtubules there was a 3 fold increase in microtubular (Figure 21). The relative scarcity of insects did, however, necessitate working with very small volumes.

Utilising the technique of one-dimensional gel electrophoresis, *Notonecta* ovary microtubules were separated into two major components. These were determined, by their molecular weights and immunoreactivities, to be α- and β-tubulin. Further separation using two-dimensional gel electrophoresis revealed that *Notonecta* ovarian α-tubulin was composed of one major and one minor isoform. On the basis of isoelectric point and post-translational modification these could be classified as α1 and α3-tubulins (L'Hernault and Rosenbaum, 1983). Two-dimensional separation of β-tubulin was not so clear. Due to the fact that the β-tubulin antibody used was in short supply, and thus had to be reused, the titre of active antibody in the serum was diluted to a level

where a single β-tubulin spot could just be defined. The two α-tubulin spots on the same nitrocellulose, however, developed to the same intensity as in the previous experiments. This faintness rendered it technically difficult to reproduce the spot on subsequent prints. Nevertheless, the results demonstrated that a single β-tubulin is expressed in *Notonecta* ovarian tissue. This is in contradiction to the work of Sharma and Stebbings (1985) who found that the ovaries of *Notonecta* had two α and two β-tubulins. In the published figure, however, the separation of the two β-tubulin spots is indistinct. It is possible that this arose due to incomplete isoelectric focussing of a single isotype producing an extended spot. The potential for incomplete separation leading to streaking was avoided here by increasing focussing to 9000Vh as opposed to the 6050Vh used by Sharma and Stebbings (1985), the increased time and voltage leading to a fuller separation. Care was also taken to ensure that isoelectric focussing was carried out with the tubes exactly vertical. Variations from the perpendicular produce artefactual smearing when run in the second dimension, this being particularly apparent in closely migrating proteins.

4.1Ib: Immunological characterisation of the nutritive tubes of *Notonecta glauca glauca*-Post-translational modifications

Investigations into post-translational modifications of *Notonecta* and *Oncopeltus* tubulins (see Sections 3.1II and 3.1III) used both monoclonal and polyclonal antibodies. These were supplied by workers who had both characterised and published well established work involving the sera. To verify this, the antibodies were tested on purified bovine brain microtubule samples separated by one-dimensional gel electrophoresis and on cultured 3T3 mouse fibroblasts. In all cases antibodies specific for the same post-translational modification gave the same results. Glu, tyr and acetylated tubulin specific antibodies recognised the same microtubule subsets that previous workers had visualised.

From two-dimensional Western blot analysis of *Notonecta* ovaries both α1 and α3 tubulins appear to exist in both the tyrosinated and

detyrosinated forms. The α3 subunit is also acetylated, the process of acetylation shifting it to a lower isoelectric point (L'Hernault and Rosenbaum, 1985a; b). The system thus contains the two most well known tubulin post-translational modifications. When considered with regard to the life-cycle of *Notonecta* this is not unexpected. *Notonecta* is an aquatic insect which lives in waters at, or approaching 0°C, for its entire life span. It can thus be assumed that the microtubules within the insect, especially the nutritive tubes, have a slow rate of turnover and hence an extended life-span. As post-translational modifications of tubulin are time-dependent, the more stable a microtubule is the more chance that its tubulin can be post-translationally modified (Bulinski *et al*, 1988). The stable microtubules of *Notonecta* nutritive tubes would, therefore, appear to be ideal substrates for these processess.

4.11c: Immunological characterisation of the nutritive tubes of *Notonecta glauca glauca*-*In situ* localisation of post-translational modifications

When the distribution of post-translationally modified tubulins of *Notonecta* ovaries was observed *in situ* some very interesting results became apparent. As expected, both α- and β-tubulin were localised in all nutritive tubes, irrespective of age. There were no exclusions of staining from nutritive tubes on the basis of their level of redundancy (Harrison *et al*, 1991). The distribution of α- and β-tubulin must therefore remain constant throughout the lifetime of a nutritive tube, up to the point of terminal redundancy, and this was apparent with all the antibodies used. Tubulin staining was also observed in the follicle cells that surround the nutritive tubes and was particularly apparent at cell junctions, presumably due to staining above and below the plane of focus. Amorphous staining was also observed within the central region of the ovarioles. This is possibly the soluble pool of tubulin that derives from the breakdown of terminally redundant nutritive tubes. There are two possible fates for the large amount of tubulin released by the breakdown of the redundant nutritive tubes: either it is transported back to the trophic region for reuse in the assembly of future nutritive tubes, or it may be used in the early divisions of the fertilised

oocytes. No evidence yet exists to confirm either hypothesis (Bennett and Stebbings, 1979).

The distribution of glu and acetylated tubulin in *Notonecta* ovarioles was identical to that of α-tubulin, all sections of nutritive tubes taken midway between the trophic region and the terminal oocyte stained completely with antibodies specific for both isotypes. This indicates that at this point all microtubules in all nutritive tubes have both glu and acetylated tubulin regardless of tube age. There was no obvious exclusion of either tubulin isotype from any part of the nutritive tubes. As noted in Section 4.1Ib, the longer a microtubule exists before breakdown, the higher the probability that it will become post-translationally modified and hence, in the case at hand, form glu and acetylated tubulins.

At a more detailed level, the effect of microtubule age on post-translational modification can be resolved by investigating the distribution of glu-tubulin at progressive sections through a *Notonecta* ovariole. In the mid-trophic region, where microtubules assemble prior to extending into nutritive tubes (Stebbings and Hunt, 1983; this paper refers to *Oncopeltus* but it is assumed that the situation is the same in *Notonecta*) the amount of glu-tubulin is relatively low. At the neck region, where the nutritive tubes proper begin, glu-tubulin is distributed throughout all nutritive tubes, both functional and redundant. This pattern continues posterially, although the levels of glu-tubulin in the nutritive tubes proper are higher than that seen in the neck region. This indicates that as the assembling microtubules are inserted into the nutritive tubes at the neck region they are still predominantly tyrosinated. However, once they actually start to grow through the nutritive tubes proper they are quickly detyrosinated. The distribution of glu-tubulin throughout the functional and redundant tubes continues through the region where the nutritive tubes enter the oocytes. This again indicates that the longevity of these microtubules allows widespread post-translational modification. Thus, moving posteriorally along the ovariole, the development of the nutritive tubes, and the increasing age of the microtubules therein, is paralled

by an increase in the level of post-translational tubulin modification. Due to the shortage of anti-acetylated tubulin specific antibodies, this analysis could not be repeated to show the distribution of acetylated tubulin. However, as the distributions of acetylated and glu-tubulin are identical in most systems investigated (Schulze *et al*, 1987; Bulinski *et al*, 1988), and in mid-nutritive tube sections (see above) it is likely that these two post-translational modifications follow the same patterns through the developing ovariole.

The distribution of tyr-tubulin in the nutritive tubes of *Notonecta* ovarioles stands in marked contrast to that of glu-tubulin and suggests a method by which the tubes grow. Sections through the mid-point of the nutritive tubes showed that tyr-tubulin is restricted to a ring inserted around the inside edge of the functional tubes. As the age of a microtubule is reflected in the level of post-translational modification (Gundersen *et al*, 1987; see above), this suggests that the microtubules around the inner edge of the functional nutritive tubes are younger than their more central counterparts which are post-translationally modified. This distribution can be further related to the known methods of construction of nutritive tubes. Hyams and Stebbings (1979b) found that when functional nutritive tubes age they increase in diameter. Despite this dimensional change, however, there is no concomitant increase in microtubule-microtubule spacing (Hyams and Stebbings, 1979b; Stebbings *et al*, 1985). This implies that there is an increase in microtubule number. It would therefore seem likely that the newly polymerised microtubules, that have yet to be post-translationally modified, are inserted at the inner edge of the enlarging nutritive tube, thus producing a ring of tyr-tubulin specific staining. As the nutritive tubes age further and expand, new, tyr-tubulin microtubules continue to be inserted at the edge. The previously tyr-tubulin microtubules are displaced to a more central position, and are concominantly post-translationally modified. The functional nutritive tube can thus be envisaged as a series of concentric rings of microtubules that get progressively younger as they move out from the centre of the tubes, a structure somewhat analogous to that of the growth rings in the trunk of a tree.

DISCUSSION

The presence of an outer ring of tyr-tubulin was found almost entirely throughout the mature nutritive tube system. In mature ovarioles the trophic region exhibited strong tyr-tubulin specific staining that was restricted to centrally located amorphous patches. This presumably represents free tubulin dimers as well as freshly assembled microtubules which have yet to be detyrosinated. The high proportion of tyr-tubulin in new microtubules is also apparent in the nutritive tubes that are in the neck region, just emerging from the trophic region. Here the tyr-tubulin of functional tubes has the characteristic ring distribution described above. There are, however, smaller tubes which also have a tyr-tubulin ring. If these were redundant tubes, as their diameter suggests, they would be expected to contain detyrosinated microtubules. A more likely explanation is that these tubes are new functional tubes which have already started to increase in diameter following the concentric circle model. The fact that the central region is devoid of tyr-tubulin staining suggests that the detyrosination mechanism is already in operation. Sections through the point where the nutritive tubes enter the oocyte illustrated that there was a decrease in tube numbers due to the breakdown of redundant tubes. Despite this, mature, functional tubes could still be observed with the tyr-tubulin ring, indicating that there is still new microtubule insertion even at this late stage in the nutritive tubes existence. As before, the redundant tubes, which become proportionally greater in number, have no tyr-tubulin. Finally, as the nutritive tubes enters the oocytes the number of functional tubes is greatly reduced, though the remaining tubes still have a tyr-tubulin ring. This indicates that the expansion of nutritive tubes, along with the concomitant insertion of microtubules occurs right up to the initiation of redundancy.

As described above, the tyr-tubulin ring structure is present throughout almost the entire ovariole system. The one exception is in the immature ovarioles of juvenile animals. Here the characteristic ovariole structure observed in more mature animals is considerably reduced with little, if any, oocyte development. Thus, the immature nutritive tubes are very short, and stain totally with tyr-tubulin

specific antibodies. The ring structure cannot be observed in either longitudinal or transverse section. It is thus assumed that, in immature *Notonecta* ovarioles, all microtubules are young.

The ubiquity of the tyr-tubulin ring throughout the mature ovariole system indicates that the inserted microtubules are probably growing to "catch up" with the older microtubules and thus maintain the constant microtubule-microtubule spacing in the expanding nutritive tubes (Hyams and Stebbings, 1979b), the new microtubules being inserted throughout the length of the nutritive tubes. This suggests that there may be microtubule organising centres at all levels of a nutritive tube, from the trophic region right up to the oocyte. These must display the unusual property of the organising centres in the trophic region, that is they must nucleate the microtubules with the + ends proximal (Stebbings and Hunt, 1983). Unfortunately, the existence of such organising centres has not been demonstrated but an obvious hypothesis is that they are restricted to the inner edge of the nutritive tubes so that "new" microtubules are inserted in their characteristic ring distribution. This is something that will have to be addressed in the future. In the meanwhile a possible model is illustrated in Figure 40. The peripheral MTOCs that are anchored through out the length of the nutritive tube could be centrioles, γ-tubulin (though microtubules nucleated in neurones, an analogous system, appear not to have such an association; Baas and Joshi, 1992) or structures like the cell-surface associated centrosomal layer found in the inner pillar cells of mouse cochlea (Tucker *et al*, 1992). As the nutritive tube expands (Figure 40a) a control mechanism initiates the nucleation of a new tyr-tubulin rich microtubule from the putative organising centre (b). The subunits for this could be carried from the trophic region by a - end directed motor such as dynein or from the oocyte by a + end directed motor like kinesin or a kinesin related protein. This is similar to the model proposed for the transport of tubulin in axonal development, though the polarity is reversed (Okabe and Hirokawa, 1988). To maintain the correct polarity with the - ends of the microtubule distal to the trophic region the new seed would then have to be translocated posteriorally along the nutritive tube, the tyr-tubulin assembling at the (+) end once the seed

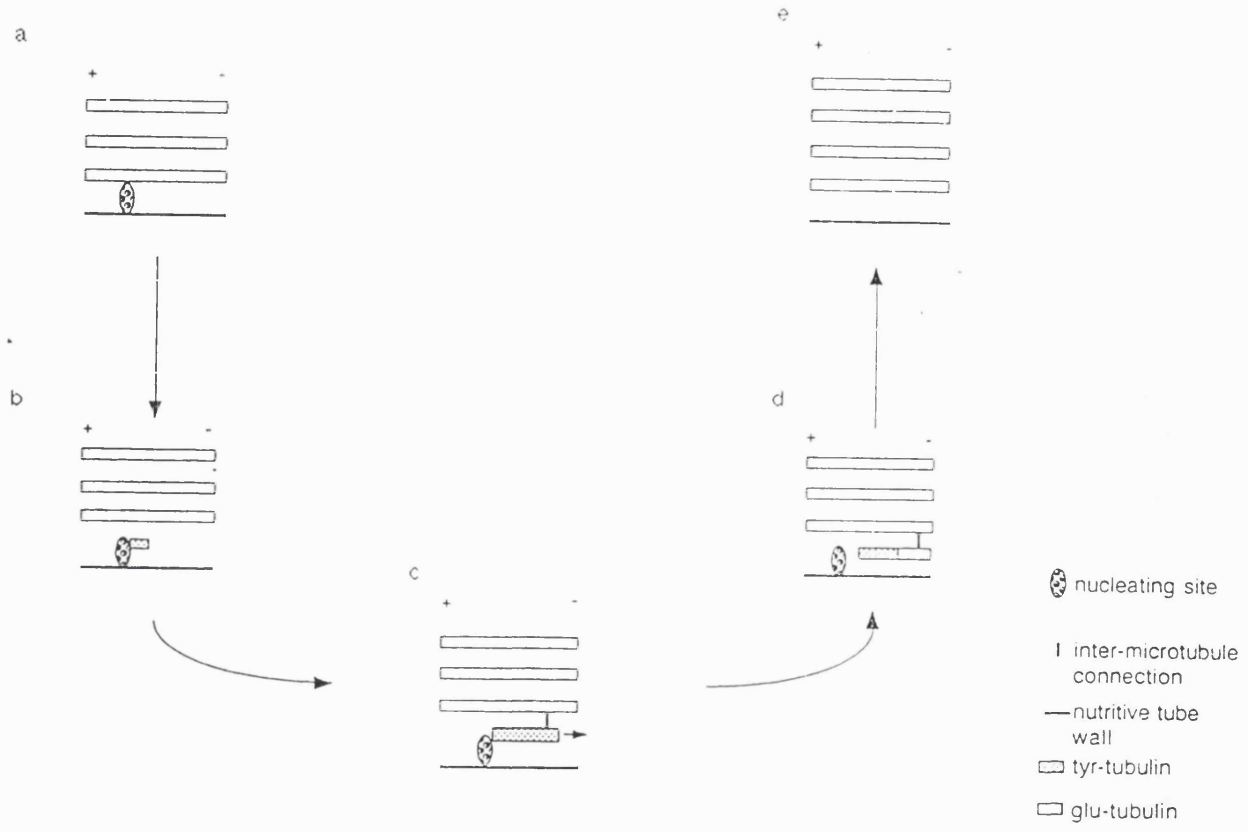

Figure 40 A Model Illustrating a Possible Method of Microtubule Insertion and Growth In Expanding *Notonecta* Nutritive Tubes For details see text

begins moving. The MTOC itself may move with the assembling microtubule or, alternatively, the microtubule's connection with the MTOC may be broken at the same time as the motor protein becomes active, its action physically moving the microtubule seed along the existing microtubule thus allowing polymerisation to occur (c,d). In both cases this indicates the presence of a control mechanism associated with the motor protein that can distinguish between the existing microtubule and the MTOC at the nutritive tube wall, the initiation of a connection with the former allowing the breaking of the connection with the latter or with the latter's connection to the nutritive tube wall. This control could be based on the phosphorylation of MAPs, as demonstrated with MAP-2 or tau (Brugg and Matus, 1991; Jameson *et al*, 1980; Burns *et al*, 1984). Alternatively binding between the microtubule and the MTOC and between the MTOC and the nutritive tube wall may be due to the same MAP with different binding repeats being involved (the first repeat of tau has a much increased binding affinity compared to the subsequent repeats; Butner and Kirschner, 1991). The identification of dynein in nutritive tubes gives further support to this model (Anastasi *et al*, 1990). It could remain inactive until conditions are correct for the beginning of the movement of the microtubule seed along the existing microtubule, the complex timing of this mechanism being controlled by either phosphorylation or the dynein activator dynactin, an insect homolog of which has recently been identified (Hyman and Mitchison, 1991; Schroer and Sheetz, 1991; Gill *et al*, 1991; Holzbaur *et al*, 1991). When the new microtubule is eventually established the need to maintain a large bundle of orientated microtubules further suggests a model similar to that proposed for microtubules nucleated in axons by Okabe and Hirokawa (1988), Baas and Black (1990) and Baas and Ahmad (1992). The newly established seeds are being used, as with the stable microtubules in the axon model, to nucleate new microtubules that will grow to "catch up" with the older, more centrally located ones. As this is occuring the nutritive tube will still be increasing in diameter so new tyr- microtubules will be initiated as outlined above, the microtubule formed in the above description moving further inwards and becoming detyrosinated (e).

4.1Id: Immunological characterisation of the ovarian tissue of *Oncopeltus fasciatus*-Initial results

As with *Notonecta* the initial work involved probing 1-D Western blots of ovarian material with α-tubulin specific antibodies. This led to the finding that *Oncopeltus* has a single α-tubulin with a molecular weight of 52kD. The scarcity of reactive β-tubulin specific antibody and the secondary nature of this investigation means that the β-tubulin content of *Oncopeltus* ovarioles was not elucidated. The prescence of only one α-tubulin was verified by 2-D PAGE. This spot had a molecular weight and isoelectric point in the same range as the α1 spot of *Notonecta* and, due to its relative size and subsequent post-translational modifications, was classified as *Oncopeltus* ovariole α1-tubulin.

4.1Ie: Immunological characterisation of the ovarian tissue of *Oncopeltus fasciatus*-Post-translational modifications

Oncopeltus α1-tubulin was found to exist only in the tyrosinated form. There were no detectable glu or acetylated isotypes present. The fact that the absence of detyrosination was not due to the inability of the glu-specific antibodies to recognise any glu-tubulin present was confirmed by *in vitro* detyrosination of *Oncopeltus* ovariole homogenate using a commercially available carboxypeptidase. This produced a spot that had the molecular weight and isoelectric point of *Oncopeltus* α1-tubulin and was also recognised by glu-tubulin specific antibodies. In concert there was a loss of tyr-tubulin specific staining. An analogous experiment using an acetyl transferase to mimic the acetylation of tubulin *in vivo* was not possible within the time constraints operating. It is possible that the *Oncopeltus* ovariole system lacks the carboxypeptidase necessary for detyrosination yet retains the transferacetylase, as occurs in HeLa cells (Bulinski *et al*, 1988). If this is the case then the relative lack of longevity of ovariole microtubules in *Oncopeltus* may be preventing the transferacteylase from acting. Alternatively the enzyme may be present yet have a very low level of activity and hence its action can not be observed in the rapidly turning over *Oncopeltus* ovariole system.

4.1If: Immunological characterisation of the ovarian and ganglion tissue of *Oncopeltus fasciatus*- *In situ* localisation of post-translational modifications

When the post-translational modifications of α-tubulin in *Oncopeltus* nutritive tube microtubules were observed *in situ*, the results corroborated the *in vitro* work and reinforced the differences between *Notonecta* and *Oncopeltus*. As expected, the nutritive tubes stained throughout with α-tubulin specific antibodies. There was no exclusion from either the functional or the redundant tubes on the basis of age or position. The nutritive tubes are, therefore, packed with microtubules that can be stained and, as before, any subsequent exclusions of staining must be due to post-translational differences and not staining artefacts. This is important when considering the distribution of glu, tyr and acetylated tubulin *in situ*. As with *Notonecta*, the presumed pool of unpolymerised tubulin in the trophic region of *Oncopeltus* stained amorphously for tyr-tubulin. It is assumed that this tubulin is subsequently polymerised into the microtubules of the nutritive tubes where it has the opportunity to be post-translationally modified. The distribution of isotypes in the nutritive tubes of *Oncopeltus* was markedly different to *Notonecta*. All *Oncopeltus* nutritive tubes, functional and redundant, are composed exclusively of tyr-tubulin. In contrast, post-translational modifications were evident in *Oncopeltus* ganglia. Thoracic and abdominal ganglia could be seen to contain a central core of tyr-tubulin surrounded by a ring of glu-tubulin. As in the ovariole, it is assumed that a pool of unpolymerised tyr-tubulin will subsequently be assembled into the microtubules of the axons. At this point the tubulin is post-translationally modified, producing a ring of microtubules at the edge of the ganglion tissue which are detyrosinated.

Overall, the absence of glu and acetylated-tubulin on immunoblots and the recognition of glu-tubulin in experimentally detyrosinated ovariole homogenate, suggests that *Oncopeltus* ovarioles either do not possess the post-translational modifing mechanisms or they have the necessary enzymes but they work with an efficiency too low to be detected.

Preliminary work suggested the former. When bovine brain microtubules, which on 1-D immunoblots have similar levels of glu and tyr-tubulin (see Figure 20), were treated with a homogenate of *Oncopeltus* ovarioles there was no detectable detyrosination, whereas complete detyrosination was achieved by commercially available carboxypeptidase. It is possible that *Oncopeltus* carboxypeptidase, if present, may not recognise bovine tubulin, but this is unlikely as bovine pancreas carboxypeptidase reacts with *Oncopeltus* microtubules and there are reports of commercially available carboxypeptidases reacting with tubulins from a number of diverse sources (Raybin and Flavin, 1977b; Gundersen and Bulinski, 1986a).

The absence of a post-translationally modifying mechanism in *Oncopeltus* ovarioles can be related to the life-cycle of the insect. Oogenesis in *Notonecta* lasts several months so an oocyte takes, on average, two weeks to mature. *Oncopeltus*, however, only lives for between 4 to 6 weeks in which time each ovariole must produce up to 6 oocytes. A nutritive tube and its component microtubules must therefore only survive for a maximum of one week (Bonhag and Wick, 1953). The predicted life of a nutritive tube microtubule in *Oncopeltus* is, therefore, significantly shorter than that in *Notonecta*. An *Oncopeltus* ovariole microtubule presumably turns over faster than a microtubule in a ganglion which is likely to be maintained for the duration of the animal's adult life. There is thus a corresponding difference in the extent of post-translational modifications, the ganglion microtubule being detyrosinated. Furthermore these post-translational modifications are confined to the microtubules at the edge of the ganglia, at the point where they extend into the axons. The post-translational modification, in this case detyrosination is, therefore, present in *Oncopeltus* microtubules and is further confined to the older of the most stable microtubules that *Oncopeltus* possess.

In summary, it is clear that *Oncopeltus* microtubules have the potential to undergo detyrosination as witnessed by the *in vivo* treatment of *Oncopeltus* ovariole homogenate with a carboxypeptidase to produce immunologically identifiable glu-tubulin. Unfortunately,

microtubules in the nutritive tubes could not be detyrosinated *in situ* presumably because the carboxypeptidase could not diffuse into the whole tissue. Treatment of tissue sections that had to be fixed and cryoprotected before sectioning was also unsuccessful. The existance of *Oncopeltus* microtubules that have been naturally detyrosinated were, however, identified in ganglia, thus showing that *Oncopeltus* has the post-translational modifying machinery. From this it is proposed that *Oncopeltus* nutritive tube microtubules have no detyrosinating mechanisms yet in the ganglia, where microtubules are relatively more long lived, the function is retained. The state of functioning of the detyrosination mechanism is thus dependent on the relative kinetics of the system it occurs in.

4.1II: Production of monoclonal antibodies to *Notonecta* microtubule proteins

The production of monoclonal antibodies is a protracted process which is prone to many pitfalls so a number of attempts were made to raise antibodies to *Notonecta* nutritive tube microtubule proteins. This met with varying levels of success

4.1IIa: Monoclonal productions 1 and 2

Initial attempts involved immunising mice each with 525µg of KLH coupled *Notonecta* ovariole microtubule proteins at a concentration of 3.5mg/ml, (Method I). These first runs produced two faint 52kD bands on the bovine nitrocellulose (Monoclonal production 1) and two bands with molecular weights of 70 and 130kD (Monoclonal production 2) which suggested that there were antibodies present in the sera that could possibly be recognising tubulin (52k), MAP2c, kinesin light chain or tau (70kD) and kinesin heavy chain (130kD). These sera, however, did not recognise microtubules or other tubulin containing structures in 3T3 cells. Despite this the well thought to be specific for α-tubulin was

cloned using cloning Method I. Unfortunately, the cloning efficiency was not very high (approximately 21% of wells grew up compared to the ideal figure of approximately 48%) and when all the wells were tested no positives were found using either immunoblotting or 3T3 cells. The experiment was therefore stopped at this point. With Monoclonal production 2 there was no subsequent recognition of these antigens after boosting the mice and fusing the spleens so it was decided to stop the run and rethink the approach used.

4.1IIb: Monoclonal production 3

It seems that among workers in the field there is a general consensus of thought that a number of proteins make very poor immunogens due to their ubiquity. If a protein is highly conserved in a large number of different organisms, including the animal that the attempt to generate sera is carried out in, it is likely that the host animal will have a tolerance to the protein, when introduced as an immunogen. Tubulin would be an ideal candidate for such a "naturally tolerable immunogen" as it is ubiquitous among all eukaryotes (Burns and Surridge, 1993). The level of conservation of the tubulin sequence is also exceedingly high, being approximately 70% between organisms as diverse as yeast and mammals (Yanagida, 1987), and this will further enhance the probability that the host animal will not produce positive antibodies to it when used as an immunogen. In an initial attempt to overcome this problem the levels of the microtubule proteins used in the injections were raised from 525μg to 2.175mg, at a concentration of 14.5mg/ml even though the former quantity is, in fact, very high (over 10 times the level suggested for particulate proteins by Harlow and Lane, 1988). It was, however, thought that increasing the amount further to 2.175mg/ml (over 40 times Harlow and lane's recommended level) would help overcome any pretolerance the mice would have to tubulin.

The increase in immunogen concentration gave an immediately apparent improvement. Even though the immunisation schedule followed that used in monoclonal productions 1 and 2 (see above), the response in the injected mouse was much stronger. Sera yielded positive bands with molecular

weights of 51, 56, 61, 115 and 175kD, molecular weights similar to that of tubulin (51kD), tau (56kD), light chain kinesin (61kD) and heavy chain kinesin (115kD). The 175kD band recognised may represent a novel MAP that is specific to *Notonecta* ovaries or possibly a poorly conserved homolog of a known MAP which has a slightly different molecular weight. Unfortunately there was no positive identification of cytoskeletal structures in 3T3 cells though particulate staining in the cytoplasm gave the suggestion of fragmented microtubules in places while the concentration of staining at the periphery of the nuclei and the generally fragmented and amorphous staining throughout the cytoplasm was similar to that found in fibroblasts incubated with a kinesin specific antibody (Neighbors *et al*, 1988: Hollenbeck, 1989; Pfister *et al*, 1989; Wright *et al*, 1991).

Despite these variable results and due to the wealth of positive bands the mouse was boosted in preparation for fusion but it unfortunately fell sick and the run was abandoned.

4.1IIc: Monoclonal production 4

After the promising results with monoclonal production 3 it was decided to again use the relatively high immunogen level of 2.175mg. This time, however, the immunisation schedule followed Method II (see Section 2.2Id). In this schedule, instead of the boosting injections being carried out interperitoneally 3 days after the tail bleed tests, they were given 11 days later, with 50% of the immunogen being administered interperitoneally, the other 50% following an intravenous route. This was deduced to be a better routine as the level of antibody in the system would, in the shorter injection time scale, be high and thus may clear the immunogen before it could bind to the primed B cells. Again there were encouraging tail bleeds, the test results being significantly higher than in the first two runs, molecular weights of 240, 220, 80, 78 and 60kD being resolved. Though this suggested the prescence of antibodies specific for MAP2-a or b (240 and 220kD) and tau (60kD) while the 80 and 78kD proteins could be specific for novel *Notonecta* MAPs or slightly different homologs of known MAPs, for example

the 80kD protein found associated with microtubules in rodent cells (Duerr *et al*, 1981), when the mice were boosted and the spleens fused none of the fusion sera could identify any bands on nitrocellulose and the subsequent cloning produced non-specific results. It was thus decided to halt the run in favour of concentrating on the following, more interesting, monoclonal experiment.

4.1IId: Monoclonal production 5

To try to eliminate the possibility that antigenic determinants in the microtubule preparation were shielded by the KLH, it was decided to ammend the immunogen-KLH fixing protocol, extensively cross-linking the KLH before fixing the microtubule sample to it. It was hoped that this would enable the antigen to be fixed onto the outside surface of the KLH protein mass. This should still stimulate a large immunological response in the mouse, while enabling the B cells to interact with the freely accessible microtubule protein and thus elicit the production of specific antibodies.

This change in the coupling protocol appeared to work, the pre-coupled sample producing a greater immunological response. As in the previous two monoclonal production attempts the nitrocellulose strips were again smeared, but indicated a strong immunological response to number of immunogens with a wide range of molecular weights. This was especially apparent when compared to the negative control, which was blank, and the positive α-tubulin control. In comparison to the tail bleed sera, blots using fusion sera were significantly clearer with a number of specific bands developing. These had molecular weights of between 29 and over 210kD which could possibly identify the presence of antibodies in the sera specific for such proteins as actin, tubulin, tau, heavy chain kinesin and MAP2-b (see Table 3). The strength of these results meant that Cloning Method I was carried out using two fusion wells that contained sera that identified the majority of the bands. The Cold Spring Harbor cloning method was abandoned as it gave such poor results in the previous run, Method I being more straight-forward and

MOLECULAR WEIGHT OF BAND (KD)	ANTIGEN THAT THE ANTIBODY IS POSSIBLY RECOGNISING
210	MAP-2b
160	-
113	HEAVY CHAIN KINESIN
93	-
87	-
71	TAU, MAP-2c, LIGHT CHAIN KINESIN
66	TAU
55	TUBULIN
48	TUBULIN
44	ACTIN
41	-
35	-
29	-
27	-

- No microtuble, microtubule-associated or microtubule interacting proteins with this molecular weight. The presence of a band is possibly due to non-specific staining

Table 3: Monoclonal Production 5-Possible bands recognised by the fusion sera

efficient. Unfortunately, a number of the specific bands identified by the fusion sera were lost in the cloning, only two bands of 54 and 114kD being identified, which again indicates that the original bands were either non-specific or the hybridomas producing the lost antibodies were at low titres prior to cloning, being outgrown by non-specific hybridomas and were subsequently lost during the cloning process. Despite these problems sera identifying 54kD and 114kD bands were of interest as the antigens recognised may correspond to *Notonecta* ovariole tubulin and kinesin.

4.1IId(i): Monoclonal production 5- Clone CL3/114

Of the cloned sera specific for antigens with the same molecular weights as known cytoskeletal proteins clone CL3/114 was of potential interest due to it binding an antigen with a similar molecular weight to kinesin heavy chain. In *Oncopeltus* a protein with the same molecular weight as kinesin has been identified in ovarioles and anterograde movement can be demonstrated *in vitro* (Anastasi *et al*, 1990). Kinesin has not, however, been identified in the nutritive tubes themselves (Anastasi *et al*, 1990). This has led to questions being raised about where exactly the *Oncopeltus* kinesin is localised. Recent work on the action of sea urchin egg kinesin *in vitro* (Cole *et al*, 1992) and the expression of human heavy chain kinesin in CV-1 cells (Navone *et al*, 1992) has implicated kinesin in microtubule bundling, a function that could be of paramount importance in the development of the massive array of bundled microtubules in nutritive tubes (see Section 4.1Ic). Kinesin may also be important in the transportation of tubulin dimers from the breakdown of redundant nutritive tubes back to the trophic region (Bennett and Stebbings, 1979) or to the new microtubules nucleating at the nutritive tube inner periphery.

The particulate staining pattern produced in 3T3 cells by CL3/114 was similar to that observed with kinesin specific sera, the staining generally being heavy and amorphous throughout the cytoplasm with punctate patches in places (Neighbors *et al*, 1988; Hollenbeck, 1989; Pfister *et al*, 1989). This was particularly apparent in methanol fixed

cells which have similar staining patterns to the cells shown in Figure 5C of Pfister *et al* (1989) and Figure 5A of Neighbors *et al* (1988), though in the latter case the staining in the published figure is not as strong as in the cells stained with CL3/114. To avoid potential fixation artefacts sera were also tested on formaldehyde fixed 3T3 cells. Staining by CL3/114 was less clear. Entire cells were filled with a dense, amorphous staining which seemed to be particularly concentrated in the region of the nucleus. Again this resembled kinesin staining and there was also a faint suggestion of microtubule-like staining at the cell margins possibly indicating co-distribution with microtubules (Hollenbeck, 1989).

The staining produced by CL3/114 on *Drosophila* B_{II} cells was clearer than for 3T3 cells. When compared with B_{II} cells stained with anti α-tubulin antibody (Figure 34) there was little difference in the staining patterns. Both the putative anti-kinesin serum and the anti-α-tubulin antibody stained predominantly amorphous patches that in some areas were more defined as microtubule-like structures. This was more pronounced with the tubulin antibody but the staining patterns of CL3/114 were close enough to suggest that an antibody in the serum stained microtubules. On frozen sections of *Notonecta* ovarioles staining occured at the inner and outer margins of the nutritive tube containing region and not in the nutritive tubes themselves. As kinesin has not been localised to nutritive tubes the reliability of these results cannot be fruitfully questioned. (Anastasi *et al*, 1990) and the staining at the margins could be artefactual due to either a concentration of antibody at the joining of two structures or out of focus staining above and below the plane of focus aggravating the problem.

Though the results are unclear it is possible that there may be an anti-kinesin antibody present in the CL3/114 serum. The number of structures staining in all the cells and tissue based assays does, however, suggest that there are several non-specific antibodies present. This will have to be overcome by further cloning runs, the sera then, hopefully being retested to give clearer results. The assays can then be made more specific for kinesin.

4.1IId(ii): Monoclonal production 5: Clones CL6/54 to CL9/54

Although potentially not as interesting as CL3/114 the putative *Notonecta* ovarioles anti-tubulin antibodies, were worth further characterisation.

On cultured cells fixed with either methanol or formaldehyde a tubulin specific monoclonal antibody would be expected to give a clear staining pattern which would identify long, smooth, relatively straight or slightly sinous, much shorter microtubules (as is the case for tyrosinated and detyrosinated α-tubulin respectively; Gundersen *et al*, 1987; Osborn and Weber, 1976; Figure 4a and 4c). Unfortunately the putative tubulin antibodies in sera CL6/54, CL7/54, CL8/54 and CL9/54 failed to produce such a clear pattern. Either the entire cytoplasm stained (for example CL6/54), or a particulate pattern which included very sparse microtubule-like staining developed (for example CL7/54 and CL8/54). The additional staining suggests that the pattern produced with the sera does not correspond to tubulin.

As with the 3T3 cells the staining pattern on *Drosophila* B_{II} cells was variable though with CL6/54 and CL7/54 it was much more like the expected 'honeycombed' pattern that an α-tubulin specific antibody produces (compare with Figure 34) but again there were no microtubule-like structures stained. This, with the additional staining observed does, however, suggest against the supposition that the sera contain tubulin specific antibodies.

The major test for the existance of the putative tubulin antibodies in the sera was their specificity for nutritive tubes in *Notonecta* ovariole sections. All the sera had a widespread staining pattern that was not confined to the nutritive tubes. If the serum contained a monoclonal antibody that was specific for *Notonecta* tubulin then it would be expected to strongly stain the nutritive tubes with, possibly, some fainter staining in the follicle cells and the centre of the ovariole (see Figure 10). It thus seems that the sera contain a number

of different antibodies, the majority of which were causing non-specific staining.

The mixed antibodies in the sera could potentially be separated by further cloning, however it is a possibility that there was no tubulin specific antibody, the amorphous staining being attributable to either background or non-specific secondary antibody staining.

4.1IId(iii): Monoclonal production 5: The final analysis

This work has developed a number of modifications from the original protocol as used in Monoclonal production 1. To overcome the natural tolerance of highly conserved microtubule proteins the level of immunogen was increased four fold. To circumvent any problems caused by the immense size of the KLH "swamping" the antigenic determinants of the immunogen pre-fixing was used which seemed to significantly improve the immune response. Finally the elapsed period between tail bleed testing and boosting was increased to prevent the newly administered immunogen being removed by specific antibodies which would be at high concentrations in the mouse's blood. Despite problems caused by having to work with such small and seasonal volumes of microtubule proteins and the development of cold sectioning of *Notonecta* ovaries at the very end of this project these changes, coupled with the original fusion and cloning methods has lead to some interesting results. Ultimately however the telling result is the absence of staining in the structures that the antibodies were raised against. This could be due to experimental failure as, by necessity, all the *Notonecta* sectioning work was carried out in one experiment and the titre of specific antibodies in the sera being tested is likely to be lower than the control sera but, unfortunately, these difficulties could not be resolved within the time scale available. It is, however, possible that with future work these problems could be circumvented and a number of useful antibodies isolated and only then may the true potential of the sera be revealed.

5.1: FURTHER WORK

Although the ovarioles of *Notonecta*, *Oncopeltus* and the hemipterans in general have many and varied uses in both cytoskeletal and cell cycle research (Hyams and Stebbings, 1979a; b; Stebbings *et al*, 1986; Stebbings and Hunt, 1987; Stebbings *et al*, 1992), the number of research groups working on them is very limited. Because of this the scope of further work that could be carried is vast. Several enticing lines of work are outlined below:

1) A suitable suite of antibodies to the microtubule proteins of the ovarioles of *Notonecta* and *Oncopeltus* have yet to be convincingly identified and characterised sufficiently for further use. The sera raised during the course of this investigation have potential but all have to be recloned at least once to produce true monoclonal antibodies. In particular, the serum containing the putative kinesin antibodies would benefit greatly from such a course of action and would be of potentially great value. After ascertaining its identity by blotting and preabsorbing against purified kinesin it would initially be used in identifying where exactly in the ovariole kinesin is localised, this being confirmed using an established kinesin antibody. Subsequently the antibody and the isolated cDNA (see point 3 below) could be used to investigate the exact action of the protein in transport within the ovariole and in development. Unfortunately within the time constraints levied by this project such a scheme was impossible. Another victim of time were the hybridomas produced from the spleens of the other mice that were sacrificed in the course of this work yet could not be used due to the labour intensity of producing antibodies. These were frozen down and may still produce potentially interesting antibodies if grown up and screened.

2) Once monoclonal antibodies to either *Notonecta* or *Oncopeltus* cytoskeletal proteins have been isolated and accurately characterised it will be possible to probe gene expression libraries prepared from the ovarioles of the respective insects, or possibly even *Drosophila*. In doing this, genes for, initially, tubulin and, more interestingly, the

microtubule-associated proteins could be isolated and characterised. Further experiments could involve *in situ* hybridisation to determine where the respective mRNAs for various MAPs are expressed and, at a more elegant level, the use of anti-sense DNA to prevent the expression of motor proteins and thus observe the effect on microtubule associated transport and ovariole development.

3) Work which would benefit enormously from the development of a specific suite of both antibodies and isolation of cDNAs to ovariole proteins would be the further characterisation of the structure of the ovarioles, with particular regard to the nutritive tubes. Initially this could involve using a battery of cytoskeletal and associated motor protein specific antibodies to systematically probe sections from throughout the ovarioles of both *Notonecta* and *Oncopeltus*. This, coupled with the development of a 3-D model using confocal microscopy, would elucidate how the systems would develop, both spatially and chronologically, using the distributions of tyr and glu tubulins as markers for microtubule age. The distributions of the microtubule associated proteins could then be localised in a similar manner and it may then be possible to define how both the transport and structural systems work. Another interesting question is what controls both the insertion points of the new microtubules in the expanding system, the use of antibodies to γ-tubulin and other MTOC components being of potentially great use, and the ordered production of the crystalline structure of microtubules as the nutritive tubes enter terminal redundancy. The elucidation of MAP distribution may also reveal if there is a relationship between the distribution of tubulin isotypes and certain MAPs. It may also reveal significant differences between the microtubules of *Oncopeltus* and *Notonecta* which are very different in ages yet exhibit similar stabilities. Furthermore, it will be interesting to use antibodies specific for tubulin-tyrosine ligase and CPA to pinpoint exactly if and where the post-translational modifications are occuring in the nutritive tubes of both *Notonecta* and *Oncopeltus*, all this antibody work combining to produce a unified model of nutritive tube development and functioning.

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